Isotype-specific Antibody Responses to *Rhodococcus equi* in Foals on a Horse-breeding Farm with a Persistent Incidence of *R. equi* Infection

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Isotype-specific antibody responses in 13 foals to Rhodococcus equi were studied during the first 20 weeks of life on a horse-breeding farm with a persistent incidence of R. equi infection. The levels of immunoglobulin (Ig) classes and IgG subclasses specific for R. equi antigens in the 13 foal sera were determined by ELISA and Western immunoblotting. Kinetics of the total amount of IgG and the number of WBC in the blood of 13 foals were also monitored. Soluble extract from R. equi strain ATCC 6939 was used as a routine ELISA antigen. During the observation period, 8 of the 13 foals showed >0.3 OD value against ATCC 6939 antigen by a routine ELISA, which was tentatively fixed to be positive on the basis of readings made of healthy horse sera in previous studies. R. equi-specific IgG_A was predominantly detected in these foals at 6 to 10 weeks after birth by ELISA with Tween 20-extracted ATCC 6939 antigens. Specific serum IgG_B, and IgA were also detected, but they showed lower titers than IgG_A antibodies in the foals. Specific IgG(T), IgG_C and IgM was present consistently at low levels in most foals. On the other hand, IgG_A and IgG_B antibodies specific for 15- to 17-kDa antigens (VapA) were detected by immunoblotting at 5 to 7 weeks of age in most foals. Out of the 13 foals, 3 foals had a serum IgG concentrations less than 1,000 mg/dl (650 to 900 mg/dl) at the first week of birth. WBC counts tended to increase by 8 weeks with age in all foals. Virulent R. equi was isolated from the feces of foals during this study. This study suggests that R. equi-specific IgG_A was predominantly measured by ELISA, and VapA-specific IgG_A and IgG_B were predominantly detected by immunoblotting in foals naturally exposed to R. equi on a farm with a persistent incidence of R. equi infection.

Key words: *Rhodococcus equi*, isotype, antibody, foals

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Rhodococcus equi is one of the most important bacterial pathogens in young foals. Infections caused by this organism are characterized by chronic, suppurative bronchopneumonia and enteritis associated with a high mortality rate [2, 13, 20]. We previously reported that the 15- to 17-kDa virulence-associated protein antigens (VapA) of *R. equi* were associated with virulence in mice, and that the presence of an 85-kb or a 90-kb plasmid was essential for

virulence and the expression of VapA [26, 30]. These virulence-associated antigens and virulence plasmids have been used as epidemiological markers to identify virulent *R. equi* isolates from horses and their environment by Western blot (immunoblot) assay with a monoclonal antibody and plasmid profiles [21, 28, 31]. In a survey of the prevalence of virulent *R. equi* at horse-breeding farms in Japan, *R. equi* was isolated from almost all soil samples, at numbers of 10² to 10⁵ per gram of soil [21]. Although the majority of these isolates did not contain virulence plasmids and were avirulent, some of these farms were contaminated to

various degrees with virulent *R. equi* in accordance with the incidence of infection [20, 21, 28].

Inhalation of dust particles laden with virulent *R. equi* is the major route of pneumonic infection [13, 34]. Ingestion of the organism is a significant route of exposure, and likely also of immunization [22], since the organism multiplies in the intestines of the foal up to about three months of age, reaching numbers up to 10^5 /g of feces [29]. Pneumonic foals swallow infected sputum, and virulent bacteria so swallowed can multiply in their intestines, so that the manure of affected foals is most likely a major source of progressive contamination of the environment with virulent organisms [20]. Foals bred on farms with endemic infection can be open to multiple exposures to large numbers of virulent *R. equi* [21, 26]. Our previous study demonstrated that serum antibody response to VapA occurred naturally in every foal in association with the quantitative changes in fecal *R. equi* during the first 1 to 3 months of life, suggesting that intestinal virulent *R. equi* might be the most important source of antigenic stimulation in foals from contaminated farms [22].

It is well established that the functional activities of antibodies are dependent on their isotypes [4], and that immunoglobulin (Ig) isotype responses to infectious organisms are not randomly distributed [8]. Recent characterization of horse IgG subisotypes by using monoclonal antibodies (mAbs) has demonstrated four well-defined equine IgG isotypes, IgGa, IgGb, IgGc and IgG(T) [19], which occupied approximately 9, 77, 1 and 13 percent of serum, respectively [15].

The purpose of this study is to analyze the Ig subclass (isotype) reactivity of anti-*R. equi* antibodies in 13 foals during the first 20 weeks of life on a horse-breeding farm with a persistent incidence of *R. equi* infection by enzyme-linked immunosorbent assay and Western immunoblotting with changes in the IgG concentration, a WBC count and the prevalence of virulent *R. equi* in the feces of foals.

Materials and Methods

Blood and sera from foals

Sera from 13 Thoroughbred foals on a farm in Aomori, which revealed recurrent incidences of *R. equi* infection, were collected 6 to 11 times at one week intervals after birth from March to August in 1998. Sera

from the dams of the 13 foals were collected once immediately after foaling.

Blood samples were collected in EDTA vacuum tubes to determine erythrocyte and total leucocyte counts. The serum IgG concentration was determined by a nephelometry assay (Nissan Gousei, Tokyo, Japan).

ELISA

ELISA antigen was prepared from *R. equi* ATCC 6939 as described previously [24]. Briefly, strain ATCC 6939 was grown on brain heart infusion agar. Bacteria were harvested after 5 days of incubation at 38°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 $20,000 \times g$ for 30 min at 4°C. The supernatant was adjusted to 1.0 µg of protein/ml in carbonate-bicarbonate buffer (pH 9.6) and used as an antigen.

The ELISA used was based on that of Takai *et al.* [25]. Optimal dilutions of reagents were obtained by checkerboard titrations. Peroxidase conjugated to anti-horse IgGa (prepared in goats, heavy chain specific, Bethyl Laboratories Inc., Montgomery, Texas), anti-horse IgGb (prepared in goats, heavy chain specific, Bethyl Laboratories Inc), anti-horse IgGc (prepared in goats, heavy chain specific, Bethyl Laboratories Inc), anti-horse IgG(T) (prepared in goats, heavy chain specific, Bethyl Laboratories Inc), anti-horse IgA (prepared in goats, α -chain specific, Bethyl Laboratories Inc), anti-horse IgM (prepared in goats, μ -chain specific, Bethyl Laboratories Inc), and anti-horse IgG (prepared in goats, heavy and light chains specific, Bethyl Laboratories Inc), were used.

Gel electrophoresis and Western immunoblot analysis

Whole-cell antigens of strain ATCC 33701, which were prepared by harvesting bacteria grown at 38°C for 48 hr in brain heart infusion broth and solubilizing the bacteria in sodium dodecyl sulfate (SDS) reducing buffer, were separated by SDS-polyacrylamide gel electrophoresis [26], and electrotransferred to nitrocellulose sheets. A strip of the sheet was incubated at 37°C for 2 hr with each test serum (diluted in PBS with 10% Block Ace 1:100). The strips were washed three times for 10 min in washing buffer. Bound foal immunoglobulin was detected by incubating the strips with monoclonal antibodies to IgGa (F12a1A5), IgGb (F12b6E8), IgGc (F14c10G12), IgG(T) (F6T3A5), IgM (F13M6D6), and IgA (F9A) which were produced by

Sugiura *et al.* [19]. After a series of titrations, the optimum dilutions were as follows: IgGa (F12a1A5), 1/4,000; IgGb (F12b6E8), 1/4,000; IgGc (F14c10G12), 1/2,000; IgG(T) (F6T3A5), 1/2,000; IgM (F13M6D6), 1/2,000; and IgA (F9A) 1/2,000. These monoclonal antibodies were detected by incubating the strips with rabbit anti-mouse immunoglobulin G conjugated with horse-radish peroxidase (diluted in PBS with 10% Block Ace 1:2,000) (Cappel Laboratories, Cochranville, Pa.). Color was developed after another series of washes with substrate solution containing DAB in 100 mM Tris (pH 8.5).

Isolation and identification of virulent R. equi from feces of foals

Fecal samples from the 13 Thoroughbred foals on the farm were collected 6 to 11 times after birth from March to August, 1998. For the selective isolation of *R. equi*, nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) medium, previously described by Woolcock *et al.* [33], was used. One gram of feces or soil sample was diluted serially with a 10-fold volume of sterile saline. Each diluent was inoculated onto two plates of NANAT medium, and the plates were incubated at 30°C for 2 or 3 days. All suspected colonies of *R. equi* were counted and the number of viable organisms per gram of feces or soil was calculated [27]. Two to ten colonies per specimen were subcultured, identified in our laboratory and then tested for the presence of 15- to 17-kDa antigens by immunoblotting and virulence plasmids by agarose gel electrophoresis, as described previously [30].

Plasmid DNA was isolated from *R. equi* by the alkaline lysis method [3], with some modifications, as described previously [30]. Samples of the plasmid preparations were separated along with the plasmids of *R. equi* 96B6 (an 87-kb type II plasmid) and L1 (a 90-kb type I plasmid) in 0.7% agarose gel at approximately 5 V/cm for 2 hr [31].

Results

Serum IgGa, IgGb, IgGc, IgG(T), IgM, and IgA antibody levels specific for Tween 20-extracted ATCC 6939 antigens in 13 foals on a horse-breeding farm with a persistent incidence of *R. equi* infection were measured during the first 20 weeks of life by ELISA. During the observation period, 8 (Nos. 2-6, 8, 9 and 13)

Fig. 1. ELISA titers against *R. equi* ATCC 6939 Tween 20-extracted antigens in the 13 foal sera obtained between 0 to 20 weeks after birth on the farm (A). WBC counts of the 13 foals (B). IgG concentrations of the 13 foals (C). Foal Nos. 1 (●); 2 (■); 3 (◆); 4 (▲); 5 (▼); 6 (○); 7 (□); 8 (◇); 9 (△); 10 (▽); 11 (⊞); 12 (◊); 13 (⊠).

of the 13 foals showed a >0.3 OD value against ATCC 6939 antigen in a routine ELISA (anti-horse IgG) (Fig. 1A), which was tentatively fixed to be positive on the basis of readings made of healthy horse sera in previous studies. Four (Nos. 2, 6, 8 and 13) of them showed clinical signs and then were treated with antibiotics. The remaining 5 foals (Nos. 1, 7, 10, 11 and 12) had an OD value less than 0.3, but 2 (Nos. 10 and 11) of them showed signs of temporal pyrexia.

Fig. 2. ELISA titers of *R. equi* specific IgGa, IgGb, IgGc, IgG(T), IgM and IgA isotypes in serum of the 13 foals collected between 0 to 20 weeks after birth on the farm. Foal Nos. 1 (●); 2 (■); 3 (◆); 4 (▲); 5 (▼); 6 (○); 7 (□); 8 (◇); 9 (△); 10 (▽); 11 (⊞); 12 (⊙); 13 (⊞).

The results of isotype-specific antibody responses were analyzed according to age and are shown in Fig. 2. *R. equi*-specific IgGa was predominantly detected in the 6 foals (Nos. 3–6, 8 and 9) at 6 to 10 weeks after birth by ELISA with Tween 20-extracted ATCC 6939 antigens. Specific serum IgGb (foal Nos. 3, 6, 8, 9 and 13) and IgA (foal Nos. 5, 9–13) were also detected, but they showed much lower titers than IgGa antibodies. Specific IgG(T), IgGc and IgM were present consistently at low levels in most foals. Low antibody responses were observed in foals Nos. 1, 11 and 12.

Western immunoblot analysis of sera from all the foals for whole cells of virulent *R. equi* ATCC 33701 was done with a set of monoclonal antibodies [18]. IgGa and IgGb antibodies specific to VapA were detected by

immunoblotting 5 to 7 weeks after birth in correlation with age in most foals except foal No. 13. Fig. 3 shows one of the typical results obtained from foal No. 3. In addition to specific IgGa and IgGb responses, foals Nos. 2 and 11 had IgGc and IgG(T) antibodies specific to VapA and foals No. 4, 6, 8, 10 and 12 had IgGc, IgG(T), IgA and IgM antibodies specific to VapA detected by immunoblotting.

WBC counts increased to 15,000/mm³ or more by 6 to 8 weeks in accordance with the age and then decreased in most foals (Fig. 1B). Out of the 13 foals, 2 foals (Nos. 1 and 7) had a serum IgG concentration less than 1,000 mg/dl (650 to 900 mg/dl) in the first week after birth, and their serum IgG concentrations gradually increased to 1,200 to 1,500 mg/dl by 20 weeks

Fig. 3. Western immunoblot analysis of sera collected from foal No. 3 at 6 to 94 days old to whole cells of virulent *R. equi*. The arrowhead on the right indicates the 15- to 17-kDa antigens. Lanes 1, 6 days old; 2, 20 days old; 3, 34 days old; 4, 47 days old; 5, 55 days old; 6, 63 days old; 7, 69 days old; 8, 76 days old; 9, 94 days old.

of age (Fig. 1C). On the other hand, serum IgG concentrations of the remaining 10 foals at first week of life were 1,500 to 2,500 mg/dl and decreased to 1,000 mg/dl by 3 to 6 weeks of age, and then gradually increased.

Virulent *R. equi* was isolated from the feces of 13 foals at prevalence ranging from 12.5 to 55.6%. Of 840 isolates from the 13 foals, 172 (20.5%) isolates were virulent *R. equi*. Of the 172 virulent *R. equi*, 94 (48.8%) contained an 87-kb type II plasmid and 88 (51.2%) contained a 90-kb type I plasmid.

Discussion

The present study revealed that IgGa antibody specific to ATCC 6393 antigens was predominantly measured by ELISA and specific IgGa and IgGb antibodies to VapA were detected by immunoblotting

in foals naturally exposed to *R. equi* on a farm with a persistent inhabitation of this organism. In *Streptococcus equi* (*S. equi*) and equine herpes virus 1 infections, IgGb or IgGa and IgGb were the predominant serum antibodies after natural infection [14, 16]. In humans, protein antigens generally stimulate IgG1 and IgG3 response [11, 12, 18], whereas polysaccharide antigens stimulate mainly IgG2 antibodies [1]. In mice, carbohydrate antigens preferentially elicit IgG3 response [17]. In contrast, IgG1 is the dominant response to protein antigens [17]. Our antigens for ELISA are soluble extracts from ATCC 6939 with Tween 20, so they may consist of capsular antigens and surface proteins of avirulent *R. equi*. On the other hand, VapA is a protein antigen which does not exist in avirulent *R. equi* [23]. We cannot yet explain the discrepancy in the results obtained from the ELISA and Western immunoblotting. It is possible that the properties of antigens used in both tests are responsible

for the difference in the isotype-specific responses.

Little is known about the variation in the isotype-specific antibody response in horses due to the nature of antigen, the route of entry, and the form in which it reaches to the immune system. A difference in serum antibody responses in vaccinated horses from those in spontaneous infection has been reported [6, 14, 16]. The levels of specific IgGa for *S. equi* were higher during and shortly after the infection, but they were very low in vaccinated horses [16]. Strong nasal IgA and serum IgGa, IgGb and IgA responses to influenza virus followed natural infection, whereas vaccinated animals showed only nasal and serum IgG(T) response [6]. The protective role of antibodies to the virulence-associated antigens of *R. equi* has been described in a mouse model [32]. The protective effect of immune plasma has also been demonstrated in experimentally and naturally infected foals [9, 10]. It might be interesting to investigate the isotype-specific antibody levels of equine hyperimmune sera or plasma and murine sera collected from mice immunized against virulent *R. equi* to study the function of IgG isotypes in the protection against *R. equi* infection.

B cells secrete IgM antibodies specific for the inducing antigen. As their response to the antigen continues, these immunoglobulin-producing B cells switch their heavy chain constant region production from μ to γ , ϵ , or α while retaining their original variable regions. In that way, they produce immunoglobulins of difference classes without altering their antigen specificity. In this study, we observed very low IgM antibody response prior to those in IgGa, IgGb, and IgA by means of ELISA and immunoblotting. With the same assays, strong *R. equi* specific IgM antibody response was observed in the foals experimentally infected with *R. equi* [25]. We cannot yet explain the difference between the IgM responses observed in the naturally exposed foals and those in the experimentally infected foals. The properties of antigens such as T-independent or -dependent, and their routes of entry such as oral or intratracheal invasion, might affect the immune response in those foals.

Recently, a complete sequence of virulence plasmid p33701 that encodes a family of seven closely related virulence-associated proteins designated VapA and VapC to VapH has been shown. VapC, -D, and -E are secreted proteins coordinately regulated by temperature with VapA [23]. Serum from foals infected with *R. equi* recognized either recombinant VapD or rVapE [5]. Further studies are needed to

clarify the isotype specific response to Vap proteins by using peptide antigens and recombinant proteins in naturally and experimentally infected foals and vaccinated foals.

R. equi is a soil organism that is ingested into the gut of many herbivores and is widespread in their environment [2]. Virulent *R. equi* showing VapA has been isolated from soil or horses at many horse-breeding farms having endemic infection [20–22, 28]. In the present study, all of the foals tested shed virulent *R. equi* in their feces at various concentrations. This means that all foals were exposed to virulent *R. equi* by inhalation and ingestion. In natural and experimental *R. equi* infection, foals often do not show any clinical changes until the later stages of the disease [34]. In our previous study [7], we demonstrated that foals having high OD values of over 0.9 in serum (total IgG) had high leukocyte counts and fibrinogen and alpha-globulin values. In accordance with the number of *R. equi* in their feces and aging, WBC counts increased in all foals with or without clinical signs. These results suggest that the foals studied might be naturally exposed to virulent *R. equi* repeatedly or continuously with various doses, and develop antibodies to the VapA without any clinical manifestations.

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