Serum Immunoglobulin, Dermal Response, and Lymphocyte Transformation Studies in Horses with Chronic Diarrhea

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Serum specimens from 12 sick and 20 normal horses were examined for levels of different classes of immunoglobulin (Ig) by a single radial immunodiffusion. The level of IgA in the sera of sick horses was about 50% lower than in the sera of normal horses. By contrast, the level of serum IgG was higher in sick than in normal horses. Phytohemagglutinin (PHA) responsiveness of blood lymphocytes showed transient suppression during the stage of severe diarrhea. The regaining of PHA responsiveness of lymphocytes was observed simultaneously with the recovery process. However, the responsiveness of lymphocytes in recovered horses was still markedly lower than in normal horses. Allergic reactions in sick and normal horses were studied by observing dermal response to the injections of saline extracts from some of the horse feeds. A delayed hypersensitivity reaction to streptokinase-streptodornase and PHA was also studied. The allergic reactions to these extracts were not induced in either sick or normal horses; however, inflammatory response to the extracts was about 50% greater in normal than sick horses. Response to the intradermal injection, either streptokinase-streptodornase or PHA, was significantly greater in normal horses than sick horses. These findings are discussed with respect to the pathogenesis of chronic diarrhea and the complexity of immunodeficiency demonstrated in this disease. The possibility that transient defects of cell-mediated immunity may predispose to chronic diarrhea is proposed.

Chronic diarrhea is a disease affecting a wide range of age and breeds of horses. Horses with persisting mild diarrhea can survive several years, but sometimes this disease becomes severe with watery stool, dehydration, and great loss of weight. Previously, Sitarska and Pytkowski (16) have reported the retardation of the growth of yearling horses caused by a persisting long-term diarrhea. The post mortem examination of experimentally sacrificed horses with chronic diarrhea did not reveal any major pathological changes in the intestines or in other organs (D. P. Gustafson, unpublished data). It is not clear whether this disease is an immunological disorder or some chronic infection. However, existing observations may suggest that this disease is correlated with immunoglobulin (Ig) deficiency (16, 17). In human medicine, it has been reported that the hypogammaglobulinemia A was sometimes associated with gastrointestinal symptoms (13). In contrast, in chronic diarrhea caused by the irritation of the colon, the level of immunoglobulins was increased (18).

The present investigation was performed to determine the immunological status of horses suffering from chronic diarrhea. I was particularly interested in the level of circulating immunoglobulins, the transformation of the lymphocytes stimulated with phytohemagglutinin (PHA), and the allergic response to some of the horse feeds as well as the delayed hypersensitivity reaction to injection of streptokinase-streptodornase (SK-SD) and PHA.

MATERIALS AND METHODS

Animals. Twelve horses from the Midwest and East Coast areas, of different breed, age, and condition, all suffering from chronic diarrhea were admitted for treatment to the Large Animal Clinic, School of Veterinary Medicine and Science, Purdue University (Table 1). Twenty normal horses were randomly selected from the herd at the University farm.

The diagnosis was established in each case by persisting diarrhea syndrome. Diarrhea ranged in intensity from severe (watery stool) to mild (pasty stool) and in duration from 1 to 6 months before the animals were admitted to the clinic. All the sick horses had undergone intensive treatment with a wide variety of antibiotics before they were admitted to our clinic. Routine hematological, bacteriological, and

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parasitological examinations did not show any abnormalities.

The sick horses were treated by the oral administration of 1 liter of normal equine serum on 3 successive days. Two weeks later, the same treatment was repeated. The horses under this treatment recovered from severe diarrhea during the next 2 or 3 weeks; however, mild diarrhea sometimes persisted for several months.

**Immunoglobulin separation and antisera.** Horse IgA and IgG were separated from colostral whey by precipitation with 40% saturated ammonium sulfate and then applied to a diethylaminoethyl-cellulose column and eluted stepwise by using nine successive buffers (22; J. P. Vaerman, Ph.D. thesis, Université Catholique de Louvain, Louvain, Belgium, 1970). Based upon immunoelectrophoresis (IEP) and immunodiffusion assays, eluants with buffers 0.04 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride + 0.08 M NaCl, pH 8, and 0.04 M Tris-hydrochloride + 0.14 M NaCl, pH 8, were IgA-rich fractions. These fractions were further purified on a Sephadex G-200 column. Sephadex fractions producing only one line with antisera to whole equine serum in IEP and immunodiffusion assays were concentrated and tested again. In addition, the protein from these fractions produced a single precipitation line with known anti-horse IgA sera.

The IgG-rich fraction was eluted from the diethylaminoethyl-cellulose column with buffer 0.005 M Tris-hydrochloride, pH 8, and further purified by separation on a Sephadex G-200 column. Fractions producing the single precipitation line with anti-equine whole serum were concentrated and tested again.

Rabbit monospecific antisera to equine IgA or IgG were obtained, absorbed, and tested as described by McGuire and Crawford (6) with one exception: rabbits were immunized with IgA or IgG that was separated from horse colostrum. Final evaluation of monospecificity of antisera was performed by IEP and immunodiffusion. The rabbit antisera producing only a single line with equine serum or colostral whey were used in the single radial immunodiffusion assay. In addition, the monospecific anti-IgA serum produced reaction of identity with the anti-IgA sera, which were kindly supplied by T. C. McGuire and J. P. Vaerman.

**Immunoglobulin determination.** Blood samples from the sick horses were collected before and 3 days after each of the two treatments. Additional blood samples were taken from some of the sick horses 2 months, 3 months, or 1 year after the treatment was completed. Blood samples were also taken from 20 normal horses. IgG and IgA levels of the horse sera were determined by modification of the single immunodiffusion technique of Mancini (12). The monospecific anti-equine IgG or IgA serum was incorporated into the agar.

Tested sera at dilutions of 1:2, 1:4, and 1:8 were applied with a 10-μl microsyringe to wells (1.9 mm in diameter and 1.2 mm deep). The plates were incubated at room temperature for 3 days; then the vertical and horizontal diameters of each ring of precipitation were measured, and the average diameter was calculated.

**Skin tests.** The injection sites were three areas each approximately 10 cm² arranged in a rough triangle on the left side and two areas on the right side of the neck of four horses with severe diarrhea and four normal horses. Each of the areas on the left side of the neck was injected intradermally with one of three saline extracts (0.2 ml) of regular horse feeds that intensified diarrhea in sick horses. The saline extracts were prepared by heating 50 g of each of the following feeds: Ruff’n Redi (Allied Mills, Inc., Chicago, Ill.); Tippecaroe Horse Feed (Lafayette, Ind.); and Tippecaroe Calf Feed (Lafayette, Ind.) with 50 ml of saline for 72 h in a water bath at 60 C. Each of these suspended feeds was centrifuged at 11,000 × g for 25 min at 4 C. The supernatant fluid from each of the suspensions was filtered by a membrane filter (0.22-μm pore size; Millipore Corp.). Each area on the right side of the neck was injected intradermally with an optimal dose of either SK-SD (100 U of streptokinase and 25 U of streptodornase) no. (1) 353-707 (American Cyanamid Co., Princeton, N.J.) or 150 μg of PHA-M (lot 0528-56, control 585786, Difco Laboratories, Detroit, Mich.). The skin thickness was measured by caliper before injection (0 h) as well as

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**Table 1. Summary of data from sick horses**

<table>
<thead>
<tr>
<th>Horse</th>
<th>Breed</th>
<th>Sex</th>
<th>Age</th>
<th>Duration of diarrhea (months)</th>
<th>Intensity of diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Appaloosa pony</td>
<td>Male</td>
<td>14 months</td>
<td>6</td>
<td>Severe</td>
</tr>
<tr>
<td>B</td>
<td>Appaloosa</td>
<td>Male</td>
<td>5 yr</td>
<td>4</td>
<td>Mild</td>
</tr>
<tr>
<td>C</td>
<td>Quarter</td>
<td>Male</td>
<td>10 months</td>
<td>2</td>
<td>Severe</td>
</tr>
<tr>
<td>D</td>
<td>Quarter</td>
<td>Female</td>
<td>13 months</td>
<td>3</td>
<td>Severe</td>
</tr>
<tr>
<td>E</td>
<td>Quarter</td>
<td>Female</td>
<td>18 months</td>
<td>2</td>
<td>Severe</td>
</tr>
<tr>
<td>F</td>
<td>Quarter</td>
<td>Female</td>
<td>2.5 yr</td>
<td>3</td>
<td>Mild</td>
</tr>
<tr>
<td>G</td>
<td>Quarter</td>
<td>Female</td>
<td>4 yr</td>
<td>4</td>
<td>Mild</td>
</tr>
<tr>
<td>H</td>
<td>Quarter</td>
<td>Male</td>
<td>2 yr</td>
<td>3</td>
<td>Mild</td>
</tr>
<tr>
<td>I</td>
<td>Standard</td>
<td>Male</td>
<td>3 months</td>
<td>1</td>
<td>Mild</td>
</tr>
<tr>
<td>J</td>
<td>Thoroughbred</td>
<td>Male</td>
<td>4 yr</td>
<td>2</td>
<td>Mild</td>
</tr>
<tr>
<td>K</td>
<td>Thoroughbred</td>
<td>Male</td>
<td>4 yr</td>
<td>1</td>
<td>Mild</td>
</tr>
<tr>
<td>L</td>
<td>Thoroughbred</td>
<td>Male</td>
<td>2 yr</td>
<td>3</td>
<td>Mild</td>
</tr>
</tbody>
</table>
15 min, 30 min, and 1, 2, 4, 5, 6, 7, 8, 12, 20, 24, 48, and 72 h after injection. The skin tests were repeated in the recovered horses.

**Lymphocyte transformation.** Blood samples were taken from four horses with severe diarrhea before treatment and before the recovered horses were dismissed from the clinic. In addition, blood samples were taken from three horses with mild diarrhea and six normal horses. Lymphocytes were separated from the blood by Ficoll-isopaque gradient as previously described (20). The mononuclear cells obtained from the blood samples were washed three times in TC medium 199. For culture, TC medium 199 was supplemented with 0.02 M L-glutamine, penicillin (100 U/ml; E.R. Squibb and Sons, Inc.), streptomycin sulfate (100 μg/ml; Pfizer Laboratories Division, New York), and 10% fetal bovine serum (Flow Laboratories, Rockville, Md.). The concentration of cells was adjusted so that each culture contained 2 x 10⁶ lymphocytes in 2 ml of the medium. Two sets of quadruplicate cultures were prepared from each of the blood samples. One quadruplicate culture from each horse was not stimulated with mitogen and served as a control. Another quadruplicate culture was stimulated with PHA (0.01 ml/culture; content of the vial with PHA-M lot 0528-56, control 585786, Difco Laboratories, was resuspended in 5 ml of distilled water). All cultures were incubated for 72 h in an atmosphere of 5% CO₂ in air, and 1 μCi of [³H]thymidine (Schwarz/Mann, Orangeburg, N.Y.) with specific activity 3 Ci/mmol was added to each culture tube 18 h before harvesting.

**Statistical analysis.** The concentration of IgG and IgA, dermal response, and index of stimulation, represented by the ratio of disintegrations per minute in stimulated and nonstimulated cultures, were expressed as the arithmetic mean with the standard error. The significance of difference between PHA-stimulated and nonstimulated lymphocyte cultures of sick, recovered, and normal horses was determined by separate analysis of variance for each of the groups (4). The Student t test was used to determine significant difference in the immunoglobulin level and in dermal response to irritants (19).

**RESULTS**

**Level of immunoglobulins.** The level of IgG in sera from sick horses was higher (P < 0.05) than the level of IgG in sera of normal horses (Fig. 1). In contrast, the average level of IgA in the sera of sick horses was about 50% lower than in the sera of normal horses (Fig. 2). However, one of the 12 sick horses had a level of IgA in the same range as the normal horses, and two of the 20 normal horses had a level of IgA in the same range as the sick horses.

There was no substantial difference in the level of IgG and IgA in serum taken from sick horses before, during, and after treatment with normal equine serum (Table 2). Furthermore, the level of immunoglobulin in sera collected from two sick horses up to 12 months after recovery was approximately the same as in sera collected before treatment. However, the level of IgA in sera and the concentration of total serum proteins in the sick horses tended to be slightly lower 3 days after oral administration of serum than before the treatment. This decrease was probably due to the rehydration of horses after treatment. In addition, the daily levels of IgG and IgA in serum from one normal horse during and after oral administration of serum were approximately the same in serum taken before treatment.

**Dermal response.** Four sick horses with severe diarrhea and four normal horses were injected intradermally with three saline extracts from different commercial horse feeds that intensified diarrhea in the sick horses. Dermal reactions to the extracts were not immediately observed in either the normal or sick
horses. However, skin reaction was gradually increased and reached the maximum response to each of these extracts at 5 h after injection and disappeared during 24 h in both normal and sick horses. This response was significantly greater in normal than in sick horses (Fig. 3). Intradermal injection of either SK-SD or PHA stimulated the greatest response at 24 h after injection. Dermal responses to these injections were very weak in sick horses, and in normal horses these responses were significantly greater (Fig. 4). Their responses on recovery were identical to those observed during illness.

**Response of lymphocytes to PHA stimulation.** The lymphocytes collected from the sick horses with severe diarrhea before treatment did not respond significantly to the stimulation with PHA. In contrast, the lymphocytes from normal horses responded very well to PHA stimulation. The lymphocytes of the recovered horses appeared to recover some ability to respond to PHA stimulation. However, their response was lower than the response of the lymphocytes from horses with mild diarrhea and of lymphocytes from normal horses (Table 3). The data were also analyzed by comparing mean indexes of stimulation among the groups of horses. Table 3 shows the mean indexes derived from individual indexes for each group of animals. The mean index of stimulation was lowest in the group of sick horses with severe diarrhea. It was about 2.5 times higher in the group of recovered horses, about 5 times higher in the group of horses with mild diarrhea, and about 7 times higher in the group of normal horses.

In addition, PHA stimulation of lymphocytes from two sick and two normal horses in sera from sick and normal horses and in fetal calf serum was measured. There were no significant changes in blast transformation of lymphocytes stimulated with PHA and incubated in either autologous, homologous, or heterologous serum. The variation among replicates from the same blood sample was smaller than among replicates from blood samples collected on different occasions from normal horses. The average index of stimulation with standard error of four replicates of the same blood sample was 11.72 ± 0.34, and the average index of replicates of the blood collected on five occasions was 12.95 ± 0.68 from one horse and 17.97 ± 0.72 from another.

![Figure 2](http://iai.asm.org/)

**FIG. 2. Average concentration of IgA in serum of 12 sick and 20 normal horses.**

<table>
<thead>
<tr>
<th>Class of Ig</th>
<th>First treatment</th>
<th>Second treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>IgA</td>
<td>179 ± 15</td>
<td>168 ± 15</td>
</tr>
<tr>
<td>IgG</td>
<td>2,460 ± 295</td>
<td>2,216 ± 215</td>
</tr>
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</table>

*a* Mean immunoglobulin levels are expressed in milligrams per 100 ml as determined by single radial immunodiffusion.

*b* Standard error of the mean.
Fig. 3. Dermal responses to each of the three saline extracts from different feeds A, B, and C in sick horses with severe diarrhea and normal horses. Extracts from Tippecanoe Horse Feed (A), Tippecanoe Calf Feed (B), and Ruff'n Redi (C). Each bar represents mean thickness of the skin ± standard error in four sick and four normal horses before (0 h) and 5 h after intradermal injection.

Fig. 4. Dermal responses to either streptokinase-streptodornase (SK-SD) or phytohemagglutinin (PHA) in sick horses with severe diarrhea and normal horses. Each bar represents mean thickness of the skin ± standard error in four sick horses and four normal horses before (0 h) and 24 h after intradermal injection.

**DISCUSSION**

In this study, hypogammaglobulinemia A was observed in 11 of 12 horses with chronic diarrhea. These horses had about a 50% lower level of IgA in their sera than randomly selected normal horses. The horses with severe diarrhea had approximately the same level of immunoglobulin in sera as the horses with mild diarrhea. Only two normal horses had a level of IgA in the same range as the sick horses. These data suggest that among normal animals there are asymptomatic animals with a deficiency of IgA and that among sick horses there are some with a normal level of IgA. Nonetheless, a high degree of correlation between hypogammaglobulinemia A and chronic diarrhea in horses was observed. However, a large survey of a random population of horses is required to support this finding. In humans, an isolated deficiency of IgA has been reported with a frequency of 0.03 to 0.5% among individuals in a normal population survey (21). In contrast to hypogammaglobulinemia A, the level of IgG was higher in the sick than in the normal horses. A similar observation was reported by Bull and Tomasi (3) as a compensatory elevation of IgG or IgM in the IgA-deficient individuals.

All sick horses treated with two series of oral administration of normal horse serum recovered from diarrhea. In contrast, treatment with antibiotics and anti-diarrheal drugs was unsuccessful. Also, intravenous administration of normal horse serum to adult horses with chronic diarr-
rhea was unsuccessful (D. P. Gustafson and E. Page, unpublished data). Further detailed study on the concentration of the different immunoglobulins in feces, in intestinal secretion, and on the distribution of immunoglobulin-containing cells in the intestinal mucosa is required. Also, isolation and characterization of pathogens is necessary in sick horses before any conclusions can be made about the mechanism of action of oral administration of the serum from normal horses to horses suffering from chronic diarrhea.

The results of this study have shown that blood lymphocytes from all the sick horses with severe diarrhea did not respond to the PHA stimulation. In contrast, the lymphocytes from the blood of normal horses responded very well, and the lymphocytes from the blood of recovered horses gradually regained the ability to respond to the PHA stimulation. Similar PHA responsiveness of lymphocytes from humans with neoplastic diseases or with leprosy has been demonstrated. A depression in response of lymphocytes was correlated with poor prognosis and a recovery of PHA responsiveness was reported in patients with tumor regression (10) or in the drug-arrested stage of the lepromatous leprosy (5). Furthermore, lymphocytes from patients with localized neoplastic disease reacted to PHA in the same way as lymphocytes from normal individuals (14). In this study, analogical observations were made that the lymphocytes from horses with mild diarrhea responded to PHA only slightly less than lymphocytes from normal horses. This may suggest that decreased capacity to respond to PHA in vitro is probably associated with a decrease in cell-mediated immunity (11) since PHA stimulation of lymphocytes reflects the immunological status of thymus-dependent lymphocytes (15).

Intradermal injections of saline extracts from horse feeds that intensified diarrhea in sick horses did not induce immediate skin reactions in sick or normal horses. However, these extracts induced about a 50% greater dermal response at 5 h after injection in the group of normal horses than in the group of sick horses. These data may suggest that the inflammatory responsiveness to exogenous material in the sick horses was depressed as compared with normal horses.

The delayed skin reaction to the injection of both SK-SD and PHA in sick horses was very weak, and in normal horses it was significantly greater than in sick horses (Fig. 4). This observation was correlated with PHA unresponsive-ness of lymphocytes from sick horses or good PHA responsiveness of lymphocytes from normal horses in vitro. A similar correlation was reported in foals (9), children, and in some cases adults (1). The horses that recovered from severe diarrhea only partially regained the ability to respond to PHA stimulation in vitro, whereas the dermal response remained the same. It is possible that the small increase in dermal response was more difficult to demonstrate than the increase in PHA responsiveness of lymphocytes from recovered horses or that different cell populations may be involved in these two processes (2).

Previously, McGuire et al. (7–9) described the combined immunodeficiency in foals characterized by an absence of T and B lymphocytes with a resultant lack of cell-mediated immunity and antibody production. The data of this study may suggest a complexity of immunological deficiencies in horses with chronic diarrhea characterized by hypogammaglobulinemia A, decrease in cell-mediated immunity, and reduction in inflammatory responsiveness. Furthermore, it seems that an increase in cell-mediated immunity plays a crucial role in the recovery process. Lymphocytes from recovered horses regained PHA responsiveness, whereas the low level of IgA in their sera and reduction in inflammatory responsiveness remained the same.

LITERATURE CITED