Serum Antibody Response to *Listeria monocytogenes*, Listerial Excretion, and Clinical Characteristics in Experimentally Infected Goats

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We induced an experimental listeriosis in five goats by oral inoculation of *Listeria monocytogenes* serovar 1/2a. The resulting generalized bacteremia was reflected by systemic illness in four of the five animals inoculated and by excretion of *L. monocytogenes* in milk by all five animals. By using an enzyme immunoassay, we recorded a markedly enhanced immunoglobulin G (IgG) antibody response in the two youngest and most seriously ill test animals. In these, the elimination of *L. monocytogenes* from the gastrointestinal tract occurred simultaneously with the development of the highest antibody levels at 14 to 15 days postinoculation. In the case of the oldest test animal, a preexisting, persistent IgG antibody response was recorded which was associated with the total absence of clinical symptoms and the shortest observed fecal carriage of *L. monocytogenes*, lasting for only 3 days. Two animals remained practically seronegative, and an IgM antibody response was not recorded for any of the animals. The findings suggest that an association exists between the humoral immune defense against *Listeria* infections, the clinical course of the infection, and the elimination of the *Listeria* organisms from the gastrointestinal tract.

*Listeria monocytogenes* is a facultative intracellular microorganism, and the essential role of cell-mediated immunity in listerial infections is well documented (10, 17, 20). The humoral immune response to facultative intracellular bacteria is generally considered ineffective, but the specific role of antilisterial humoral immunity is still incompletely understood. Serological tests, such as the most widely used agglutination methods and the complement fixation test, lack the sensitivity to detect the weak antibody response frequently seen in confirmed listerial infection. As such, they are unsatisfactory both in experimental studies and as diagnostic tests for listeriosis.

Most of the experimental studies with *Listeria* have been carried out with mice, in which listeriosis differs in several aspects from that occurring in both domestic animals and humans (7, 17). Moreover, most of the previous experiments have been obscured by the use of inoculum sizes and routes not comparable with natural infection. Therefore, the time course of the antibody response to *L. monocytogenes* and its relation to the course of and sequelae to naturally occurring listeriosis are poorly characterized.

The present work was undertaken to study the humoral immune response to *L. monocytogenes* in goats experimentally infected with the organism. Natural listeriosis in domestic animals is most common among ruminants (3, 8), and the overt disease in these animals bears close resemblance to human listeriosis. With respect to the firmly established role of *L. monocytogenes* as a foodborne pathogen (6, 13, 23), the oral route was used for inoculation. The serum antibody responses were determined with an enzyme immunoassay, and the relations between the antibody levels, the clinical characteristics, and the fecal carriage were determined.

MATERIALS AND METHODS

**Animals.** Five nonpregnant dairy goats of Finnish origin were used as test animals. The characteristics of the animals are shown in Table 1. Before the inoculation, all goats were observed for 2 weeks, during which the milk and fecal samples were examined for *Listeria* spp. twice a day. The animals were housed separately in pens from which the fecal material and litter were cleaned daily. Hay and oats used for feeding were monitored for the presence of *L. monocytogenes* twice a month.

**Inoculum.** A serological reference strain of *L. monocytogenes* serovar 1/2a (SLCC 2371) was used. The strain was obtained from the Special Listeria Culture Collection of the Institute of Hygiene and Medical Microbiology, University of Würzburg, Würzburg, Federal Republic of Germany.

Before the experimental infection of the goats, the virulence of the infecting strain was restored by a passage through mice. The virulent strain was then cultured in tryptose broth (Difco Laboratories, Detroit, Mich.) at 37°C for 18 h. The culture was sedimented by centrifugation (6,000 × g for 15 min) and suspended in 0.01 M phosphate-buffered saline solution (PBS), pH 7.2, to the targeted inoculum level of 10⁹ CFU/ml. The number of viable organisms was determined by duplicate plating on tryptose agar (Difco). Each animal was dosed perorally with 6 × 10⁹ *L. monocytogenes* cells in 2 ml of PBS. The inoculum was administered with a tube into the esophagus.

**Clinical examination.** The animals were observed and their rectal temperatures were recorded three times a day for the first week postinoculation and once a day for a total of 6 months thereafter. Fecal samples were obtained from the rectum and were cultured for *Listeria* spp. daily for the first 4 months and once a week thereafter. The animals were milked daily, the milk yield was weighed, and cultures for *Listeria* spp. were made daily for the first 4 months and once a week thereafter. Blood samples for the serum antibody

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TABLE 1. Clinical characteristics of the test animals used for experimental listeriosis

<table>
<thead>
<tr>
<th>Goat no.</th>
<th>Age (yr)</th>
<th>No. of past deliveries</th>
<th>Duration of fever (days)</th>
<th>Duration (in days) of listerial shedding in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Feces</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>5</td>
<td>0*</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>5*</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>27*</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

* Goats were inoculated orally with $6 \times 10^9$ L. monocytogenes serovar 1/2a.
* No fever observed postinoculation.
* Animal received 1,200,000 U of procaine penicillin intramuscularly on day 5 postinoculation.
* Undulant fever with temperatures ranging from 38.8 to 40.1°C.

Determinations were obtained daily for the first 5 days postinoculation, twice a week for the first month postinoculation, once a week for the following 2 months, and at 2-week intervals for a total of 6 months thereafter.

**Bacteriological examination.** The occurrence of L. monocytogenes in the milk and feces was determined by direct plating on selective media and by a selective enrichment procedure. For quantitative analysis, the milk and fecal samples were serially (1:10) diluted in PBS, pH 7.2, and each dilution was plated in duplicate on Oxford agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). The plates were incubated at 37°C for 48 h, and the colonies typical of listeriae were counted. The milk samples were enriched by a modified procedure of Lovett et al. (16) by adding (per liter) 10 mg of acriflavine hydrochloride, 40 mg of naldixic acid, and 50 mg of cycloheximide to the enrichment broth. The incubation time was 48 h at 30°C. The fecal samples were enriched according to the procedure of McClain and Lee (18). After the enrichment procedures, the samples were cultured on Oxford agar (Oxoid Ltd.) as described above. The positive isolation of L. monocytogenes was verified biochemically as described by Seeleger and Jones (25) and serologically by using tube agglutination with Listeria type O1 antisera (Difco).

**Preparation of antigen.** L. monocytogenes (1-liter culture) grown in tryptose broth (Difco) at 20°C for 18 h was harvested by centrifugation at 5,000 x g for 30 min, washed twice in PBS, pH 7.2, and resuspended in 5 ml of PBS. The cells were lysed by periodic ultrasonic treatment on ice with a Vibra-Cell sonicator (Sonic & Materials, Inc., Danbury, Conn.) at full power for 10 min and centrifuged (5,000 x g for 30 min) to clear remaining whole bacteria. The supernatant was dialyzed against PBS for 18 h at 4°C, and the resulting antigen was stored at -20°C.

**Antibody determination.** Tests were done on microdilution plates (Linbro Microtiter multiwell plate; Flow Laboratories, Inc., McLean, Va.). The plates were sensitized with the antigen diluted 1:100 (30 μg/ml) in PBS, pH 7.2, by overnight incubation at 37°C. The plates were then washed with PBS containing 0.5% (vol/vol) Tween 20. The serum samples were serially diluted in PBS containing 5% (vol/vol) fetal calf serum and 0.01% (vol/vol) Tween 20 and NaCl (final concentration, 0.5 M). They were then incubated in 100-μl volumes per well for 90 min at 30°C. After washing, 100 μl of rabbit anti-goat immunoglobulin G (IgG) or IgM serum (RAG/IgG H+L or RAG/IgM Fc; Nordic Immunology, Tilburg, The Netherlands) diluted in the PBS diluent mentioned above was added to each well. After an incubation of 45 min at 37°C followed by washing, horseradish peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins (DAKO-Immunoglobulins A/S, Copenhagen, Denmark) diluted in the PBS diluent were added (100 μl per well). The incubation time was 60 min at 37°C. Then, after washing, α-phenylenediamine at a concentration of 0.4 mg/ml in 0.1 M citrate-Na2HPO4 buffer, pH 5.5, activated by 0.002% (vol/vol) H2O2, was added. After 30 min of incubation at 37°C, 2 M H2SO4 was added to stop the reaction. The color reaction was measured with a spectrophotometer (Titertek Multiscan; EFLab, Helsinki, Finland) at 492 nm. The antibody titers were determined as the highest dilutions of the sera showing twice the absorbance values of the corresponding preinoculation sera in the enzyme immunoassay. Normal goat serum (batch no. 08B-121; Janssen Life Sciences Products, Olen, Belgium) was included as a standard in each assay to control the interassay variation.

**RESULTS**

Four of the five goats developed a febrile illness within 36 h postinoculation (Table 1). The highest rectal temperatures, ranging from 41.0 to 41.8°C, were observed at 1 and 2 days postinoculation. Poor appetite in these animals resulted in hypogalactia from 3 days postinoculation, reducing the milk yield for approximately 2 weeks. The most severe infections occurred in the youngest, primiparous test animals, goats 3 and 4. Because of critically severe infection, goat 3 received 1,200,000 U of procaine penicillin intramuscularly at 5 days postinoculation. In the other animals, no such intervention was needed and there was complete clinical recovery in all animals. In goat 4, however, an undulant fever continued for a total of 4 weeks (Table 1), with temperatures ranging from 38.8 to 40.1°C.

Goats 2 and 3, with four and two past deliveries, respectively, developed only mild clinical symptoms 2 to 4 days in duration. No fever, other clinical signs of infection, or decrease in daily milk yield was recorded in the oldest test animal, goat 1, which had had five past deliveries.

Within 24 h postinoculation, all animals started to excrete L. monocytogenes in milk, indicating a bacteremic infection. The excretion in milk lasted for 2 days postinoculation in the youngest animals, goats 3 and 4, and for 1 day in the others. The colony counts of L. monocytogenes in the milk ranged from $10^2$ to $10^4$ CFU/ml.

Three different patterns of serum IgG antibody response were observed (Fig. 1). The youngest test animals, goats 3 and 4, developed an enhanced antibody response. The antibody levels started to rise from day 6 postinoculation, and the highest enzyme immunoassay titers of $10^4$ to $10^5$ were reached at 14 to 15 days postinoculation. Therefore, the antibody titers continued to fall but reached the preinoculation titers only 2 months postinoculation. The oldest test animal, goat 1, which lacked clinical symptoms, was found to be seropositive from the onset of the infection. Besides slight initial fluctuation in the antibody levels at 2 to 5 days postinoculation, no major alterations in the antibody levels occurred in this animal. Goats 2 and 5 remained practically seronegative in the enzyme immunoassay throughout the clinical infection and thereafter. An IgM response was not detected in any of the animals.

The duration of the postinoculation fecal carriage of listeria and the numbers of L. monocytogenes cells isolated from the feces are shown in Table 2. The longest carriage periods of 14 to 15 days postinoculation were observed in the...
youngest animals, goats 3 and 4, in which enhanced antibody responses were recorded. The disappearance of *L. monocytogenes* from the feces of these animals occurred simultaneously with the reaching of the highest antilisterial antibody titers in serum. The shortest fecal carriage period of only 3 days was observed for the oldest test animal, goat 1, which was found to be initially seropositive. The serologically weak or nonresponding animals, goats 2 and 5, carried *L. monocytogenes* for 10 and 7 days, respectively.

**DISCUSSION**

In the present study, an experimental listeriosis was induced in goats by an oral inoculation of *L. monocytogenes* serovar 1/2a. Foodborne infection is considered the major mode of natural listeriosis transmission in both humans and animals (22, 28). The majority of cases are caused by serovars 1/2 and 4b (24), with serovar 1/2a being the most common serovar among animal strains in Finland (12). The size and variation of the infective dose are still largely unknown, although in some cases of human listerial food poisoning the contamination level of the consumed food has been determined (2, 11). High numbers of *L. monocytogenes*, comparable with the dose used in the present study, have been detected in contaminated silage associated with listeriosis in ruminants (5). Before the current experiment, the infective potential of the dose was pretested in two adult goats. Generalized bacteremia after dissemination of the organisms was reflected by systemic illness in four of the five animals inoculated and by excretion of *L. monocytogenes* in milk by all five animals. Since the incubation period and the clinical signs of the test animals used here were similar to those reported for natural outbreaks of listeriosis in goats (15), the current findings should be relevant to some forms of naturally occurring listeriosis.

The most severe infections occurred in the youngest test animals in the present study. In general, young animals appear to be more susceptible to *Listeria* infection than old animals (8). The increased capacity of adults to withstand listeriosis has been associated with the activity of the cell-mediated immune response (4). Moreover, age-related differences in the normal microbial flora of the gastrointestinal tract are probably involved (19, 29). In the current series consisting of five adult goats, we recorded a markedly enhanced IgG antibody response for the two youngest animals, whereas the oldest animal was initially seropositive and remained seropositive throughout the study. The preexisting antibodies in serum were associated with rapid clearance of *L. monocytogenes* from the gastrointestinal tract and the absence of clinical symptoms, suggesting that they were related to or even contributed to the resistance against the organism. In the two youngest animals, a similar association was found between the formation of high levels of specific IgG antibodies in serum and the disappearance pattern of *L. monocytogenes* from the feces. These findings suggest that humoral defense is involved in the elimination of *Listeria* infections.

The lack of severe clinical signs and the relatively short duration of fecal shedding in two principally seronegative animals indicate the major role of cell-mediated defenses. However, the possible role of secretory antibodies was not addressed in the present study and calls for further investigation. The possibility of an effective secretory antibody response even in the absence of detectable serum antibody response cannot be excluded. In ruminants, the secretory antibodies belong mainly to the IgG1 isotype (27).

The absence of detectable IgM antibodies, which are usually produced during primary immune response, may indicate previous contacts with *listeriae* or antigenically related bacteria. The predominance of IgG antibody response in experimental listeriosis of adult sheep has been previously demonstrated by Aalund et al. (1).

The implication of the persistent high levels of antilisterial antibodies in serum demonstrated for one goat in the present study is not clear. Acquired immunity to facultative intracellular parasites is generally short lived (27), and the enhanced cellular and humoral reactions are generally thought to persist only as long as viable organisms remain in the body. Persistence of infection is common with certain other intracellular bacteria, such as *Brucella abortus* and *Mycobacterium bovis* (26). In *Brucella* infections, high titers of IgG antibodies without opsonizing activity have been
suggested to indicate chronicity of the infection (21). In this study, however, no preinoculation fecal carriers of listeriae were detected, and the possibility of an extraintestinal preexisting infection remains questionable.

Although healthy animals may be carriers of listeriae when exposed to these bacteria through feed, the carriage is much more common after overt listeriosis (9, 14; R. G. Dijkstra, Ph.D. thesis, Rijksuniversiteit, Utrecht, The Netherlands, 1965). In the present study, however, the clinical recovery of the animals was associated with the rapid disappearance of the *Listeria* organisms from the feces. Therefore, the fecal shedding seems to be of short duration and should be considered to indicate a reinfection of the animal rather than a chronic carrier state.

In conclusion, the results of the present study suggest that antibodies, in addition to cell-mediated mechanisms, are implicated in the resistance against listerial infections. In some cases of clinical listeriosis, the serum antibody levels reflect the clinical course of the infection and are associated with the elimination of *L. monocytogenes* from the gastrointestinal tract. The possible role of secretory antibodies in the immune defense against listeriosis needs more detailed investigation. The diversity in the serum IgG antibody response in generalized listeriosis, the absence of an IgM antibody response, and the possibility of a persistent serum IgG antibody response without a detectable carrier state decrease the diagnostic value of the enzyme immunoassay technique described here. Further studies of the antigenic specificity and determinants of the persistent and temporary IgG antibody responses are required.

**LITERATURE CITED**


