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Kinetics of Antibody Production against Listeriolysin O in Sheep with Listeriosis

SYLVIE LHOPITAL,[1] JOSÉ MARLY,[2] PIERRE PARDON,[2] AND PATRICK BERCHE[1]*

Laboratoire de Microbiologie, Faculté de Médecine Necker-Enfants Malades, 156, rue de Vaugirard, 75730 Paris Cedex 15,[1] and Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, Centre de Tours, 37380 Nouzilly,[2] France

Received 30 October 1992/Accepted 16 March 1993

The kinetics of antibody production against listeriolysin O (LLO), a major virulence factor of the intracellular bacterial pathogen *Listeria monocytogenes*, was studied by dot blot analysis with highly purified LLO during oral infection of sheep. Specific antibodies appeared as soon as day 9 of an oral infection and peaked by day 20 of infection; specific antibody levels then remained almost stable for at least 4 months. A subclinical infecting dose ($\sim 10^6$ viable bacteria) was capable of eliciting a significant antibody response to LLO, almost at the same level as that observed with a high-dose oral challenge ($\sim 10^{10}$). Antibodies to LLO were mostly constituted by immunoglobulin G (IgG), since an IgA response was not detectable and only a transient and inconstant IgM response was observed between day 9 and day 20 of an oral infection. These results show that antibodies to LLO are constantly produced during oral infection even with a low infecting dose, thus confirming that LLO is highly immunogenic. Detection of antibodies to LLO can therefore be used to detect sheep that have been previously exposed to *L. monocytogenes*.

Listeria monocytogenes is a gram-positive intracellular bacterium that is responsible for severe infections in a large array of animal hosts, including humans (11). This ubiquitous pathogen is usually transmitted via contaminated food (5). The virulence of this pathogen is based on its capacity to survive and to multiply in host cells (18). It is established that intracellular bacteria escape the phagolysosomal compartment to gain access to the cytosol (6, 13). Genetic evidence (7, 14, 21) indicates that this evasion is due to the secretion of listeriolysin O (LLO), a 56-kDa protein (9) belonging to the group of SH-activated exotoxins produced by all pathogenic strains of *L. monocytogenes* (10, 15). LLO therefore appears to be a crucial virulence factor which also induces T-cell recognition during the course of an acute infection in the mouse (3). Indeed, there is evidence that a specific epitope, LLO 91-99 peptide, is the target for $CD8^+$ T cells (12, 20) and that LLO might be a dominant target of the immune T cells (1). This is in agreement with a cytosolic pathway of presentation of LLO epitopes following intracytoplasmic production of this exotoxin in antigen-presenting cells (23).

Although the humoral immune response in animals with listeriosis is not involved in acquired immunity (18), it has been shown in human patients that LLO is also the target for specific antibodies (4), thus indicating that activated macrophages that destroy the bacteria can process LLO through the lysosomal class II-dependent pathway of antigenic presentation (23). This observation has been used to design a useful serodiagnostic method for patients with listeriosis (4), especially when bacteria cannot be isolated from clinical specimens (8). This is the case, for example, for pregnant mothers or for some human patients with encephalitis because of the intermittent presence of bacteria in the blood or the inaccessible foci of bacterial replication. However, more information is needed to interpret this new serodiagnostic method since nothing is known about the level of induction or the kinetics of this antibody response after oral contamination. We investigated this question with sheep, because listeriosis is an important problem in these animals, which experience severe meningoencephalitis epidemics and spontaneous abortions as a result of infection with *L. monocytogenes*. The kinetics of antibody production to LLO was therefore studied during the course of an experimental oral infection in sheep.

MATERIALS AND METHODS

Infection of sheep. Male "Pré-Alpes du Sud" lambs (ages, 2 to 3 months) bred at the Institut National de la Recherche Agronomique station (Nouzilly, Tours, France) were orally infected with various doses of *L. monocytogenes* F13, a spontaneous mutant resistant at a high level to spectinomycin (>1,000 μg/ml) derived from strain LO28 (22). F13 expressed the same level of virulence in mice as that expressed by LO28 (20a). Animals were then monitored for 128 days, when they were sacrificed. The sheep's rectal temperatures were determined every day for 10 days and then twice a week. At the same time, the presence of *L. monocytogenes* in bacteriological samples (stool, blood, and buccal and nasal swabs) was determined on specific culture media. The following liquid and solid media were used for the stools and buccal and nasal specimens: (i) agar selective medium, which consisted of 40 g of trypticase soy agar (bioMérieux, Marcy-l'Etoile, France), 1 g of esculin (Sigma Chemical Co., St. Louis, Mo.), 0.5 g of iron ammonium chloride (Sigma), 15 g of lithium chloride (Sigma), 400 mg of cycloheximide (Sigma), 5 mg of acriflavine (Sigma), 2 mg of cefotetan (Apacef; ICI Pharma), 10 mg of fosfomycin (Sigma), and 400 mg of spectinomycin (Sigma); (ii) liquid enrichment broth, which consisted of 5 g of proteose peptone 3 (Difco Laboratories, Detroit, Mich.), 5 g of tryptone (Difco), 5 g of beef extract (Oxoid), 5 g of yeast extract (Difco), 20 g of sodium chloride, 12 g of $Na_2HPO_4 \cdot 2H_2O$, 1.35 g of KH_2PO_4, 1 g of esculin, 12 mg of acriflavine, and 20 mg of nalidixic acid (Sigma). *L. monocytogenes* was quantified in

* Corresponding author.

feces by surface plating 10-fold dilutions in 0.15 M NaCl on selective agar medium and incubation at 37°C for 3 days. Hemocultures were made on brain heart infusion broth (bioMérieux), and hemocultures were incubated at 37°C for 5 days.

Purification of LLO. Highly purified LLO was obtained by a new purification procedure with preparative isoelectric focusing, as follows. Briefly, 10 ml of an overnight culture of strain EGD (7) in brain heart infusion broth was grown at 37°C in 1,000 ml of charcoal-treated medium (10). After a 12-h incubation, the bacteria and charcoal were removed by centrifugation at 5,000 × *g* for 20 min. The supernatant was filtered through a 0.22-μm-pore-size filter (Nalgene Co., Rochester, N.Y.) and was concentrated by ultrafiltration at 4°C in an stirred cell apparatus (Amicon, Lexington, Mass.) equipped with an Amicon YM 10 membrane to eliminate material with M_rs of less than 10,000. The crude concentrate, adjusted to pH 6.0, was passed twice through a DEAE column, which was prepared as follows. An agarose gel containing DEAE exchange groups (Bio-Rad Laboratories, Richmond, Calif.) was poured into a Pharmacia column (2.5 by 30 cm) and was equilibrated at pH 6.0 by washing with 100 ml of buffer A (0.005 M Tris-HCl buffer [pH 6.0]). Proteins with a pI of <6.0 were retained by DEAE exchange groups, whereas the majority of LLO (pI 6.7) was eluted. The resulting effluent, containing LLO, was concentrated to approximately 10 ml on a YM 10 membrane (Amicon). The final step of purification was a preparative isoelectric focusing in a Rotofor cell (Bio-Rad). Rotofor runs were performed by using 2% Biolyte ampholytes (pH 5 to 7; Bio-Rad) and 10% (vol/vol) glycerol in a total volume of 45 ml. To minimize protein precipitation, the Rotofor cell was prefocused by using 12 W of constant power at 4°C for 1 h to establish the pH gradient before injecting the protein sample. The initial conditions of focusing were 30 mA and 360 V, and then focusing continued for another 3 h at 17 mA and 645 V. Fractions were harvested and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (mini-gels); this was followed by Western blot (immunoblot) analysis by using as a control a rabbit anti-LLO polyclonal antibody, as described previously (9). Fractions containing LLO were pooled and dialyzed overnight against 100 volumes of phosphate-buffered saline (PBS; 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer salts [pH 7.2]) supplemented with 0.5 M NaCl to remove ampholytes, and finally, aliquots were stored at −80°C.

Titration of antibodies against LLO. Serum samples were obtained from sheep before infection and regularly during the entire course of infection. Aliquots were stored at −80°C until required. Antibodies to LLO were titrated by dot blot analysis with highly purified LLO as antigen, according to a procedure previously described in detail (4). Briefly, 0.1 μg of purified LLO was absorbed onto 0.1-μm-pore-size nitrocellulose filters (Schleicher & Schuell, Dasnel, Germany) with a dot blot SF apparatus (Bio-Rad). Filters were then incubated for 1 h at 22°C with PBS–10% Tween 20 supplemented with 5% (wt/vol) skim milk (Regilait; France-lait, Saint Martin-Belle Roche, France) and then incubated for 1 h at 22°C with dilutions of lamb sera ranging from 1/100 to 1/10,000. After extensive washings in PBS–0.15% Tween 20, filters were incubated for an additional 1 h at 22°C with a peroxidase-labelled affinity-purified rabbit anti-sheep immunoglobulin G (IgG; Fc fragment, γ-chain specific; Cappel, Malvern, Pa.) or anti-sheep IgM (Fc fragment, μ-chain specific; Cappel) diluted 1:500. We also used a mouse IgG1 monoclonal antibody anti-sheep IgA (M_cM_{10}), obtained from K. J. Beh (Glebe, Australia), which was described previously (2). This ascitic fluid was diluted 1:10,000 in buffer. After the washings, the enzymatic activity was detected with 0.06% 3,3′-diaminobenzidine tetrachloride (Sigma)–0.075% hydrogen peroxide. The titer was the highest dilution that gave a visible precipitation on nitrocellulose filters.

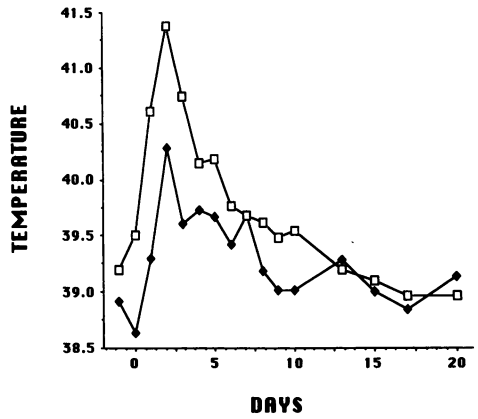

FIG. 1. Rectal temperatures of lambs orally infected with *L. monocytogenes*. ◆, 6 × 10^6; □, 6 × 10^{10}.

RESULTS AND DISCUSSION

Groups of five lambs each were orally infected with either 6 × 10^6 or 6 × 10^{10} *L. monocytogenes*. No clinical signs were observed in any lamb receiving the low or the high infecting dose (6 × 10^6 or 6 × 10^{10}), including the absence of diarrhea. However, the mean rectal temperature of the lambs increased significantly by days 1 to 2 of infection, particularly in lambs infected with the highest infecting dose. After this early peak, the temperature curves returned to normal within 8 to 10 days (Fig. 1). Bacteriological study revealed that samples from lambs infected with 6 × 10^6 *L. monocytogenes* were all negative (stools, hemocultures, and nasal and buccal swabs), even after culture enrichment. In contrast, *L. monocytogenes*-positive cultures were found in lambs infected with 6 × 10^{10} *L. monocytogenes*. This pathogen was isolated from nasal and buccal samples from four of five lambs at a low rate (<20 colonies per sample) during a short period of time (days 0 to 5), indicating that *L. monocytogenes* did not efficiently colonize the oropharynxes of infected lambs. With regard to the coprocultures, *L. monocytogenes* was isolated in low numbers from the feces during the first 4 to 5 days of infection. The amount of *L. monocytogenes* did not exceed $10^{2.5}$ bacteria per g (except for the feces of one lamb at day 1, which contained $10^{3.6}$ bacteria per g). Another lamb in this group had positive hemocultures every day between days 7 and 10 at significantly higher levels ($10^{3.2}$ to $10^{4.7}$ *L. monocytogenes* per g). The reemergence of *L. monocytogenes* in the feces of these lambs strongly suggests the occurrence of a septicemic dissemination with hepatic shedding of bacteria to the gut. After this phase, *L. monocytogenes* was never again found in multiple bacteriological samples. Unexpectedly, one lamb of this group died by day 60. Postmortem samples (including brain, liver, and spleen) were negative for *L. monocytogenes*. This death was therefore apparently unrelated to the experimental infection.

The kinetics of IgG anti-LLO titers observed in the two

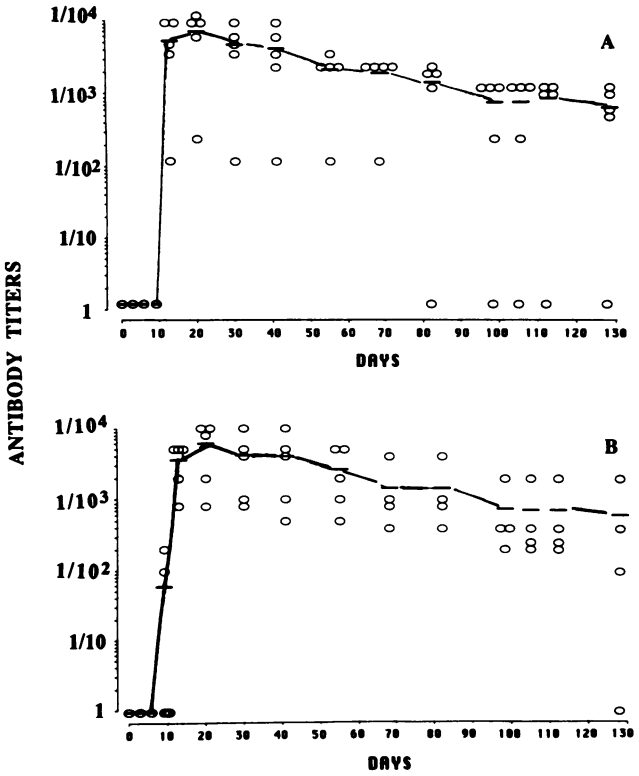

FIG. 2. Kinetics of anti-LLO antibodies in lambs orally infected with 6×10^6 (A) or 6×10^{10} (B) *L. monocytogenes*.

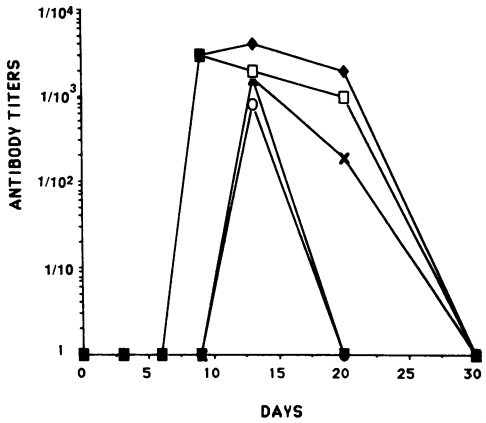

FIG. 3. Individual kinetics of IgM anti-LLO antibodies in orally infected lambs. ◆, ×, ▼, 6×10^6; ○, □, 6×10^{10}.

groups of orally infected lambs is illustrated in Fig. 2. Antibodies to LLO were not detectable in any serum sample obtained 1 day before infection. It was found that antibodies to LLO appeared as soon as day 9 of infection in two lambs receiving 6×10^{10} *L. monocytogenes*, although they were present at low titers (1:100 and 1:200) at this early stage of infection. Most infected lambs produced detectable IgG antibodies to LLO by day 13, with titers ranging from 1:100 to 1:8,000. Then, IgG anti-LLO titers reached a peak by day 20 which was followed by a plateau, with a slow decline over the next 4 months. By day 128, the anti-LLO titers ranged from 1:100 to 1:2,000 except in one lamb infected with the high-dose challenge; no antibodies to LLO were detectable in that lamb (Fig. 2). We also found that a low infecting dose (6×10^6 *L. monocytogenes*) was surprisingly sufficient to trigger the production of IgG antibodies to LLO, which were present at almost the same level as that seen for a high bacterial challenge (Fig. 2). Although colonization with *L. monocytogenes* could not be bacteriologically documented, even with liquid enrichment medium, this observation strongly suggests that an active, replicative, subclinical infection occurred in the intestinal tracts of these lambs. This might also explain the presence of IgG antibodies to LLO in about 15% of healthy persons (4) who might have been orally infected with low infecting doses of *L. monocytogenes* associated with contaminated food but who have no clinical symptoms.

Although the kinetics of the humoral immune response observed in the sheep cannot be directly extrapolated to infections in humans, we previously found that the IgG anti-LLO titers were higher in sheep than in humans, in whom they did not exceed 1:800 (4). However, in our previous work (4), most patients were immunocompromised and the infecting dose was unknown. The fact that antibodies to LLO were detectable as soon as the clinical onset of human listeriosis (4) might indicate that patients were contaminated at least 10 to 14 days before, as indicated by the present data. As in humans, IgG anti-LLO levels persisted for several months in lambs (Fig. 2). Low and Donachie (17) have shown that the IgG anti-LLO predominantly belongs to the IgG1 subclass during listeriosis in sheep, as observed in humans (2a).

The production of IgA and IgM anti-LLO antibodies in the sera of infected lambs was also studied. We could not detect IgA anti-LLO antibodies in any serum sample during the entire course of infection. In contrast, IgM anti-LLO antibodies were transitorily found, between days 9 and 20, in three of five lambs that received 6×10^6 *L. monocytogenes* and one of five lambs that received 6×10^{10} *L. monocytogenes*. The kinetics of IgM production in these animals is illustrated in Fig. 3. Anti-IgM was found at titers of as high as 1:4,000 but disappeared very rapidly after days 13 to 20 of infection. This confirms the data in a previous report on infected lambs (17) but is in contrast to our own observations in humans, in whom IgM remained undetectable (4). To explain this discrepancy, it can be hypothesized that the transient IgM antibody response might occur in humans before the clinical onset of listeriosis or might be rarely induced in immunocompromised patients.

The results presented here indicate that antibodies to LLO are consistently produced during the course of oral listeriosis, in contrast to the variable antibody response against crude *Listeria* antigens, as observed in goats (19) and humans (4). These results are in agreement with a recent observation showing that antibodies to LLO can be successfully detected in the sera of silage-fed sheep among which listeric enteritis and spontaneous abortions occurred (16). These findings confirm that LLO is highly immunogenic and induces a strong humoral immune response during infection, even when animals were infected with subclinical infecting doses of as low as 6×10^6 *L. monocytogenes*. The knowledge of the kinetics of antibodies to LLO will be helpful for interpreting the serodiagnosis in patients and for studying the exposure of human or animal populations to *L. monocytogenes*.

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