INFECTION AND IMMUNITY, June 1977, p. 923-927
Vol. 16, No. 3

Printed in U.S.A.

Kinetics and Maintenance of Acquired Resistance in Mice to *Listeria monocytogenes*

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Received for publication 3 February 1977

In the mouse system, acquired resistance to *Listeria monocytogenes* can only be demonstrated after immunization with viable microorganisms. A successful state of immunity cannot be elicited with formalin-killed organisms or bacterial cell-derived products. Viable, serologically cross-reactive organisms (not mouse pathogenic) do not induce a state of immunity as measured by acquired resistance. The duration of immunity, once established, is dose independent, and the absolute interval of its existence is not extended by secondary challenge with large numbers of viable organisms. The decline of immunity in actively immunized animals is not altered by antigenic challenge with formalin-killed cells or cell products. This indicates that the cellular requirements for the development of host resistance are similar for induction as well as maintenance. In vitro measurements of cellular immunity by migration inhibition indicate that formalin-killed organisms as well as cell products were recognized by actively sensitized lymphocytes obtained from immune animals.

Acquired resistance in mice to infection with the facultative intracellular parasite *Listeria monocytogenes* is mediated by immunologically committed lymphocytes that function in the activation of macrophages essential for the expression of immunity (3-5, 7). That resistance to *L. monocytogenes* is not dependent on humoral antibodies is established by the fact that neither passive transfer of immune serum nor production of high-titer circulating antibodies induced by killed vaccines protects an animal against infection by *L. monocytogenes* (6).

After an immunizing infection in mice with viable *L. monocytogenes*, two immunologically distinct but important events are generated: a short-lived state of immunity essential for the rapid removal of the infecting microorganism from affected tissues and a long-term cellular immunity enabling the host to more efficiently defend against a secondary infection (10, 11). The cells important in the establishment of acquired resistance in mice to *L. monocytogenes* are thymus-derived lymphocytes (3, 13, 14).

Stimulation of immunologically committed lymphocytes is generated in the spleen 2 days postinfection; peak production is reached by day 6 and then declines rapidly after apparent removal of infecting microorganisms from affected tissue (9). Thus, although immunity in the *Listeria* system is apparently cell mediated, the control of this response limits its expression to a short time interval.

This report investigates two aspects associated with immunity in mice to *L. monocytogenes*: first, the decay rate of acquired immunity to *L. monocytogenes*; and secondly, an examination of various antigen preparations as to their effectiveness in inducing acquired resistance in mice. Throughout these investigations, viable cells, formalin-treated cells, or cell-derived products of two serologically cross-reactive strains of *L. monocytogenes* were employed.

MATERIALS AND METHODS

Animals. In all studies, 6- to 8-week-old Swiss-Webster mice of either sex were obtained from a colony maintained by the Department of Bacteriology and Public Health, Washington State University. Animals were housed under standard conditions and offered feed and water ad libitum.

Cultures and maintenance. *L. monocytogenes* strain 10403, serotype 1 (obtained from M. L. Gray, Montana State University), has been maintained in a virulent state by repeated passage in mice and has a mean lethal dose (LD_{50}) of approximately 1.5×10^4. *L. monocytogenes* strain 19113, serotype 3 (obtained from American Type Culture Collection), was maintained on brain heart infusion (Difco) semisolid agar at 4°C. The cultures were periodically checked for purity and transferred at monthly intervals to freshly prepared media. *L. monocytogenes* strain 10403 is pathogenic for mice, whereas the serologically cross-reactive strain 19113 is not. These strains of *L. monocytogenes* were employed for use either as killed or viable immunogens.

LD_{50} determination. Swiss-Webster mice were

injected intravenously with 4.5×10^3 to 4.5×10^8 viable *Listeria*. Groups of 10 mice, between the ages of 4 to 6 weeks and of both sexes, were inoculated with each bacterial concentration suspended in 1% peptone water. The mice were observed for 1 week, and the LD_{50} was determined by the method of Reed and Muench (16).

Immunization. Suspensions for immunization or challenge were prepared from log-phase cultures grown at 37°C in brain heart infusion broth while on a rotary shaker. Mice were infected with a log-phase culture in brain heart infusion broth, which was diluted in 1% peptone water to contain the desired number of bacteria in 0.1 ml of inoculum. Administration of approximately 4.5×10^3 bacteria was used for primary immunization, whereas the intravenous injection of either 1 LD_{50} or 5 LD_{50} was used for the secondary injection. The dose used to challenge the various populations of mice was approximately 1.5×10^5. All injections were administered intravenously.

Antigen preparation. Whole-cell antigen was prepared by inoculating a 2-liter Erlenmeyer flask containing 1 liter of brain heart infusion broth with 10 ml of an 18-h broth culture of *L. monocytogenes* serotypes 1 and 3 and grown at 37°C. After a 24-h incubation on a rotary shaker at 37°C, formalin was added to a final concentration of 0.6% and allowed to react at room temperature for 72 h. This formalin-treated preparation was then tested for any residual viability. The cells were then centrifuged and washed twice with sterile phosphate-buffered saline and resuspended in a final volume of 20 ml of sterile phosphate-buffered saline. After being checked for bacterial sterility, the antigens were stored at 4°C in sterile vaccine bottles. The suspension used for injection contained approximately 3×10^9 bacterial cells ml.

Soluble antigen was prepared from culture filtrates of *L. monocytogenes*. Protein-free medium was prepared by dialysis of a 10× concentrated solution of Trypticase soy broth (Difco) against 10 volumes of distilled water for 24 h. The dialysate was then autoclaved and seeded with 10 ml of inoculum from an 18-h broth culture of *L. monocytogenes* serotype 1 or 3. This was incubated at 37°C for 72 h. Bacteria were removed by centrifugation at 7,000 × *g* for 30 min. The supernatant material was filtered through a 0.45-μm membrane filter (Millipore Corp.) to yield the broth antigen. The soluble antigen preparations were then concentrated with negative-pressure dialysis using the Prodicon apparatus (Bio Molecular Dynamics). The protein concentration of the broth antigen was determined using the method of Groves et al. (2). The soluble antigen preparations were placed in sterile vials and stored at -20°C. The same batch of broth antigen was used throughout the investigation.

Macrophage migration inhibition. The capillary tube method for determination of migration inhibition factor was employed (1). Each chamber was filled with RPMI 1640 supplemented with 15% filtered normal mouse serum and antigen when indicated. Whole-cell antigens were added at a concentration of 9×10^6 cells/ml. The soluble antigen preparation was added at a concentration of 10 μg/ml. The chambers were incubated at 37°C in 5% CO_2 for 24 h. After this period of incubation, the migration patterns were traced, and the fan size was quantitated by use of a polar planimeter.

RESULTS

Development of acquired resistance in normal Swiss-Webster mice. To confirm that immunity to *L. monocytogenes* strain 10403 develops after primary infection, a population of mice was injected intravenously with approximately 4.5×10^3 (0.3 LD_{50}) bacteria. These mice were subsequently challenged at weekly intervals with approximately 1.5×10^5 (10 LD_{50}) bacteria to determine the percent survival. Immunity to *L. monocytogenes* strain 10403 began to decline after 2 weeks, and by the fifth week immunity was no longer evident (Fig. 1).

Effectiveness of alternative methods of antigenic exposure in establishing anti-*Listeria* immunity. To determine whether alternative methods of antigenic exposure affect the primary acquisition of anti-*Listeria* immunity, formalin-treated *L. monocytogenes* strain 10403 and viable *L. monocytogenes* strain 19113 were employed as immunogens. Groups of 10 mice each were injected intravenously with either of the two antigen preparations. Mice in each tested group received either 3×10^8 organisms/ml of formalin-treated *L. monocytogenes* strain 10403 or 4.5×10^8 organisms/ml of viable *L. monocytogenes* strain 19113. The immunizations continued weekly for 3 weeks. Ten days after the final injection, each group was challenged with 10 LD_{50} of *L. monocytogenes* strain 10403 or assayed for the existence of cell-me-

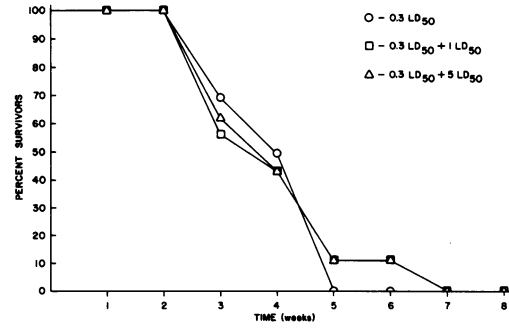

FIG. 1. *Survival of mice that had received a primary immunization of 4.5 × 10³ (0.3 LD_{50}) viable L. monocytogenes strain 10403 or a secondary immunization of either 1 LD_{50} or 5 LD_{50} 1 week after primary immunization. All grouped mice were subsequently challenged intravenously with 10 LD_{50} of L. monocytogenes strain 10403 to determine population levels of acquired immunity. Each tested group consisted of 10 mice.*

diated immunity by macrophage migration inhibition. All mice in each tested group succumbed after challenge, and the in vitro correlate of cell-mediated immunity, macrophage migration inhibition, was negative. Thirty days after the final injection with the two aforementioned antigenic preparations, additional groups were treated in an identical fashion. Data obtained from this experiment indicated that neither antigen preparation was effective in inducing immunity to *L. monocytogenes* strain 10403, suggesting that only viable *L. monocytogenes* strain 10403 is capable of establishing acquired resistance in mice (data not shown).

Influence of antigenic dose on the longevity of anti-*Listeria* immunity. Having established that anti-*Listeria* immunity develops after a primary injection of viable *L. monocytogenes* strain 10403, consideration was focused on the longevity of anti-*Listeria* immunity and whether the decay of this response could be altered. To ascertain the importance of antigenic dose on the longevity of immunity to *L. monocytogenes*, a large population of mice was grouped according to the dose of antigen they were to receive. Initially, all mice received a sublethal dose of antigen, which was approximately 4.5×10^3 (0.3 LD_{50}) bacteria. One week later, one group of mice received 1 LD_{50} of strain 10403, whereas the other group of mice received 5 LD_{50}. The degree of anti-*Listeria* immunity established in these mice was monitored in vivo by weekly challenge of a 10-animal sample from both groups with 10 LD_{50} and in vitro by employing the macrophage migration inhibition technique. By the third week after challenge with either 1 LD_{50} or 5 LD_{50}, immunity to strain 10403 had begun to diminish, and by the seventh week, both groups were no longer protected (Fig. 1). It is evident that the duration of immunity was dose independent and that the absolute interval of its existence was not extended by secondary challenge with large numbers of viable organisms. We have extended these observations to include secondary injection with 10 LD_{50}, which failed to alter the kinetics of the immune response.

The data obtained from macrophage migration inhibition, using both formalin-treated cells and *L. monocytogenes* product as antigen, are presented in Fig. 2. Although inhibition of migration was achieved in some groups, there was a great deal of variation observed from week to week. Thus, only animals that were exposed to viable 10403 organisms successfully resisted challenge, and only animals exposed to viable 10403 organisms developed positive migration inhibition responses. Antigens in the

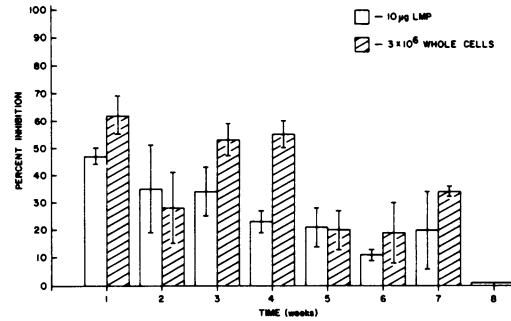

FIG. 2. *Migration inhibition of peritoneal exudate cells when challenged with either 10 μg of L. monocytogenes product (LMP) or 3 × 10^6 whole cells derived from L. monocytogenes strain 10403. Peritoneal exudate cells were obtained from mice that received a secondary injection of 5 LD_{50} 1 week after a primary immunization with approximately 4.5 × 10^3 (0.3 LD_{50}) viable L. monocytogenes strain 10403. Indicated values represent the mean ± standard deviation of separate migration inhibition experiments. Each tested group consisted of 10 mice.*

form of whole cells or *L. monocytogenes* product could elicit lymphokine production in assays of sensitized animals, although these same preparations could not inititate the development of a sensitive population of lymphocytes in vivo.

Effectiveness of various immunogens in maintenance of acquired resistance. This experiment was designed to determine whether the immunogen used in establishing acquired resistance after primary immunization was also essential in achieving protection at a time when the survival of the animal population had approached 50% or whether other closely related immunogens would function in maintaining a state of specific resistance. Mice were immunized with approximately 4.5×10^3 viable *L. monocytogenes* strain 10403, and groups derived from the immune population were challenged at weekly intervals with 10 LD_{50} of *L. monocytogenes* strain 10403 until such time that survival approached 50% of the challenged sample groups. At this point, the remaining population of immune mice was divided into groups of 30 mice each, and groups received a secondary intravenous injection of either a sublethal dose of viable *L. monocytogenes* strain 10403, 9×10^8 formalin-treated *L. monocytogenes* strain 10403, 6×10^8 viable *L. monocytogenes* strain 19113, or saline. Only mice that received a second sublethal dose of *L. monocytogenes* strain 10403 survived after challenge with 10 LD_{50} of *L. monocytogenes* strain 10403 (Fig. 3), indicating that the decline of immunity in actively immunized animals was not altered by antigenic challenge with formalin-treated 10403 cells, cell products, or viable

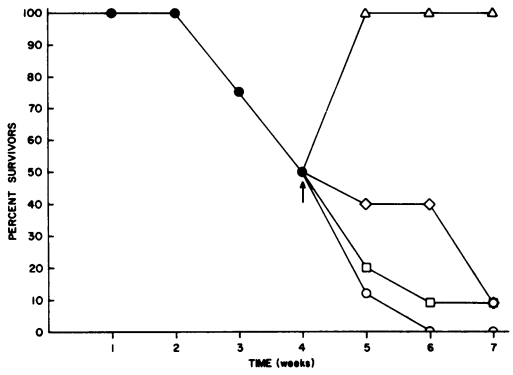

FIG. 3. *Effectiveness of various immunogens in maintaining acquired resistance in mice after primary immunization with approximately 4.5 × 10^3 (0.3 LD_{50}) viable L. monocytogenes strain 10403* (●). *The arrow indicates approximately 50% survival of the mouse population after primary immunization and the time at which the remaining mice received a secondary intravenous injection of either: a sublethal dose (0.3 LD_{50}) of viable L. monocytogenes strain 10403* (△); *9 × 10^8 formalin-treated L. monocytogenes strain 10403* (◇); *6 × 10^8 viable L. monocytogenes strain 19113* (□); *or saline* (○). *All mice were challenged intravenously at weekly intervals with 10 LD_{50} of L. monocytogenes strain 10403.*

19113. These results imply that the cellular requirements for the development of host resistance are similar for induction as well as maintenance.

DISCUSSION

Acquired resistance to *L. monocytogenes* strain 10403 develops in mice after a primary intravenous injection of 4.5 × 10^3 (0.3 LD_{50}) bacteria. By challenging these mice at weekly intervals with 10 LD_{50}, it was found that this immunity persists for several weeks (Fig. 1). These results seem to correlate well with those of Mackaness (4), who established that after an infection the bacterial population increases in the spleen and liver during the first 3 days and that, on day 4 postinfection, the mouse becomes hypersensitive to *Listeria* antigens and growth of bacteria ceases. This period of absolute resistance to *Listeria* was found to last for 3 weeks; however, after this time period, the animal remains hypersensitive to *Listeria* but unable to resist a challenge dose of *L. monocytogenes*.

In an effort to explore the possibility that other antigenic preparations may be effective in establishing immunity to *Listeria*, either formalin-treated *L. monocytogenes* strain 10403 or viable *L. monocytogenes* strain 19113 was used. Neither of these two immunogens was effective in the development of acquired resistance to *L. monocytogenes*. Although these strains are serologically cross-reactive, the antigens shared between these two strains are obviously not those important in establishing immunity to *L. monocytogenes*. This might suggest that viable *L. monocytogenes* strain 10403 possesses or produces material lacking in the nonpathogenic cross-reactive strain that may be unique to the establishment of acquired resistance. Thus, in the methodology employed, formalin treatment or disruption destroys the protective antigen. Several investigators have fractionated *L. monocytogens* in an effort to isolate those factors important in the establishment of acquired immunity, but to date the chemical makeup of these substances is not apparent (15, 17, 18).

North, in his investigations of the cellular response of mice to infection with *Listeria*, has determined that the various parameters associated with anti-*Listeria* immunity peak on about day 6 postinfection and that shortly after this time period the response begins to decay, suggesting that immunity to infection with *L. monocytogenes* is short-lived (9). The development of antibacterial immunity is established at a rate that corresponds to the appearance of delayed-type hypersensitivity to listerial antigens. The uncertainty of the relationship between these two phenomena is still an area of considerable interest, and it has been suggested that antimicrobial cellular immunity may occur as a phenomenon independent of delayed hypersensitivity (12). Our report has approached the problem of longevity by determining whether the immune response established in mice to *L. monocytogenes* can be augmented by increasing the antigenic dose after primary immunization. As can be seen from the data presented in Fig. 1, regardless of the antigenic dose received in the second injection, the temporal existence of acquired immunity in these groups of mice was indistinguishable.

Macrophage migration inhibition, as an in vitro assay, was used throughout this investigation to observe the degree of correlation between in vivo and in vitro assays in ascertaining cell-mediated immunity to infection with *L. monocytogenes*. In general, our observations would indicate that if migration inhibition is an in vitro correlate of developed hypersensitivity, then the appearance and disappearance of anti-*Listeria* immunity coincide with positive and negative migration inhibition determinations.

The importance of sensitizing immunogen in maintaining resistance to lethal challenge with *L. monocytogenes* strain 10403 in mice that had previously been immunized with a sublethal dose of *L. monocytogenes* strain 10403 was in-

vestigated. Both homologous and cross-reactive antigens were administered intravenously after the initial decline of the immune response. Data presented in Fig. 3 show the response of these mice after the intravenous injection of various immunogens, and it is significant to note that only those mice that received a secondary intravenous injection of viable *L. monocytogenes* strain 10403 were effective in maintaining acquired resistance. Apparently, the cell-mediated response has certain unique requirements, since recall of specific immune maintenance cannot be achieved by either an intravenous injection of formalin-treated homologous antigen or with a viable cross-reactive nonpathogenic strain. In a similar fashion, the initial host-parasite reaction that occurs when viable organisms are introduced in vivo may importantly predict the immune response that is eventually generated. The requirements, therefore, for a successful anti-*Listeria* immune response would appear to be similar for both the primary and secondary event in cell-mediated immunity and would seem to involve a necessary parasitic event that only occurs after host interaction with virulent strains of this bacterium.

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