Recall of Acquired Cellular Resistance in Mice by Antigens from Killed Brucella

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Mice infected with Brucella abortus 19 were challenged intravenously with Listeria monocytogenes. Spleen assays to determine the number of viable Listeria cells present revealed that these mice were highly resistant to Listeria when challenged on day 17 of the Brucella infection. Resistance was absent in mice challenged on the 5th day and was declining in mice challenged on the 33rd day. Resistance could not be detected by day 49 of the Brucella infection but could be recalled by the injection of antigens from smooth B. abortus 2308. Thus, extracted antigens appeared to be as effective in recall as the live cells used in earlier studies. Similar injections of extracts from rough B. abortus 45/20, or from B. ovis REO 198, were also effective in recalling resistance; this suggests that the smooth surface agglutinin may be relatively unimportant in recall.

Resistance to diseases caused by facultative intracellular parasites is believed to be mediated primarily by cellular, rather than humoral, mechanisms (7, 17). Cellular immunity or acquired cellular resistance appears to be the result of an immunologically specific reaction which is nonspecific in its antimicrobial effects (15). The establishment of resistance endows the host with macrophages which are effective, for a time, against organisms which are antigenically (4-6, 13), taxonomically (2, 3, 15, 24), or even phylogenetically (20-23) unrelated to the original infecting parasite.

The duration and degree of immunity appear to be influenced by the persistence of antigen (3-6, 14-16, 20, 23). In mice, resistance to challenge with Listeria monocytogenes begins to decline as the previously established Brucella infection enters the latent phase but can be recalled by the injection of live Brucella (15). Although Mackaness and Blanden (17) reported that injection of dead Brucella during the latent phase of a Brucella infection caused a sudden drop in the number of residual brucellae persisting in the tissues, quantitative data were not given to support this statement. This report presents the results of a study on the use of antigens prepared from killed brucellae to recall acquired cellular resistance in Brucella-infected mice. A brief report covering portions of this work was given earlier (R. D. Hinsdill and B. L. Halliburton, Bacteriol. Proc., p. 101, 1971).

MATERIALS AND METHODS

Bacterial cultures. All cultures of Brucella were provided by L. M. Jones, Department of Veterinary Science, University of Wisconsin. Brucella abortus smooth strain 2308 and the rough strain 45/20 were fresh isolates from experimentally infected guinea pigs. B. ovis REO 198 and the smooth B. abortus 19 were received as lyophilized cultures. B. abortus 19 was a subculture of a freeze-dried vaccine originally obtained from the central Veterinary Laboratories, Weybridge, England. B. abortus strains 2308 and 45/20 were maintained on slants of brucella agar (Albimia Laboratories, Flushing, N.Y.) and stored at 4 C. Cultures of B. abortus 19 and B. ovis REO 198 were prepared by using brucella broth and were incubated on a shaker at 37 C until the culture reached an optical density of 0.6 to 0.7. A portion of each broth was centrifuged for 20 min at 7,710 x g, and the pellet was suspended in a freeze-dry medium consisting of 2.5% Casitone (Difco Laboratories, Detroit, Mich.), 5.0% sucrose, and 1.0% L-glutamic acid in distilled water. Small amounts of the suspended culture were placed in vials and lyophilized on an Edwards Freeze Dryer (model SPS, Edwards High Vacuum, Inc., Grand Island, N.Y.). The vials were sealed while under vacuum and stored at 4 C.

L. monocytogenes (serotype undetermined) was provided by Shirley Johnson of the Animal Health Laboratory, Wisconsin State Department of Agriculture, Madison. The culture was then mouse-passaged twice, and a fresh isolate was recovered from the spleen of a moribund mouse. This isolate was used to prepare lyophilized stock cultures in the manner described above. Brain Heart Infusion (BHI) agar and broth (Difco Laboratories) were used as the growth media. The intravenous 50% lethal dose (LD50) for mice was determined several times by
using the method of Reed and Muench (19). In each experiment, the most recently calculated dosage was used, ranging from a high of $4.0 \times 10^4$ at the start of the research to a low of $8.5 \times 10^4$ at the finish. One-half of one LD$_{50}$ was always used for challenge, as indicated in each figure by an arrow.

Mice. Outbred Swiss-Webster mice were obtained from Rolfsmeier Rat and Mouse Farm or A. R. Schmidt Co., Madison, Wisconsin. Mice were all of one sex and were 6 to 8 weeks old at the beginning of any one experiment.

Preparation of bacteria for inoculation. A lyophilized stock culture of B. abortus 19 was suspended in brucella broth and used to inoculate a brucella agar slant. After incubation at 37°C for 2 to 4 days, growth on the slant was washed off with 2 ml of brucella broth and added to 75 ml of broth in a side arm flask. The flask was placed in a reciprocal water bath shaker at 150 strokes per minute (150 rpm) and the broth culture was incubated at 37°C. When the broth culture was in late log phase (optical density, 0.6 to 0.7), 0.1 ml of the culture was centrifuged at 7,710 g for 20 min. The pellet was washed once by suspending in Hanks balanced salt solution containing 0.1% bovine albumin fraction V (HBSS-V; Grand Island Biological Co., Grand Island, N.Y.); after centrifugation, the cells were resuspended in an equal volume of HBSS-V. After appropriate dilution in HBSS-V, 0.1-ml amounts were injected intravenously (iv) into mice. The exact number of viable bacteria injected was determined by plate counts of the immunizing suspension. Tests were done to verify the colonial smoothness of the Brucella (10, 25).

Suspensions of L. monocytogenes used for inoculation were prepared and handled in the same manner; BHI agar and broth were used as the growth media, and the slants were incubated for 24 to 36 hr.

Spleen assays. The number of viable bacteria per milliliter of spleen homogenate from mice was determined by the method of Mackaness (14). In assays for Brucella, spleens were homogenized in 5.0 ml of brucella broth, and appropriate decimal dilutions were plated in duplicate on well-dried brucella agar plates. Colonies were counted and checked for smoothness after 4 to 5 days of incubation at 37°C. BHI broth and agar were used in the assays for Listeria. Colonies were counted after 24 to 36 hr of incubation.

Antigens. The water extracts of sonically disrupted B. abortus 2308, strain 45/20, and B. ovis REO 198 were prepared by using the method of Hinsdill and Berman (11). The staphyloccocal antigen, mostly cell walls and debris, was obtained by disrupting Staphylococcus aureus in a Ribbi Cell Fractionator. The method for growing, harvesting, and breaking the cells was described by Gagliano and Hinsdill (9). The suspension of broken cells was centrifuged at 7,000 $\times$ $g$ for 45 min. The pellet was suspended in distilled water and freeze-dried. All antigens were suspended in HBSS-V for injection into mice.

Recall experiments. For any one experiment, the general procedure was as follows. A large group of mice was obtained, half of which were set aside to be used as normal controls and the other half inoculated iv with B. abortus 19. When the Brucella infection was in the latent phase (day 44 or later), half of the infected mice and half of the normal mice were injected subcutaneously (sc) in two sites with the antigen (5.0 mg/ml dry weight), 0.1 ml per site, for a total of 1 mg per mouse. Five days later, all of the mice were challenged iv with L. monocytogenes. Spleen assays for Listeria were done each day for the next 5 days, beginning 24 hr after challenge, on randomly selected mice from each group. The groups were designated as follows: group I, normal mice; group II, normal mice injected with antigen; group III, Brucella-infected mice which received no antigen; group IV, Brucella-infected mice injected with antigen early in the latent phase (days 44 to 51); and group V, Brucella-infected mice injected with antigen on day 93.

RESULTS

Demonstration of cellular immunity. A large group of mice was infected with $4.7 \times 10^4$ B. abortus 19. Twenty-four hours later and then at 4-day intervals, five mice were killed and their spleens were assayed to determine the number of viable Brucella present. The bacterial count rose from $6.3 \times 10^6$ Brucella per ml of spleen homogenate at 24 hr to $9.4 \times 10^6$ Brucella per ml at 9 days (Fig. 1, top). After this point, the Brucella population steadily decreased. By day 30, the infection had entered the latent phase, and there were only small differences in spleen counts during the following 20 days.

On days 5, 17, and 33 of the Brucella infection, 25 infected mice and 25 normal mice were challenged with L. monocytogenes. Each day for

![Fig. 1. Top: growth of B. abortus 19 in the spleens of normal mice. Bottom: growth of challenge organism L. monocytogenes injected at stages (arrows) during the Brucella infection shown above. Arrows also indicate total challenge dose. Symbols: normal mice (□) Brucella-infected mice (△). Each point represents the mean of five mice and indicates the viable counts per milliliter of spleen homogenate. Bars indicate standard deviation of the mean.](http://iai.asm.org/Downloaded from http://iai.asm.org/ on September 30, 2017 by guest)
on day 44. Growth of Brucella-infected mice represented a challenge to the Brucella-infected phase (day 93). The mean of five mice was determined (Fig. 1, bottom).

The upper curves in the bottom half of Fig. 1 represent the spleen counts for normal mice. In each case, the spleen counts increased for 2 to 3 days and then declined. The bottom curves represent Brucella-infected mice challenged with Listeria. On day 5 of infection, the challenge resulted in a typical growth curve for the Listeria. On day 17, a time when the Brucella population was declining, the Listeria challenge was rapidly eliminated. By day 33 of infection, the Listeria challenge resulted in growth approaching a typical curve.

Recall with antigens from B. abortus 2308. Published growth curves (15) for a primary Brucella infection in mice do not extend beyond 40 days, a time when there are still large numbers of Brucella present in the spleen. Therefore, spleen assays to determine the number of Brucella persisting from a primary infection were carried out to day 99. In most mice, the number of residual bacteria had dropped to less than $10^6$ per ml of spleen homogenate by day 90. On the basis of these data, recall of immunity was attempted by injecting antigen early in the latent phase (day 44) and also at a time when only a few Brucella could be detected in the spleens of infected mice (day 93). A dose of $9.0 \times 10^6$ Brucella was used to initiate the infection, and approximately 200 mice were used for the entire experiment. The antigen selected was a partially characterized (11) water extract of sonically disrupted B. abortus 2308 which has been shown to act in skin tests and tissue culture studies much the same as whole killed cells (12). Each mouse received a total of 1 mg (dry weight) as described above.

The growth curves of the Listeria in each of the five groups (see above) are shown in Fig. 2. The results indicate that extracted antigens of Brucella can effectively recall a state of enhanced nonspecific resistance. Injection of antigen on day 44 recalls resistance more effectively than injection of antigen on day 93, as would be expected.

Recall with antigens from B. abortus 45/20. A surface lipopolysaccharide of smooth virulent Brucella (antigenic component IX), believed to be the agglutinogen, was suspected by Hinsdill, unpublished data). The absence of enhanced resistance to a rough strain were substituted. We used a sonic extract of the rough, avirulent B. abortus 45/20 which contains no component IX detectable by immunoelectrophoresis (R. D. Hinsdill, unpublished data). The absence of enhanced resistance to challenge would indicate that component IX, the agglutinogen, was important in recall.

One hundred mice were inoculated with 1.3 $\times$ 10^8 of Brucella and were challenged with Listeria. The results of those challenged are shown in Fig. 2. The recall was obtained by injecting antigen (12) on day 45. The antigen was prepared as a water extract of sonically disrupted B. abortus 2308. Each mouse received a total of 1 mg (dry weight) as described above.

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10⁸ B. abortus 19. On day 48 of the Brucella infection, half of the mice were injected with 1.0 mg of 45/20 antigen. Five days later, all of the infected mice were challenged with L. monocytogenes. Daily spleen assays were done on 10 mice per group. One hundred normal (groups I and II) control mice were assayed in a similar manner 1 week earlier. The results (Fig. 3) indicate that a sonic extract of the rough, avirulent B. abortus 45/20 is effective in recalling cellular immunity. A sonic extract from B. ovis, another rough Brucella, somewhat more distant taxonomically from strain 19, has been tested in similar experiments with the same results.

**Specificity of recall.** To insure that there was some antigenic specificity to the recall phenomenon, cell walls and debris obtained from disrupted S. aureus were used as an antigen. One hundred mice were inoculated with 9.8 × 10⁸ B. abortus 19. Half of the mice were injected with 1.0 mg of the staphylococcal antigen on day 52 of the Brucella infection. On day 57, all of the mice were challenged with L. monocytogenes. The 100 normal control mice were assayed in the same manner 6 days later. The results (Fig. 4) show that this antigen did not recall a state of nonspecific resistance.

**DISCUSSION**

The growth pattern (Fig. 1) of B. abortus 19 in normal mouse spleens is similar to that reported by Mackaness (15). The infection may be separated into three distinct phases: a period of multiplication, a period of bacterial inactivation, and a period of latency. Enhanced resistance to the heterologous organism L. monocytogenes is observed only when mice are challenged during the period of bacterial inactivation (middle Listeria curves, Fig. 1). Brucella-infected mice show no resistance to Listeria when the heterologous challenge is given during the period of Brucella multiplication (Fig. 1, left-hand Listeria curves). Nonspecific resistance begins to decline early in the latent phase (Fig. 1, right-hand Listeria curves) and disappears by day 49 of the Brucella infection (Fig. 2–4, group III).

The rapid decay of antibacterial immunity shown here for a Brucella infection is typical of diseases caused by facultative intracellular parasites. Other workers have demonstrated a similar decay in mice infected with Listeria (14) or Salmonella (5). The development of a critical antigenic mass seems to be an essential step in the establishment of cellular immunity (16, 17). As the level of antigen declines, the level of immunity also declines. However, a sudden increment in antigenic material to which the host is sensitized can recall the nonspecific phase of immunity. In previous studies, recall has been elicited by the injection of live homologous organisms (2, 5, 17). The resistance to Listeria challenge in group IV mice shown in Fig. 2–3 indicates that extracted antigens are as effective as live cells in recalling immunity.

Collins (5) found that the rate of recall in salmonellosis was related to the persistence of antigen. As the number of residual vaccinating microorganisms declined, the rate of recall also declined. A similar observation was made with Brucella in this study. Early in the latent phase of infection, approximately 10⁸ viable bacteria per ml of spleen homogenate could be recovered from the spleens of Brucella-infected mice (Fig. 1). By day 90 of infection, the number of residual bacteria had dropped to less than 10⁸ per ml of spleen homogenate. Brucella-infected mice injected with antigen on day 44 were highly resistant to Listeria challenge 5 days later (Fig. 2, group IV). Mice injected with antigen on day 93 (Fig. 2, group V) also developed resistance to the heterologous challenge, but at a reduced rate.

Virulence in Brucella is normally associated with smooth strains. The injection of antigen from B. abortus 45/20, a rough avirulent strain, was an indirect test to determine the importance of the smooth-surface antigen, believed to be the agglutinin, in the recall of immunity. The extract from strain 45/20 (Fig. 3, group IV) was as effective as the extract from strain 2308 (Fig. 2, group IV) in recalling the nonspecific phase of immunity. Similar results were obtained with antigens from B. ovis. These results suggest that
smooth-surface antigens may not be of major importance in recall. We cannot rule out the possibility, however, that strain 45/20 and \textit{B. ovis} contain a small, difficult to detect quantity of the smooth-surface lipopolysaccharide which is acting to recall acquired cellular resistance. Diaz et al. (8) found that water-soluble antigens obtained by ultrasonic treatment of \textit{Brucella} showed extensive cross-reactions within the genus, including \textit{B. ovis}, and further experiments are planned to determine the identity of the antigen(s) responsible for recall. We note that Ralston and Elberg (18) have recently shown that a protein fraction from \textit{B. melitensis} Rev I when used in Freund's adjuvant can elicit macrophages with enhanced resistance against challenge with the virulent strain 6015.

The \textit{Brucella} antigens were effective in \textit{Brucella}-infected mice only. Normal mice injected with antigen (Fig. 2–4, group II) responded to a \textit{Listeria} challenge in the same manner as normal mice which were not injected with antigen (Fig. 2–4, group I). These results are in contrast to those of Berger et al. (1), who reported increased resistance to infection with \textit{Salmonella typhi-murium}, \textit{Salmonella typhosa}, \textit{Diplococcus pneumoniae}, or \textit{Streptococcus mastidis} in normal mice treated with lipopolysaccharides extracted from the rough \textit{B. abortus} 19-9R. We have no explanation for this, except that the experiments may not be directly comparable because of differences in dosages, time periods, and criteria of immunity. Injections of staphylococcal antigens (Fig. 4) did not recall a state of nonspecific resistance in \textit{Brucella}-infected mice. This, however, is not a rigorous test of specificity.

Our experiments support the idea (15) that recall can be elicited only by antigens to which the host is specifically sensitized. Further experiments are necessary to determine the effect of smaller single doses of antigen. If persistence of immunity is indeed dependent on the persistence of antigen, it may be possible to sustain a high level of immunity by sustaining the level of antigen; repeated injections of small doses of antigen, started early in the latent phase of infection, might serve this function. In assessing the effects of various antigen treatments on recall, the conditions under which primary sensitization takes place should also be considered. Failure to reach a critical mass of the necessary antigen(s) initially may preclude later recall even with the live homologous organism. For example, in one set of experiments, Ralston and Elberg (18) found that an intraperitoneal injection of 10^4 \textit{B. melitensis} Rev I was incapable of recalling immunity, as measured by in vitro challenge of macrophages, in guinea pigs which had been vaccinated earlier with strain Rev I. That these animals had been primed to some degree, however, was evident from the fact that these animals did exhibit recall when injected with a more virulent strain.

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**LITERATURE CITED**