

Role of the Macrophage in Natural Resistance to Salmonellosis in Mice

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In vitro, macrophages from normal strain BRVR mice killed *Salmonella* more quickly than did macrophages from normal strain BSVS mice. *Salmonella* injected intraperitoneally multiplied more quickly in BSVS mice than in BRVR mice. BRVR macrophages injected intraperitoneally into BSVS mice protected against *Salmonella* multiplication better than did BSVS macrophages. The populations of peritoneal cells that could be washed from the peritoneal cavity of normal BRVR and BSVS mice were morphologically and numerically identical. In vitro, BSVS macrophages were as efficient as BRVR macrophages in phagocytizing virulent *Salmonella*. These findings all support the concept that the greater natural resistance of BRVR mice to *Salmonella* infection may be explained by the greater ability of normal BRVR macrophages to kill ingested *Salmonella*.

In the 1930's, Webster succeeded in selecting two inbred strains of mice which differed markedly in their natural susceptibility to oral infection with *Salmonella enteritidis* (10). Strain BRVR was relatively more resistant than strain BSVS. Subsequently, Webster showed that strain BRVR mice were also more resistant to *S. enteritidis* introduced by the intravenous (iv), intraperitoneal (ip), or subcutaneous (sc) routes (11).

Later, Groschel et al. showed that strain BRVR mice were more resistant than strain BSVS mice to iv infection by a highly virulent strain of *S. typhimurium* (3). After iv infection, multiplication of *Salmonella* in the liver and spleen occurred more quickly in BSVS than in BRVR mice. In contrast to iv infection, BRVR mice were only slightly more resistant than BSVS mice to ip infection (3). However, BRVR but not BSVS mice became much more resistant to ip infection after a 24-hr pretreatment with live, avirulent *Salmonella*, live *Listeria monocytogenes*, or heterologous endotoxin (3).

These findings suggested that some difference in the macrophage function may be responsible for the difference in the natural resistance exhibited by BRVR and BSVS mice. In the present investigation, normal BRVR and BSVS macrophages were studied with respect to phagocytosis and killing of virulent *Salmonella*.

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MATERIAL AND METHODS

Animals and bacteria. *Salmonella*-resistant BRVR mice and *Salmonella*-susceptible BSVS mice were obtained from the inbred colonies maintained by the Skin and Cancer Hospital, Philadelphia. They were descendants of original breeding pairs selected by Webster. Both male and female mice, between 6 and 12 weeks old, were used. These inbred mice lacked demonstrable antibody to *Salmonella*, as shown by micro-passive hemagglutination (7) and the technique of Diener (2) for the assay of single antibody-forming cells.

A virulent strain of *S. typhimurium*, SR-11, was obtained through the courtesy of Dieter H. M. Groschel (M. D. Anderson Hospital, Houston, Texas). Overnight cultures were washed and suspended in balanced salt solution prior to use. Both animals and bacteria have been previously described (3).

Immune sera. High-titer rabbit antiserum was induced by the sc injection of 10⁹ heat-killed *S. typhimurium* SR-11 every week for 3 mo. The titer determined by micro-passive hemagglutination was 1:9000. Immune guinea pig serum, titer 1:360, was obtained from D. H. M. Groschel.

Peritoneal cell collection. Mice were sacrificed, and peritoneal cells were washed from the peritoneal cavity in medium 199 (GIBCO), collected in siliconized centrifuge tubes, and washed twice in medium 199 before use. Cells were counted in a hemocytometer, and viability was determined by trypan blue exclusion. For the experiments using non-glass-adherent cells, the peritoneal cells were incubated in petri dishes at 37 C for 1 hr. The nonadherent cells then were washed free, centrifuged at 70 × g for 10 min, and resuspended in medium 199.

Phagocytosis. From a peritoneal cell suspension collected as described, samples containing 5×10^5 cells were pipetted into Sykes-Moore tissue culture chambers, and 5×10^7 *S. typhimurium* SR-11 cells, previously opsonized by 10% normal or immune serum, were added. The cell-bacteria mixture was incubated at 37 C for 1 hr. The medium was then removed, the chambers were disassembled, and the bottom cover slips fixed in methanol and stained in May-Grunwald Giemsa. The cover slips were then examined microscopically; 300 to 500 cells were enumerated and scored for the number of bacteria appearing in the cytoplasm.

Bactericidal activity of macrophages. Phagocytosis was obtained without preopsonization in vitro by injecting 10^7 cells of strain SR-11 into the peritoneal cavity. Groups of at least four mice were used. After 15 min, the mice were sacrificed and peritoneal cells were collected as described. Fractions of the suspension, each containing 5×10^5 peritoneal cells, were distributed into small petri dishes, 44 mm in diameter. The dishes were incubated 40 min at 37 C, after which nonadherent cells and unphagocytized bacteria were removed by five washes.

Fresh medium at 37 C was added, and, at accurately timed intervals, three sample dishes were chilled rapidly to 4 C and then washed five times. Sterile distilled water was added to lyse the cells and release the intracellular bacteria (4). The number of viable intracellular bacteria was determined by plating a 0.1-ml fraction of the lysate on Trypticase soy agar. The results were expressed graphically as the \log_{10} (percentage of *Salmonella* surviving) versus time. The first sample was taken as 100% *Salmonella* surviving.

Transfer of peritoneal macrophages. Groups of at least four mice were injected intraperitoneally with 10^7 cells of strain SR-11. After 15 min, these donor mice were sacrificed and their peritoneal cells were collected. Phagocytized cells of strain SR-11 were separated from unphagocytized strain SR-11 with three washes by centrifugation ($70 \times g$ for 10 min). A sample of the washed cells was used to determine the concentration of viable cells of strain SR-11 in the suspension, as well as the number and viability of the peritoneal cells. The remaining cell population was used to inoculate groups of recipient mice (8-12 mice per group). Each mouse received 5×10^5 peritoneal cells containing 5×10^3 to 5×10^4 cells of strain SR-11. One-half of each group of mice was sacrificed at 24 hr, and the remaining half was sacrificed at 72 hr. The entire liver and spleen of each mouse were assayed for viable SR-11 cells.

Assay of viable *Salmonella* in tissue. Livers and spleens were removed aseptically, rinsed in rapidly boiling water to remove bacteria on the surface of the organ, pooled, and kept at 4 C. The organs were homogenized in Ten-Broek tissue grinders, and suitable dilutions in distilled water were assayed on Trypticase soy agar.

RESULTS

Peritoneal cell populations of BRVR and BSVS mice. There was no significant difference in the

total number of peritoneal cells that could be routinely washed from the peritoneal cavity of BRVR and BSVS mice (by three washes of 2 ml each). This number varied between 10^6 and 2×10^6 cells. By morphological characteristics, the peritoneal cell populations of the two mouse strains were indistinguishable. Both contained 10% polymorphonuclear leukocytes (PMN), 40 to 60% lymphocytes, and 30 to 50% mononuclear phagocytes (macrophages).

Phagocytosis of strain SR-11 in vitro by BRVR and BSVS macrophages. BRVR and BSVS peritoneal macrophages phagocytized strain SR-11 in vitro with equal facility after preopsonization by either normal BRVR or normal BSVS serum (Fig. 1). As can be seen from the distribution of phagocytized *Salmonella* within macrophages, there was no significant difference in total number of bacteria phagocytized. Phagocytosis was greatly increased by specific preopsonization by either immune rabbit or immune guinea pig serum (Fig. 2). However, the amount of phagocytosis by BRVR and BSVS macrophages was still comparable. With both the BRVR and BSVS macrophages, preopsonization of strain SR-11 with immune serum resulted in a twofold increase in the number of macrophages phagocytizing *Salmonella* as well as a 300-fold increase in the number of *Salmonella* phagocytized.

***Salmonella*-cidal activity of normal BRVR and normal BSVS macrophages.** As measured in vitro, BRVR macrophages killed ingested *Salmonella* more quickly than did BSVS macrophages (Fig. 3). The half-life for *Salmonella* survival within BRVR macrophages was 10 to 11 min. BRVR macrophages progressively killed virulent *Salmonella* until about 1 to 3% remained viable. Since at this point the number of unremovable extra-



FIG. 1. Opsonization and phagocytosis of SR11 in vitro by BRVR macrophages in normal serum, ■; BSVS macrophages in normal BRVR serum, □; BRVR macrophages in normal BSVS serum, ▨; BSVS macrophages in normal BSVS serum, ▩.

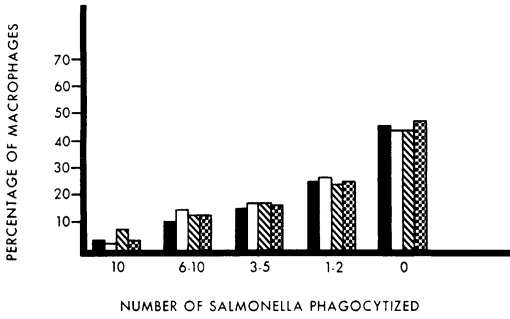


FIG. 2. Opsonization and phagocytosis of SR-11 in vitro by BRVR macrophages in immune rabbit serum, ■; BSVS macrophages in immune rabbit serum, □; BRVR macrophages in immune guinea pig serum, ▨; BSVS macrophages in immune guinea pig serum, ▩.

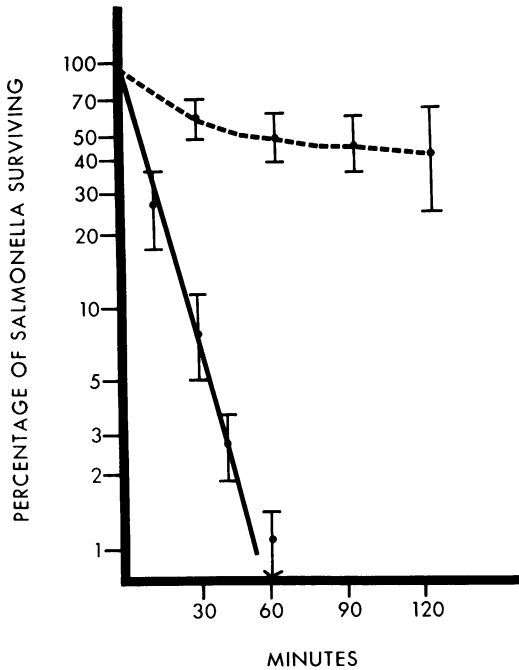


FIG. 3. Bactericidal activity of normal BRVR (—) and BSVS (----) macrophages in vitro. Vertical lines delimit two standard deviations from the mean.

cellular bacteria approached the number of surviving intracellular bacteria, the technique was not able to detect killing below the level of 1%. The initial half-life for *Salmonella* survival within BSVS macrophages was 50 min. The rate of killing within BSVS macrophages gradually decreased with time and approached zero. The final extent of killing, at this time, was between 50 and 65%.

Reticuloendothelial system function of BRVR and BSVS mice—liver and spleen assay. BRVR and BSVS mice were injected ip with 5×10^8 cells of virulent *Salmonella*. At 24 and 72 hr, the number of viable *Salmonella* in the liver and in the spleen was assayed (Fig. 4). In both BRVR and BSVS mice, even by 24 hr, there was a larger number of bacteria in the liver and spleen than was contained in the inoculum. The increase was about fourfold in BRVR and 12-fold in BSVS mice at 24 hr and increased to 10- and 1,000-fold, respectively, by 72 hr.

In the next experiments, the ability of phagocytized *Salmonella* to multiply within macrophages in BRVR and BSVS mice was studied. BRVR or BSVS peritoneal macrophages which had previously phagocytized *Salmonella* in vivo were collected, washed free from extracellular bacteria, and injected ip into groups of BRVR and BSVS recipients. One-half of the recipients were sacrificed at 24 hr and one-half at 72 hr after inoculation, to assay the number of viable *Salmonella* in the liver and spleen (Fig. 5). Both BRVR and BSVS peritoneal cell inocula contained 6×10^8 cells of viable *Salmonella*. At 24 hr, BSVS recipients of BRVR macrophages contained eightfold fewer *Salmonella* in the liver and spleen as compared with BSVS recipients of BSVS macrophages. From 24 to 72 hr, *Salmonella* multiplied in both groups of BSVS recipients at about the same rate.

At 24 hr, BRVR recipients contained about equal numbers of *Salmonella* in their livers and spleens after receiving either BRVR or BSVS

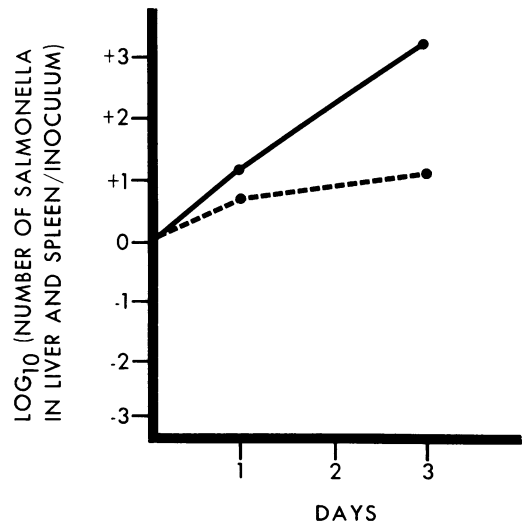


FIG. 4. Growth of SR-11 in the liver and spleen in BRVR (----) and BSVS (—) mice injected intraperitoneally with 5×10^8 cells of strain SR-11 (groups of 8 mice).

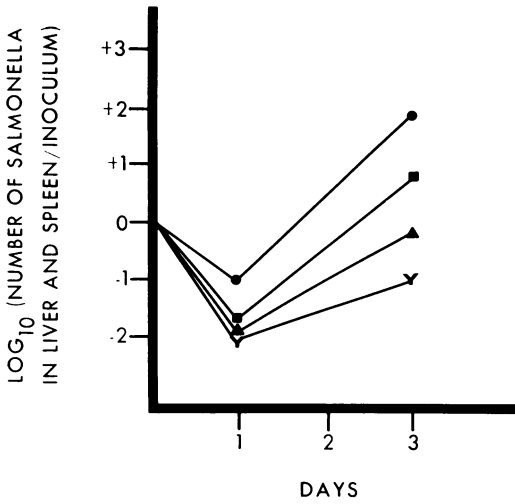


FIG. 5. Growth of SR-11 previously phagocytized by BRVR or BSVS macrophages after intraperitoneal injection into BRVR or BSVS recipients; BSVS cells into BSVS mice (●), BRVR cells into BSVS mice (■), BSVS cells into BRVR mice (▲), and BRVR cells into BRVR mice (Y). All recipients received 6×10^6 peritoneal cells containing 6×10^3 cells of strain SR-11, after washing three times before transfer.

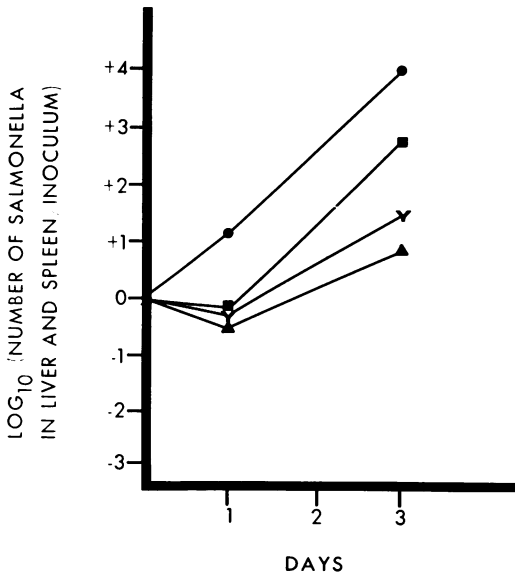


FIG. 6. Growth of SR-11 previously phagocytized by BRVR or BSVS macrophages after intraperitoneal injection into BRVR or BSVS recipients; BSVS cells into BSVS mice (●), BRVR cells into BSVS mice (■), BSVS cells into BRVR mice (Y), BRVR cells into BRVR mice (▲). All recipients received 3×10^6 peritoneal cells containing 6×10^3 cells of strain SR-11, after washing once before transfer.

macrophages ip. All groups of recipients possessed, at 24 hr, fewer *Salmonella* in the liver and spleen than were contained in the inoculum. This is in sharp contrast to the results shown in Fig. 4, in which an equivalent viable *Salmonella* inoculum (but one not contained within macrophages) resulted in liver and spleen counts 100-fold higher for all groups.

The cell transfer experiment was repeated with one modification. After phagocytosis in vivo, donor macrophages were washed only once (instead of three times) before transfer. This modification resulted in a significant number of free extracellular bacteria being transferred along with the phagocytized bacteria. At 24 hr, BSVS recipients of BRVR macrophages exhibited the same degree of protection as BRVR recipients of BRVR macrophages (Fig. 6). However, the ratio of bacterial number in the liver and spleen to inoculum is higher in all groups in this experiment than in the preceding one (Fig. 5).

The antibacterial activity of non-glass-adherent peritoneal cells (lymphocytes and PMN species) was tested. Three groups of BSVS and three groups of BRVR mice received *Salmonella* alone or *Salmonella* mixed with either BRVR or BSVS nonadherent cells. Figure 7 shows that the addition of nonadherent cells in any donor-recipient combination did not produce results significantly different from those obtained by injection of virulent *Salmonella* alone.

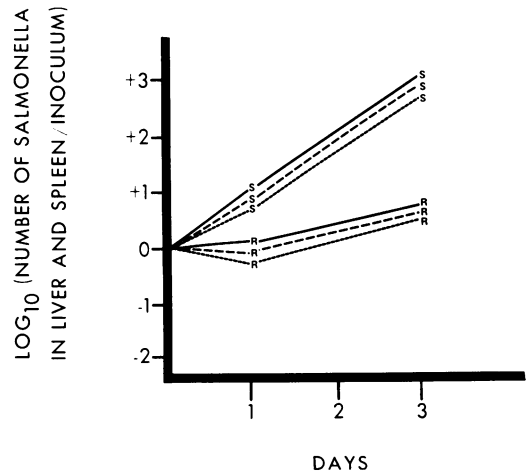


FIG. 7. Growth of 5×10^8 intraperitoneally injected cells of strain SR-11 in the liver and spleen of BRVR and BSVS mice receiving 5×10^6 nonadherent peritoneal cells (NAC); BSVS NAC into BRVR mice (R—R), BRVR NAC into BRVR mice (R- -R), BRVR controls (R----R), BSVS NAC into BSVS mice (S—S), BRVR NAC into BSVS mice (S- -S), BSVS controls (S----S).

BRVR and BSVS mice were injected ip with homologous normal serum prior to challenge with virulent *Salmonella* (Fig. 8). Normal mouse serum temporarily limited (at 24 hr) the number of *Salmonella* found in the liver and spleen. The degree of effectiveness was 50-fold better than with no opsonic protection (Fig. 4). As expected, after 24 hr multiplication occurred faster in BSVS mice than in BRVR mice.

DISCUSSION

BRVR mice have been shown to possess greater natural resistance than BSVS mice to *Salmonella* infection (1, 3, 10). There is evidence that neither BRVR or BSVS mice possess preformed antibody to *Salmonella* (3) and that both BRVR and BSVS mice form antibodies in response to immunization to *Salmonella* antigens (4).

As shown in Fig. 1, BRVR and BSVS macrophages phagocytized *Salmonella* with equal facility in vitro. This in vitro result is in agreement with the in vivo results of Groschel et al. (3) who showed that normal BRVR and BSVS mice clear *Salmonella* from the blood at the same rate. Rowley et al. have shown that very small amounts of cytophilic antibody increased phagocytosis by macrophages (8, 9). It could be proposed that normal BRVR mice possess cytophilic antibody to *Salmonella* and that BSVS mice do not. Contrary to the prediction of this proposal, BRVR macrophages do not phagocytize *Salmonella* more efficiently than do BSVS macrophages. Although

not in itself definitive, this argues that the greater natural resistance of BRVR mice is not attributable to cytophilic antibody.

Although no difference between the two mouse strains was apparent with respect to phagocytic activity, a striking difference was observed when the bactericidal activity of the macrophages was examined. BRVR macrophages killed ingested *Salmonella* in vitro much more quickly and completely than did BSVS macrophages (Fig. 3). The half-life for survival of strain SR-11 was 10 to 11 min in BRVR macrophages, as compared with 50 min in BSVS macrophages. By comparison, Jenkin and Benacerraf (6) reported that virulent *Salmonella* were killed by normal Swiss macrophages with a half-life of 45 min, and that macrophages from BCG-immunized mice killed *Salmonella* with a half-life of 16 min. It is apparent that, whereas normal BSVS macrophages resembled normal macrophages from Swiss mice in their ability to kill ingested *Salmonella*, normal BRVR macrophages resembled the activated macrophages from BCG-immunized Swiss mice.

As compared with BSVS macrophages, BRVR macrophages were better able to contain the growth of ingested strain SR-11 when injected ip into BSVS recipients (Fig. 5). This resulted in lower numbers of *Salmonella* in the liver and spleen at both 24 and 72 hr in BSVS recipients of BRVR macrophages. However, in both groups of BSVS recipients, the number of *Salmonella* in the liver and spleen increased at the same rate between 24 and 72 hr. Thus, protection was only temporary, indicating that no transfer of systemic resistance was involved. The importance of the intracellular containment for the full demonstration of protection can be seen by comparison of Fig. 5 and 6. In Fig. 6, more extracellular bacteria were transferred. This resulted in a decreased degree of protection but was still better than injection of virulent *Salmonella* alone.

Normal homologous serum injected into the peritoneal cavity also resulted in temporary protection (Fig. 8). Since it was shown that normal serum promoted phagocytosis in vitro (Fig. 1), normal serum may effect its temporary in vivo protection by promoting phagocytosis in the peritoneal cavity.

Salmonella plus nonadherent cells (Fig. 7) did not produce results significantly different from those obtained by injection of *Salmonella* alone. This further supports the concept that the protective effect of transferred peritoneal cells was attributable to the bactericidal activity of the glass-adherent, phagocytic cells or macrophages and was not attributable to lymphocytes, and PMN species which are nonadherent,

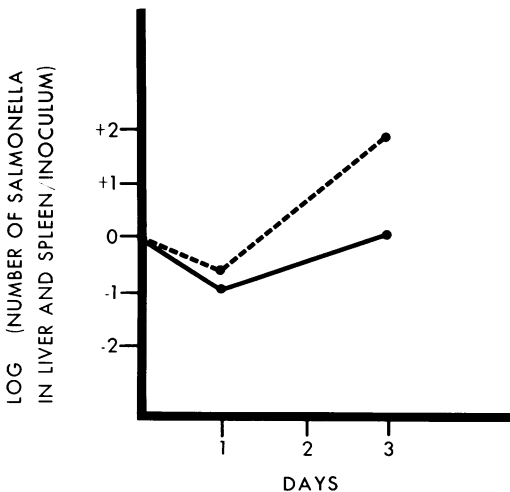


FIG. 8. Growth of 5×10^8 intraperitoneally injected strain SR-11 cells in the liver and spleen of BRVR (—) and BSVS (----) mice after receiving 0.2 ml of homologous normal serum intraperitoneally.

The finding that BRVR macrophages kill *Salmonella* more efficiently than do BSVS macrophages naturally engenders further inquiry. The nature of the difference in macrophage bactericidal activity and its relationship to delayed hypersensitivity are still speculative. These broader aspects of cell-mediated immunity are currently under investigation.

In conclusion, the concurrence of *in vitro* and *in vivo* results argue strongly that the resistance of BRVR mice to *Salmonella* infection may be explained by the greater ability of BRVR macrophages to kill ingested *Salmonella*.

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