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Ability of Mononuclear Phagocytes from Cattle Naturally Resistant or Susceptible to Brucellosis To Control In Vitro Intracellular Survival of *Brucella abortus*

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The role of bovine mammary macrophages and peripheral blood monocyte-derived macrophages in natural resistance to bovine brucellosis was evaluated. A group of 11 naturally resistant and 10 chronically infected susceptible cows was studied following challenge with *Brucella abortus*. Macrophages from a greater proportion ($P < 0.026$) of naturally resistant cows were significantly superior to macrophages from susceptible cows in their ability to inhibit the in vitro intracellular replication of *B. abortus* after challenge exposure. Studies of a second group of cows used mammary macrophages from 12 heifers and blood monocyte-derived macrophages from 22 bulls and heifers. These tests were completed before exposure to *B. abortus*, using mammary macrophages ($P < 0.039$) and blood monocyte-derived macrophages ($P < 0.045$), and also showed that macrophages from naturally resistant cattle were significantly superior in their ability to control the in vitro intracellular replication of *B. abortus*. Our data indicate that the mononuclear phagocytes from more than 80% of the resistant cattle controlled intracellular replication of *B. abortus* significantly better than did mononuclear phagocytes from susceptible cattle. Mononuclear phagocyte function appears to be an important factor in determining natural resistance to bovine brucellosis.

Brucella abortus, the causative agent of bovine brucellosis, is a gram-negative facultative intracellular bacterium that is strictly parasitic and produces chronic infections in cattle consisting of persistent or recurrent bacteremias manifested typically by abortion (36). Persistent infections of the mammary gland and supramammary lymph nodes occur in 80% of infected cows (14, 45). Many of these chronically infected cows continue to shed *B. abortus* in milk (41, 43, 45).

Control programs that include *B. abortus* 19 vaccination, isolation, testing, and slaughtering of infected cattle have been costly and relatively ineffective in eradicating brucellosis (3, 5, 13, 22, 27, 45, 62). Undetected latent infections, prolonged incubation periods, varied serologic responses, persistent infections, and similar antibody responses in vaccinated and naturally infected cattle are all features of bovine brucellosis that complicate eradication by conventional procedures (13, 33, 45). Strain 19 vaccination does not protect all cattle from subsequent challenge. As many as 30% of cows in vaccine trials were not protected by strain 19 vaccination (17, 20, 26). Interestingly, in vaccine trials it has also been found that up to 30% of the cows that were not vaccinated with strain 19 were naturally resistant to *B. abortus* infection (17, 20, 26).

In cattle, macrophages appear to be important in resolving an infection with *B. abortus*, although opsonification of the bacterium by *Brucella*-specific antibody has been shown to contribute to increased phagocytosis and an increased chemiluminescence response by mammary macrophages in vitro (30–33). Mammary macrophages from naturally resistant cattle produce significantly higher oxidative bursts and bacteriostatic activities than do macrophages from susceptible cattle (32).

Susceptible and resistant cattle react to *B. abortus* by both cell-mediated and humoral immunity (4, 21, 33, 38, 58). The blastogenic response patterns of oligoclones of *B. abortus*-specific bovine lymphocytes from resistant cattle to four species of *Brucella* differed from those of oligoclones from susceptible cattle (R. Smith III, J. C. Kapatsa, S. J. Sherwood III, T. A. Ficht, J. W. Templeton, and L. G. Adams, Am. J. Vet. Res., in press). Susceptible cows were found to have initially higher and more prolonged total antibody responses, significantly higher immunoglobulin G1 (IgG1) (10) responses, and a preferential expression of the IgG2a (A1) allotype (D. M. Estes, Department of Veterinary Pathology, Texas A&M University, College Station, Tex., personal communication, 1989). Differences in lymphocyte reactivity and immunoglobulin allotype expression were measured after exposure, making it impossible to determine whether the differences observed in these adaptive immune responses were causes or effects of innate resistance or susceptibility to bovine brucellosis. It appears that both arms (cell-mediated and humoral) of the immune system are important in resistance to bovine brucellosis, but the relative importance of each is unknown.

In the present study we evaluated the relationship between the ability of bovine mononuclear phagocytes to control the intracellular replication of *B. abortus* and the resistance and susceptibility of cattle to infection. Previous studies have already demonstrated a correlation between mammary macrophage control of intracellular survival and phenotypic classification of the cow as resistant or susceptible. However, those were short-term assays of intracellular survival of *B. abortus*, and the rate of growth of the organisms during that period was rather slow even in the absence of mammary macrophages (32). Our first experiment was designed to confirm the previous findings, but was

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done with an assay in which there was at least 1 log unit of growth of the organism during the period of the assay. Previous studies tested mammary macrophage activity after exposure to *B. abortus*. The chronic presence of *B. abortus* in the mammary glands of most of the susceptible cows may have been responsible for the lower activity of their mammary macrophages in this assay. Our second experiment was designed to address this problem and to ask a more general question: Are the differences in the ability of mammary macrophages to inhibit the intracellular replication of *B. abortus* in resistant or infected cows intrinsic to the mammary macrophage of an individual cow or a result of the presence or absence of chronic infection? Finally, previous studies used mammary macrophages, thus precluding the possibility of testing bulls. In our last experiment, we extend the previous findings with mammary macrophages to peripheral blood monocyte-derived macrophages and included bulls in the analysis. In the present study, a significantly greater proportion of the macrophages from resistant cattle controlled the intracellular replication of *B. abortus* both pre- and postexposure than did macrophages from susceptible cattle. Our data suggest that differences in the ability of macrophages to control replication of *B. abortus* are determined before exposure to the organism and that macrophages may play a central role in determining the final outcome of exposure to *B. abortus* in cattle.

MATERIALS AND METHODS

Cattle. A group of 21 crossbred (*Bos taurus* × *Bos indicus*) cows and 34 crossbred [*Bos taurus* × (*Bos taurus* × *Bos indicus*)] yearling bulls ($n = 15$) and first-calf heifers ($n = 19$) were studied. The cattle were unvaccinated and had not been exposed to *B. abortus*. Nonexposure to *B. abortus* was confirmed on at least three separate occasions in the 8-month prechallenge period by card, Rivanol agglutination, complement fixation, enzyme-linked immunosorbent assay, and hemolysis-in-gel tests (2, 11, 33). The cattle were challenged at midgestation of their first pregnancy by conjunctival administration of 10^7 CFU of live *B. abortus* 2308. The bulls were from 12 to 16 months of age at the time of challenge. The resistant cattle developed low transient serologic titers and did not abort their fetuses, and bacteriologic cultures of material from the postpartum uterus, lacteal secretions, and calf meconium were negative for *B. abortus* (33). Susceptible cows, conversely, developed high titers and aborted their fetuses, and *B. abortus* was isolated from their lacteal secretions, uterus, placenta, or fetuses (33). The bulls were classified as resistant or susceptible based on whether or not *B. abortus* was obtained from cultures of 50 different tissues tested after the bulls were slaughtered 90 days postchallenge.

The postchallenge classification of the mammary macrophage bacteriostatic activities was done on the 21 crossbred (*Bos taurus* × *Bos indicus*) cows 3 to 5 years postchallenge. Classification of the prechallenge mammary macrophage bacteriostatic activities of 12 first-calf heifers and the prechallenge blood monocyte-derived macrophage bacteriostatic activities of 4 first-calf heifers and 18 yearling bulls were conducted during the 2 months before they were challenged.

Collection of mammary gland macrophages. Bovine mammary gland macrophages were collected by irrigating the lactiferous sinuses of the involuted mammary glands of cows with 50 ml of endotoxin-free sterile saline (18). The cells were washed three times in Hanks balanced salt solution without calcium and magnesium (GIBCO Laboratories, Life Technologies Inc., Grand Island, N.Y.). The macrophage population was enriched by centrifugation at 1,600 × *g* for 30 min over a Ficoll-metrizoate sodium (Lymphoprep; Nycomed A S, Oslo, Norway) density gradient (specific gravity, 1.079). These macrophages were washed three times with 20 ml of Hanks balanced salt solution. More than 95% of the cells in this suspension were macrophages, as determined by their morphology, adherence to plastic, phagocytosis of zymosan, and nonspecific esterase staining (37, 59). Viability was 80 to 90%, as determined by exclusion of 0.2% trypan blue. One hundred microliters of a suspension of 10^6 macrophages per ml of supplemented RPMI 1640 medium (GIBCO Laboratories) was added to each of six wells of a flat-bottom, 96-well microdilution plate (Linbro Laboratories Inc., McLean, Va.). Another 250 μl of medium was added to each well, and the plates were centrifuged at 75 × *g* for 1 min to speed contact and to more evenly distribute the macrophages on the bottoms of the wells. The RPMI 1640 medium was supplemented with 20% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), 4 mM L-glutamine (GIBCO Laboratories), 10 μl of nonessential amino acid solution (Hazelton Research Products Inc., Lenexa, Kans.) per ml, 1 mM sodium pyruvate (GIBCO Laboratories), 100 μg of streptomycin (GIBCO Laboratories) per ml, and 100 IU of penicillin (GIBCO Laboratories) per ml. The microdilution plates containing the macrophages were maintained in an incubator at 37°C in a 7.5% CO_2 atmosphere. Three hours after the macrophages were added, 250 μl of medium was removed from each well and replaced with 250 μl of fresh medium. At 10 and 20 h after the macrophages were added, 250 μl of medium was removed from each well three times and replaced by fresh RPMI 1640 medium without penicillin and streptomycin. This process concurrently washed the adherent macrophages, removed nonadherent cells, and reduced the concentrations of penicillin and streptomycin in the wells to subinhibitory levels for *B. abortus*. Approximately 8×10^3 to 3×10^4 adherent macrophages remained in each well following the wash procedure.

Collection of blood monocyte-derived macrophages. Blood monocytes were collected by separation of heparinized blood in a Ficoll-metrizoate sodium density gradient (specific activity, 1.079) at 1,600 × *g* for 30 min. The interface cells, which contained an enriched population of monocytes and lymphocytes, were suspended in 20 ml of RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 10 μl of nonessential amino acid solution per ml, 1 mM sodium pyruvate, 100 μg of streptomycin per ml, and 100 IU of penicillin per ml in 250-ml tissue culture flasks (Costar, Cambridge, Mass.). Mononuclear cells were allowed to adhere to the plastic surface for 24 h while they were incubated at 37°C in a 7.5% CO_2 atmosphere. Nonadherent cells were first removed by gently rocking the tissue culture flasks and pouring the medium into a second flask. Fresh medium was added to the first flask, and both flasks were incubated for an additional 24 h. The remaining nonadherent cells then were removed from both flasks by one vigorous wash with warm (37°C) Hanks balanced salt solution. The adherent cells were incubated in 20 ml of Hanks balanced salt solution without calcium and magnesium for 12 min to detach the cells (6, 28). The detached cells were centrifuged at 75 × *g* for 5 min and suspended to a concentration of 10^6 cells per ml in supplemented RPMI 1640 medium containing penicillin and streptomycin. One hundred microliters of the suspension was

added to each of six wells of a flat-bottom, 96-well microdilution plate. The resulting monocytes were allowed to mature to macrophages in culture for an additional 5 days at 37°C in a 7.5% CO_2 atmosphere (64). During the 24 h before inoculation of the bacteria, a wash procedure similar to that described above for the mammary macrophages was used to remove unattached cells and reduce the concentrations of penicillin and streptomycin in each well. Following the wash procedure, 1×10^4 to 2.9×10^4 adherent blood monocyte-derived macrophages remained in each well.

Bacteria. *B. abortus* 2308 (kindly provided by B. L. Deyoe, National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa) was grown on Farrell medium plates (23, 24), suspended in barbital buffer, diluted to approximately 10^8 bacteria per ml, and stored at −70°C. A suspension of 1.3×10^5 *B. abortus* 2308 bacteria per ml was incubated for 30 min at room temperature with a subagglutinating (1:100) dilution of heat-inactivated serum pooled from five infected cows (33). The positive pooled serum yielded an optical density value of 1.345 determined by the standard *Brucella* enzyme-linked immunosorbent assay (11), and its background negative control optical density was 0.110. Immediately following opsonification of the bacteria by *Brucella*-specific antibody, the bacterial suspension was incubated for 5 min in a sublytic 1:100 dilution of normal bovine serum (to provide complement) that was negative in the standard *Brucella* enzyme-linked immunosorbent assay. The sera were collected from two unvaccinated and unexposed cows (33).

Bacteriostatic assay. In the postchallenge experiments, 24 h after the mammary macrophages were added to the wells, 1.3×10^4 opsonified bacteria (*B. abortus* 2308) were added to each well of a microdilution plate (including control wells without macrophages). The plates were then centrifuged at $75 \times g$ for 10 min. The resulting initial macrophage-to-bacterium ratio was approximately 0.6:1 to 2.3:1. In the prechallenge experiments, 2.8×10^3 bacterium were used in the assays conducted on the mammary macrophages of first-calf heifers and on the blood monocyte-derived macrophages, resulting in an initial macrophage-to-bacterium ratio of 3.5:1 to 10.7:1. Extracellular bacteria were killed by adding 250 μl of medium containing streptomycin to a final concentration of 14.3 μg/ml in each well 30 and 45 h following bacterial inoculation. Streptomycin is commonly used in vitro to inhibit the extracellular growth of bacteria during the period of intracellular bacterial replication (35, 50, 52). The number of adherent macrophages in the wells was determined immediately before each harvest by using an inverted microscope with a calibrated eyepiece reticle to count three randomly selected representative fields in three different wells. The final ratio of the number of bacteria per macrophage was calculated. The macrophages and *B. abortus* were harvested at 52 h following bacterial inoculation (day 2) and 76 h following bacterial inoculation (day 3) by removing the medium in the wells and adding 250 μl of endotoxin-free distilled water for 10 min to rupture the macrophages. The contents of each well were serially diluted with distilled water, and 100 μl of the appropriate dilution was spread evenly on a tryptose soy agar plate. After a 4-day period of incubation at 37°C, the number of bacteria was determined by counting individual bacterial colonies (CFU) on each tryptose soy agar plate and recording the data based on the dilution factor. The percent survival for each cow was determined by dividing the median number of bacteria obtained from the 3-day harvest by the median number of bacteria obtained from the 2-day harvest for each cow. Macrophages that were able to effect a reduction in the intracellular survival of *B. abortus* to <100% were designated restrictive, and those that were unable to reduce the intracellular survival to <100% were designated permissive.

Three sets of triplicate control wells containing bacteria but not macrophages were used to monitor bacterial growth, the effectiveness of streptomycin in killing the extracellular bacteria, and the effectiveness of the wash procedure in eliminating the penicillin and streptomycin from the wells before the inoculation of bacteria. At the same time as streptomycin was added to kill the extracellular bacteria in the wells containing cells, 250 μl of medium containing streptomycin was added to each of three wells in the first set of control wells to a final concentration of 14.3 μg/ml. These control wells were used to demonstrate that the streptomycin added was adequate to kill all extracellular bacteria. For the second set of control wells, supplemented medium containing penicillin and streptomycin was added to each well, and the wells were washed by the same procedure that was used to reduce the level of penicillin and streptomycin in the wells containing macrophages. For the third set of three control wells, supplemented medium containing penicillin and streptomycin was not added to them. The growth of *B. abortus* in the second set of control wells was compared with the growth of *B. abortus* in the third set of control wells to demonstrate that the concentrations of penicillin and streptomycin were reduced by the wash procedures used on the macrophages to levels that were not inhibitory to the growth of *B. abortus*. A group of each set of control wells was harvested at 30 h, when streptomycin was first added to kill the extracellular bacteria, and at 52 and 76 h, when the macrophages and intracellular *B. abortus* were harvested.

Data analysis. Data are reported as percent survival. Because the populations of cattle were not normally distributed, data were analyzed for significance by the nonparametric Mann-Whitney U test for two samples from Statworks software (Data Metrics, Inc., Philadelphia, Pa.).

RESULTS

Initial studies both with and without antibiotics confirmed that the extracellular bacteria grew much more rapidly than did the intracellular bacteria (data not shown). When no antibiotic was present in the medium, the more rapid extracellular replication of *B. abortus* 2308 obscured any inhibition of the intracellular bacterial replication and resulted in highly varied numbers of bacteria within sets of macrophages. In the studies described herein, at 30 h postinoculation, the number of bacteria in the control wells had increased an average of 2.9 log units (range, 2.9 to 3.0 log units) in each set of experiments (Table 1). The bacteria continued to grow to approximately 4.5 log units (range, 4.4 to 4.7 log units) at 52 h postinoculation, when the first harvest was taken (day 2). The growth of the bacteria slowed during the next 24 h, and the total growth was approximately 4.9 log units (range, 4.6 to 5.1 log units) at 76 h postinoculation (day 3). At the 2- and 3-day harvests no growth of bacteria from control wells to which streptomycin was added in each experiment occurred on tryptose soy agar plates, indicating that all extracellular bacteria were killed by the time the bacteria were harvested from the wells. Preliminary experiments showed that opsonified *B. abortus* (anti-*B. abortus* antibodies and complement) grew as well as nonopsonified *B. abortus* did (data not shown). Preliminary experiments also showed that the distilled water that was used to

TABLE 1. Growth of *B. abortus* 2308 opsonified with specific antibody and bovine complement in control wells without macrophages or antibiotics

Day[a]	No. of bacteria present in wells after the following expt[b]:		
	Postchallenge macrophage (n = 10)[c]	Prechallenge macrophage (n = 4)[d]	Prechallenge monocyte (n = 7)[e]
0	4.09 ± 0.16	3.43 ± 0.05	3.43 ± 0.10
1	7.06 ± 0.35	6.28 ± 0.99	6.31 ± 0.31
2	8.45 ± 0.19	8.17 ± 0.51	7.95 ± 0.27
3	8.67 ± 0.16	8.55 ± 0.21	8.35 ± 0.19

[a] Number of days following inoculation of test plates with *B. abortus* 2308.
[b] Values are the base 10 logarithm of the mean (±1 standard error of the mean) number of bacteria present in the wells.
[c] Mammary macrophages were collected from cows that were previously challenged by conjunctival inoculation of 10^7 CFU of live *B. abortus* 2308.
[d] Mammary macrophages were collected from cows that had no history of prior exposure to *B. abortus* confirmed by standard serology tests.
[e] Blood monocyte-derived macrophages were collected from cows that had no history of prior exposure to *B. abortus* confirmed by standard serology tests.

rupture the macrophages did not cause osmotic rupture of the bacteria (data not shown).

Macrophages were collected from groups of five to seven cows for individual assays. The results from one of these assays are described in detail in Table 2. The mean number of bacteria present in each well represented the total number of intracellular bacteria. Variations in the mean total number of bacteria harvested from each set of macrophages depended on (i) the phagocytic abilities of the macrophages in that set, (ii) the total number of adherent macrophages present in the well, and (iii) the number of bacteria added to macrophages. Occasionally, one or two of the replicates of each set of macrophages had values dissimilar to those of the remaining replicates. As a result some of the means presented in Table 2 have relatively large standard deviations. By using the median value for the replicates, we were able to minimize the effect of extreme values.

TABLE 2. Results of a single representative experiment depicting in vitro intracellular survival of *B. abortus* 2308 in bovine mammary macrophages after opsonification with specific antibody and bovine complement

Cow no.[a]	No. of bacteria (mean + SD)[b]		No. of bacteria/cell[c]		Survival (%)[d]
	Day 2	Day 3	Day 2	Day 3	
524R	3,612 ± 690	175 ± 162	0.87	0.04	5
622R	6,068 ± 1,793	2,402 ± 873	0.59	0.19	32
618R	20,625 ± 4,667	11,770 ± 1,168	2.66	1.45	55
610S	31,662 ± 9,479	9,423 ± 5,994	1.55	0.49	31
530S	95,590 ± 20,691	117,168 ± 28,133	4.73	4.79	101
616S	2,805 ± 524	3,502 ± 1,028	0.04	0.07	157
614S	2,060 ± 777	5,182 ± 4,650	0.20	0.49	243

[a] The R or S after the cow number represents the classification of the cattle as resistant or susceptible, respectively, based on the results of challenge by conjunctival administration of 10^7 CFU of live *B. abortus* 2308.
[b] The wells containing macrophages and control wells without macrophages were initially inoculated with 2.84×10^3 CFU of live *B. abortus* 2308. Wells without macrophages on day 3 postinoculation contained 4.26×10^8 CFU. The mean and standard deviation of the total number of bacteria per well were based on six individual wells per animal.
[c] The number of bacteria per macrophage was calculated by dividing the mean number of total bacteria in six wells by the mean number of viable macrophages in those wells.
[d] The percentage of bacteria surviving at 3 days postchallenge was determined by dividing the mean number of bacteria per cell present at 3 days postchallenge by the mean number of bacteria per cell present at 2 days postchallenge and multiplying the result by 100.

TABLE 3. Results of multiple experiments on individual cows assessing the ability of mammary macrophages to control the intracellular survival of *B. abortus* 2308

Expt date (mo/day/yr)	% Intracellular survival for the following cows[a]:							
	22S	192R	202R	210R	218R	49VF	53VF	341S
1/19/88	—[b]	21	43	53	62	112	148	111
1/11/89	—	23	—	—	—	127	—	—
1/30/89	—	75	—	—	—	107	—	—
5/7/89	20	—	51	42	—	158	110	—
5/12/89	25	—	50	—	54	180	148	116

[a] The R or S after the cow number represents the classification of the cattle as resistant or susceptible, respectively, based on the results of challenge by conjunctival administration of 10^7 CFU of live *B. abortus* 2308. The VF (vaccine failure) indicates that the cow was classified as having a susceptible phenotype when challenged after vaccination with strain 19.
[b] —, Missing data indicate experiments for which the macrophages from that cow were not tested.

In multiple experiments in which the same cows were tested repeatedly over a 2-year period, there was variation in the intracellular survival for each cow from experiment to experiment, but the intracellular survival was almost always in a range that would not alter the macrophage classification of the cow as restrictive or permissive (Table 3).

The in vitro bacteriostatic activities of mammary macrophages from resistant and susceptible cows were compared postchallenge (Fig. 1). The median percent survival of *B. abortus* 2308 in 11 resistant cows was 55 (range, 14 to 216%), whereas the median percent survival of *B. abortus* 2308 in 10 susceptible cows was 104 (range, 32 to 329%). The abilities of the macrophages from the two groups of cows to inhibit the intracellular replication of *B. abortus* 2308 were significantly different ($P < 0.026$). Macrophages from resistant cattle were generally more capable than those from suscep-

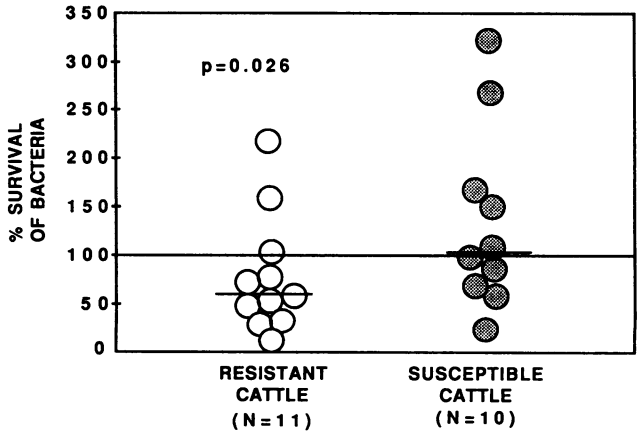

FIG. 1. Ability of bovine mammary macrophages from a group of 21 cows after exposure to *B. abortus* to control the in vitro intracellular replication of *B. abortus* 2308 opsonified with specific antibody and bovine complement compared with the postchallenge *B. abortus* infection statuses of the cows. Percent bacterial survival at 3 days is based on colony counts at 2 days post-tissue culture inoculation. The control of replication of *B. abortus* was significantly different ($P < 0.026$) between the macrophages from resistant and susceptible cows. The short horizontal lines signify the median percent survival for each group.

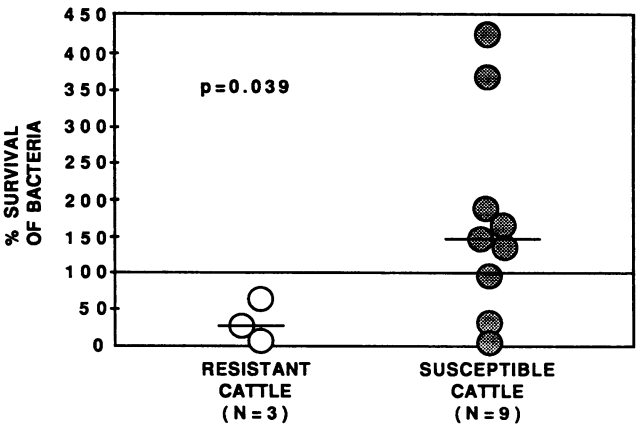

FIG. 2. Ability of bovine mammary macrophages from a group of 12 heifers previously unexposed to *B. abortus* to control the in vitro intracellular replication of *B. abortus* 2308 opsonified with specific antibody and bovine complement compared with the postchallenge *B. abortus* infection statuses of the cows. Percent bacterial survival at 3 days is based on initial counts at 2 days post-tissue culture inoculation. The control of replication of *B. abortus* was significantly different ($P < 0.039$) between the macrophages from resistant and susceptible cows. The short horizontal lines signify the median percent survival for each group.

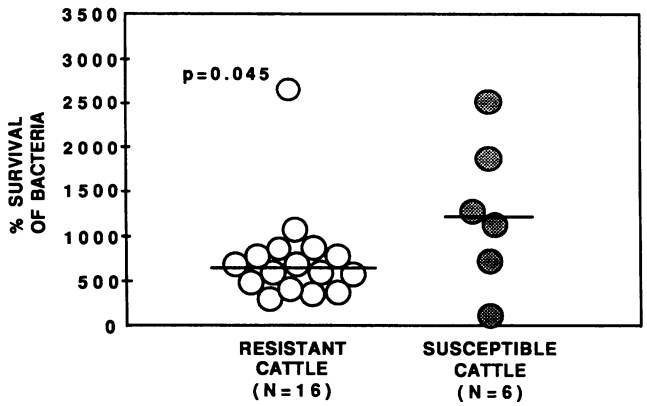

FIG. 3. Ability of bovine blood monocyte-derived macrophages from yearling bulls ($n = 18$) and first-calf heifers ($n = 4$) that were previously unexposed to *B. abortus* to control the in vitro intracellular replication of *B. abortus* 2308 opsonified with specific antibody and bovine complement compared with the postchallenge *B. abortus* infection statuses of the cattle. Percent bacterial survival at 3 days is based on colony counts at 2 days post-tissue culture inoculation. The survival of *B. abortus* was significantly different ($P < 0.045$) between blood monocyte-derived macrophages from resistant and susceptible cattle. The horizontal lines signify the median percent survival for each group.

tible cattle of reducing the percent intracellular survival of the organisms. Mammary macrophages from 9 of 11 resistant cattle (82%) were able to inhibit the intracellular replication of *B. abortus* during the 24-h interval they were studied. Those macrophages that were able to inhibit the intracellular replication of *B. abortus* were considered to be restrictive. During the same time interval, the macrophages from only 5 of 10 susceptible cows (50%) were able to inhibit the intracellular replication of the bacteria. The macrophages that were unable to inhibit the intracellular replication of *B. abortus* were considered to be permissive. The percentages of bacteria that survived within macrophages from resistant and susceptible cows were consistent with the classification of the cattle into those two groups 67% of the time. Although we did not consider it likely, we could not rule out the possibility that the restrictive macrophages were better at internalizing the streptomycin and delivering it to the phagosome.

Experiments with mammary macrophages collected from groups of heifers before challenge were designed to determine whether the ability of the mammary macrophage to inhibit intracellular growth was intrinsic to the macrophage or was a result of the presence or absence of chronic infection (Fig. 2). The median percent survival of *B. abortus* in macrophages from a group of three resistant cows was 31 (range, 3 to 61%), whereas the median percent survival of *B. abortus* in macrophages of nine susceptible heifers was 148 (range, 7 to 432%). The resistant and susceptible cows were significantly different ($P < 0.039$) in the prechallenge ability of their mammary macrophages to inhibit the intracellular replication of *B. abortus* 2308. The mammary macrophages from all three resistant heifers were able to reduce the numbers of intracellular organisms over a 24-h period, but the macrophages from only 3 of 9 cows (33%) which were classified as susceptible were able to inhibit the intracellular replication of the bacteria. By using mammary macrophages from heifers before they were exposed to *B. abortus*, the survival assay was able to correctly predict the eventual classification of the cattle as resistant or susceptible 75% of the time.

Subsequent prechallenge experiments were designed to extend the findings with mammary macrophages to blood monocyte-derived macrophages. Survival of *B. abortus* in blood monocyte-derived macrophages collected from the yearling bulls ($n = 18$) and first-calf heifers ($n = 4$) before challenge inoculation was significantly different ($P < 0.045$). The median percent survival of *B. abortus* in blood monocyte-derived macrophages from a group of 16 resistant cows was 566 (range, 271 to 2,670%), whereas the median percent survival of *B. abortus* in blood monocyte-derived macrophages of 6 susceptible heifers was 1,260 (range, 80 to 2,560%) (Fig. 3). Although the survivability of the intracellular bacteria in blood monocyte-derived macrophage cultures overall was 10-fold greater than that in mammary macrophage cultures, monocyte-derived macrophages from resistant cattle were better able to regulate the intracellular replication of the bacteria than were those from susceptible cattle. The ability of the monocyte-derived macrophages to restrict the intracellular survival of *B. abortus* 2308 appears to be grouped below 1,000%. If the data are arbitrarily divided at 1,000%, 14 of 16 resistant cattle (88%) but only 2 of 6 susceptible cattle (33%) were able to restrict the replication of the intracellular bacteria to less than 1,000%. The in vitro assignment of monocyte-derived macrophage function as permissive or restrictive correlated 82% of the time with the postchallenge phenotypic classification as resistant or susceptible.

In addition to the susceptible cows that had permissive mammary macrophages which were unable to control the replication of the bacteria, there were some susceptible cattle (e.g., 610S) with restrictive mammary macrophages that were able to restrict the survival of the bacteria to less than 100%. Although not shown in the experiment for which the results are depicted in Table 2, there were also examples of resistant cows with permissive mammary macrophages that were unable to control the replication of the bacteria. In

some cows that had intracellular bacterial survival percentages near 100%, the variation in the values resulted in no significant difference between the 2- and 3-day harvests.

DISCUSSION

Our in vitro studies demonstrated that the mammary macrophages and blood monocyte-derived macrophages of resistant and susceptible cows are significantly different in their ability to control the intracellular survival of *B. abortus*. This ability may be important in the natural resistance or susceptibility of the animal to bovine brucellosis.

Earlier studies (32, 33) were conducted with cattle that were previously inoculated with *B. abortus*; therefore, macrophage function suppressed by concurrent brucellosis in the susceptible cows could not be eliminated as a possible cause for differences in macrophage function between the two groups. In the present study, we addressed that question by using mammary gland- and blood monocyte-derived macrophages obtained from cattle before challenge with *B. abortus* 2308. The assays were performed before the cattle were challenged. There were significant differences in the bacteriostatic activities of macrophages from resistant and susceptible cattle (Fig. 2 and 3), and the data demonstrate a tighter grouping of the resistant cattle, with the macrophages from a majority of resistant cattle having superior bacteriostatic abilities.

Testing of mammary macrophages limited our results to adult female animals. In order to evaluate the resistance of the male offspring and include them in future genetic studies, we also assayed blood monocyte-derived macrophages. Although the blood monocyte-derived macrophages from the cattle did not control intracellular bacterial replication, there were significant differences in the replication-inhibiting activities of blood monocyte-derived macrophages obtained from resistant and susceptible cattle prechallenge (Fig. 3). There are several possible explanations for the reduced ability of blood monocyte-derived macrophages to control the intracellular replication of *B. abortus*. Monocytes are less differentiated cells and, possibly (despite the 7 days in culture to allow them to mature), were not fully differentiated under the conditions of these assays. It is also considered possible that the extended period that the monocytes were in culture compared with that of the mammary macrophages in our experiments resulted in a loss of the surface receptors that are necessary for phagocytizing the organisms. Further investigation into the harvesting of blood monocytes and the culturing of the blood monocyte-derived macrophages may provide more stable functional cells. The blood monocyte-derived macrophages should be a better source of macrophages for evaluating the genetically predetermined abilities of the mononuclear-phagocyte system against brucellosis in cattle for a number of reasons. First, the mammary gland is a terminal compartment for infiltrating inflammatory cells (60). The leukocytes in this compartment can die and disintegrate gradually. Therefore, phagocytic cells in the mammary gland can possess a wide range of phagocytic and bactericidal activities (60). Second, the ability of the mammary phagocytes to phagocytize and inhibit the replication of intracellular organisms is affected by proteins and immunoglobulins of bovine colostrum (19), inhibiting factors in lacteal secretions from nonlactating cows (25), and phagocytized milk fat globules and casein (51, 53). Factors that cause variations in the phagocytic abilities of mammary phagocytes include the age of the cow, mammary gland quarter, and lactation stage (42). In addition, differences are attributed to variations introduced into phagocytic assays as a result of isolation of the macrophages, repeated lavages, and variations in samples harvested on different days (46–48). The blood monocyte-derived macrophages thus appear to be a more uniform, albeit less differentiated, population of macrophages that have not been modified by conditions in the local microenvironment of the mammary gland.

The mononuclear phagocyte is a central cell in the resistance to facultative intracellular pathogens in mice. Natural resistance to *B. abortus* and other intracellular parasites in mice is a result of the control of the early survival and growth of the organisms. This resistance is genetically determined and is mediated at the level of macrophage function (7, 12, 16, 34, 39, 40, 44, 55, 56). The natural resistance of outbred cattle to brucellosis may parallel that which has been demonstrated in the inbred strains of mice; however, further characterization of these cattle is necessary to show that the functions are homologous. The cattle used in this study were completely outbred, whereas in all the mouse studies inbred strains were evaluated.

The role of macrophages in natural resistance to infectious diseases in mice has been shown to be under the control of a single gene or gene complex for four infectious organisms (8, 15, 51): *Mycobacterium lepraemurium* (9), *Salmonella typhimurium* (*Ity*) (49), *Listeria donovani* (*Lsh*) (8), and *Mycobacterium bovis* (*Bcg*) (29). This *Ity/Bcg/Lsh* gene, or gene complex, is located on chromosome 1 of the mouse. Natural resistance to brucellosis in cattle is thought to be determined by the actions of several interacting genes (61). Genetic studies (in pedigreed families) that evaluate the macrophage function (of resistant and susceptible progeny of resistant and susceptible cattle) will further define the heritability of these observed differences in resistance to infectious diseases. Studies that test the ability of macrophages from cattle that are resistant and susceptible to brucellosis to control the intracellular survival of other primary intracellular pathogens, such as *Salmonella dublin*, *S. typhimurium*, and *M. bovis*, may further demonstrate that cattle have an innate natural resistance to facultative intracellular pathogens like that observed in mice. Encouragement to search for genes that control natural resistance to intracellular pathogens in cattle has come from the recently reported conservation of synteny of the genes for isocitrate dehydrogenase-1 (*Idh*-1), gamma crystallin (*Cryg*), fibronectin (*Fn*), and villin (*Vil*) in cattle (1; L. C. Skow, Department of Veterinary Anatomy, Texas A&M University, College Station, Tex., personal communication), mice (54, 57), and humans (54, 63). The *Ity/Bcg/Lsh* gene, or gene complex, is linked to *Vil*, *Fn*, *Cryg*, and *Idh*-1 in mice.

It is clear that the macrophage is not the sole determining factor in the final outcome of the infection. In each experiment, there were exceptions. Some resistant cattle were able to clear the infection despite the presence of permissive macrophages that could not inhibit the intracellular replication of *B. abortus*, and several susceptible cattle became chronically infected despite the presence of restrictive macrophages that efficiently inhibited the intracellular replication of *B. abortus*. Additionally, even with repeated washings to remove nonadherent cells, we were not able to completely eliminate the possibility of some T-cell contamination in some sets of macrophages. The possibility of some effect on the ability of the mammary macrophages used in our studies to control the intracellular replication of *B. abortus*, resulting from the interactions between macrophages and T cells, could not be entirely dismissed. How-

ever, the prechallenge studies would suggest that T-cell contamination is not an important factor in these experiments, because these prechallenge experiments were conducted before an adaptive immune response to *B. abortus* was developed.

Recent studies have demonstrated that the expression of immunoglobulin allotypes (Estes, personal communication) and the reactivity of oligoclones of *B. abortus*-specific lymphocytes (Smith et al., in press) were significantly different between resistant and susceptible cattle. If each of these three immune components is controlled by a single gene, then natural resistance to brucellosis would be controlled by a minimum of three genes in cattle. Of course, this does not rule out the possibility that a single gene could be influencing the response of all three immune components or that additional immune components (and genes) that are as yet unstudied could be involved.

The observation of significant differences in the bacteriostatic capabilities of mononuclear phagocytes in genetically diverse crossbred cattle, which were grouped entirely by their resistance or susceptibility to brucellosis, is remarkable because of the genetic uniqueness of each individual animal. Our data suggest that although the mononuclear phagocytes are not the sole determining factor in the outcome of *Brucella* infection, they are likely central players that interact with other immune components in determining whether cattle are resistant or susceptible to brucellosis. If a functional equivalent of the *Ity/Bcg/Lsh* gene or gene complex demonstrated in mice is conserved in cattle and the gene could be identified with a DNA sequence probe or a defined gene product, a strategy could be devised to increase the frequency of operative genes that could be used to enhance natural disease resistance to intracellular pathogens in cattle, which should be a helpful addition to overall herd health and also the brucellosis control and eradication programs.

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