Opsonin-dependent stimulation of bovine neutrophil oxidative metabolism by *Brucella abortus*

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SUMMARY

Nonopsonized *Brucella abortus* and bacteria treated with fresh antiserum, heat-inactivated antiserum, or normal bovine serum were evaluated for their ability to stimulate production of superoxide anion and myeloperoxidase-mediated iodination by neutrophils from cattle. *Brucella abortus* opsonized with fresh antiserum or heat-inactivated antiserum stimulated production of approximately 3 nmol of O_2^-/10^6 neutrophils/30 min. Similarly treated bacteria also stimulated the binding of approximately 4.3 nmol of NaI/10^7 neutrophils/h to protein. Significant ($P < 0.05$) production of O_2^- and iodination activity by neutrophils were not stimulated by nonopsonized bacteria or organisms treated with normal bovine serum. Seemingly, *B abortus* stimulated oxidative metabolism in bovine neutrophils; however, the stimulation was dependent on the presence of bacterial associated opsonins.

The etiologic agent of bovine brucellosis is the facultative intracellular bacterium *Brucella abortus*. Mechanisms responsible for the organism's ability to survive within polymorphonuclear neutrophils (PMN) are not fully understood. Smooth and rough strains of *B abortus* are readily ingested by bovine and human PMN.[1,2] Virulent *B abortus* also does not possess surface-associated substances that inhibit the ability of PMN to ingest radiolabeled *Staphylococcus aureus*.[3] Ingestion of *B abortus* by human PMN did not appear to stimulate hexose monophosphate shunt activity, as measured by oxidation of [^{14}C]glucose.[5] Bacteria used in that study were treated with heat-inactivated homologous serum. Ingestion of nonopsonized *B abortus* did not stimulate production of O_2^-, as measured by quantitative nitroblue tetrazolium dye reduction, because of a lack of stimulation of oxidative metabolism on ingestion.[6] Seemingly, a lack of stimulation of the oxidative metabolic burst enhances the intracellular survival of *B abortus*. However, this interpretation may apply only to nonopsonized *B abortus*, because neither of previous studies[5,6] used specific antibody as an opsonin. Opsonized *B abortus* is capable of eliciting an oxidative response by bovine mammary gland macrophages.[4] The observation that opsonized zymosan-stimulated production of O_2^- by bovine PMN is not inhibited in the presence of *B abortus* indicates that the bacteria do not specifically inhibit O_2^- production by neutrophils.[3]

Virulent *B abortus* inhibits the myeloperoxidase (MPO)-H_2O_2-halide antibacterial reaction of PMN.[3] Fractions 3b and 10, responsible for this inhibition, were isolated from the < 1,000-dalton fraction of supernatant preparations of heat-killed *B abortus*. The inhibition of the MPO-H_2O_2-halide activity is the result of specific suppression of peroxidase-positive granule degranulation.[7] Fraction 3b has been identified as 5'-guanosine monophosphate, and fraction 10 has been identified as adenine.[8] The purpose of the study reported here was to determine whether different opsonins (C3b, antibody) altered the oxidative metabolic response of bovine PMN to ingestion of *B abortus*.

Materials and Methods

Preparation of bacteria—Procedures used to grow *B abortus* have been described.[3] Briefly, smooth virulent strain 2308 of *B abortus* was grown in a fermentor. After 48 hours' incubation at 37 C, bacteria were harvested in log phase, washed 3 times with saline solution (0.85% NaCl), and suspended in Earle balanced salt solution (EBSS)[a] without phenol red at a concentration of 5 × 10^9 bacteria/ml.

A culture of *S aureus* strain 305 was obtained[b] for use as a control organism that is known to be killed readily on ingestion by bovine PMN. Bacteria were inoculated into flasks containing 100 ml of Trypticase soy broth.[c] After 18 hours' incubation at 37 C with agitation, cells were harvested by centrifugation, washed 3 times with saline solution, and suspended in EBSS at a density of 5 × 10^9 bacteria/ml.

Opsonization of bacteria and zymosan—Suspensions of *B abortus* were opsonized with 1 of 3 serum preparations. Serum containing anti-*B abortus* 2308 antibody (standard agglutination titer = 1:3,200) was obtained from a *B abortus*-infected cow. This antiserum preparation also was used after heating at 56 C for 30 minutes to inactivate complement components. A pool of normal bovine serum was prepared from freshly collected blood obtained from 3 steers seronegative for anti-*Brucella* antibodies. Suspensions of *S aureus* were opsonized with a subagglutinating titer of a pool of fresh antiserum preparations from 6 cows infected with *S aureus* 305.

Aliquots (1 ml) of standardized bacterial suspensions were added to 1.5-ml microcentrifuge tubes and were mixed with 0.25 ml of the desired opsonizing serum. Tubes were placed in a 37 C water bath and were incubated for 15 minutes. After incubation, bacteria were pelleted by centrifugation at 9,000 × g for 5 minutes. The supernatant was discarded, and bacteria were suspended in 1 ml of EBSS.

Opsonization of zymosan particles with a pool of normal bovine serum from 3 steers was performed as described.[9] This procedure results in the deposition of C3b on the surface of the zymosan particles via the alternate pathway of complement fixation.

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[a] Grand Island Biological Co, Grand Island, NY.

[b] Obtained from the Mastitis Research Unit, National Animal Disease Center, Ames, Iowa.

[c] BBL Microbiology Systems, Cockeysville, Md.

PMN preparation—Eight healthy adult mixed-breed steers free of detectable anti-*Brucella* antibodies, as determined by the standard tube agglutination assay, were used as a source of PMN. These steers were housed in outdoor pens and were fed hay and grain once daily. Neutrophils were isolated as described[9] and were suspended in RPMI-1640 medium[a] to a concentration of 1×10^6 cells/ml.

Complement fixation by B abortus—The ability of live *B abortus* 2308 to fix complement was evaluated by an indirect fluorescent antibody technique. Suspensions of *B abortus* were treated with 1 of the 3 serum preparations used to opsonize the bacteria as described. Preparations consisting of bacteria treated with EBSS rather than with the primary opsonizing serum were included as negative controls.

Aliquots (0.1 ml) of the standardized bacterial suspensions were added to 1.5-ml microcentrifuge tubes and were mixed with 0.1 ml of the desired primary opsonizing serum. Tubes were placed in a 37 C water bath and were incubated for 30 minutes. After incubation, bacteria were washed twice with EBSS by centrifugation at 9,000 × g for 5 minutes. Bacteria were suspended in 0.1 ml of a 1:10 dilution of anti-bovine C3 antibody conjugated with fluorescein isothiocyanate.[d] This antiserum reacted with bovine C3b on the surface of zymosan-particles treated with normal bovine serum. Treatment of bacteria with heat-inactivated anti-*B abortus* serum was used as a control to check for cross-reactivity of the anti-C3 antibody with other serum components, such as immunoglobulins or albumin. The conjugated antibody was allowed to react with the bacteria for 30 minutes at 37 C. After incubation, bacteria were washed twice with EBSS by centrifugation and were suspended in 0.1 ml of mounting medium containing a 9:1 solution of glycerin and EBSS, respectively. Preparations were examined by reflected fluorescence microscopy.

PMN function assays—Cytochrome C reduction—This test measures the amount of O_2^- produced by PMN during the oxidative metabolic burst. The assay was performed in 96-well plates with membrane filters attached to the bottom of the wells.[e] Samples were evaluated in duplicate, and the average of duplicate values was used for calculation as described.[8] The ability of bacterial preparations to stimulate O_2^- production was evaluated by replacing opsonized zymosan in the standard reaction mixture with 0.025 ml of a standardized bacterial suspension (1.25×10^6 cells). Control wells containing 10 μg of superoxide dismutase (SOD) suspended in 0.01 ml of EBSS in addition to the usual reaction mixture were included to evaluate the specificity of the reaction for O_2^- production. After incubation at 37 C for 30 minutes with agitation, culture filtrates were collected, and the optical density of the solutions at 550 nm was determined, using a micro-ELISA spectrophotometer.[f] The nmoles of O_2^- produced by 10^6 PMN in 30 minutes were determined, using the extinction coefficient of 2.1×10^4 cm^{-1}.

Iodination assay—The iodination assay (a measure of MPO-H_2O_2-halide activity by neutrophils)[10] was performed as described[8] in duplicate, and the average of duplicate values was used for calculations. This assay was also performed in 96-well plates with membrane filters attached to the bottom of the wells.[e] To determine effects of the mixtures of the bacterial preparations on MPO-H_2O_2-halide activity of opsonized zymosan-stimulated PMN, 0.05 ml of a bacterial preparation (2.5×10^8 organisms) or 0.05 ml of EBSS as a control was added to the standard reaction mixture. The ability of bacterial preparations to stimulate iodination activity was evaluated by replacing the opsonized zymosan in the standard reaction mixture with 0.025 ml of a standardized bacterial suspension (1.25×10^6 cells). After incubation at 37 C for 20 minutes with agitation, cells were washed twice by filtration with 0.25 ml of EBSS, and filters were transferred to 12 × 75-mm tubes. The amount of entrapped radioactivity was determined, using a gamma counter,[g] and results were expressed as nmoles of NaI/10^7 PMN/h.

Statistical evaluation—To determine effects of different preparations of opsonized bacteria on each PMN function, the value obtained when a bacterial preparation was added to PMN was compared with control (EBSS-treated) PMN from the same steer. An analysis of variance procedure (blocked by day) or a Student *t* test was used to determine significance of differences in PMN function.

Results

Complement fixation by B abortus—Results of the complement fixation assays involving live *B abortus* indicated that bacteria treated with normal bovine serum or anti-*B abortus* 2308 serum fixed C3b at or near the cell surface. Conversely, cell-associated C3b was not observed on bacteria treated with heat-inactivated antiserum or EBSS-treated control preparations. The lack of staining noticed with the heat-inactivated antiserum indicated that the anti-C3 antibody did not cross-react with serum components other than C3 (immunoglobulin or albumin).

Stimulation of superoxide anion production—Significant ($P < 0.05$) production of O_2^- was stimulated by opsonized zymosan, mixtures of *B abortus* or *S aureus* with opsonized zymosan, *B abortus* or *S aureus* opsonized with fresh antiserum, and *B abortus* opsonized with heat-inactivated antiserum (Fig 1). Production of O_2^- was not stimulated by nonserum-treated *B abortus* or *B abortus* incubated with normal bovine serum. Neutrophils stimulated in the presence of SOD did not produce detectable reduction of cytochrome C. Neither *B abortus* nor *S aureus* induced reduction of cytochrome C in the absence of PMN. Opsonized zymosan-stimulated production of O_2^- by PMN was not significantly ($P > 0.05$) affected in the presence of *B abortus* or *S aureus*.

The amount of O_2^- produced by PMN stimulated with *B abortus* or *S aureus* opsonized with fresh antiserum was significantly ($P < 0.01$) less than that produced by cells stimulated with opsonized zymosan (3 nmol/10^6 PMN/30 min vs 13 nmol/10^6 PMN/30 min, respectively; Fig 1). There was no significant ($P > 0.05$) difference between the amount of O_2^- produced by PMN stimulated with *S aureus* opsonized with fresh antiserum and the amount produced by PMN stimulated with similarly treated *B abortus*. The amount of O_2^- produced by PMN stimulated with *B abortus* opsonized with fresh antiserum was similar to the amount produced by cells exposed to bacteria opsonized with heat-inactivated antiserum (3.2 ± 0.3 nmol/10^6 PMN/30 min vs 2.8 ± 0.3 nmol/10^6 PMN/30 min, respectively).

Effects of bacterial preparations on iodination—Significant ($P < 0.01$) increases in iodination activity occurred when PMN were stimulated with opsonized zymosan (32.4 ± 1.8 nmol of NaI/10^7 PMN/h; Fig 2). Opsonized zymosan-stimulated iodination was significantly ($P < 0.01$) suppressed to 66.7 ± 8.0% (mean ± SEM) of control in the presence of live *B abortus* (Fig 2). Opsonized zymosan-

[a] Cooper Biomedical, Malvern, Pa.
[e] Model SV, 0.45 μm pore size, Millipore Co, Bedford Mass.
[f] Model MR 600, Dynatech Corp, Alexandria, Va.
[g] Multi-Prias 1, Packard Instruments Co, Downers Grove, Ill.

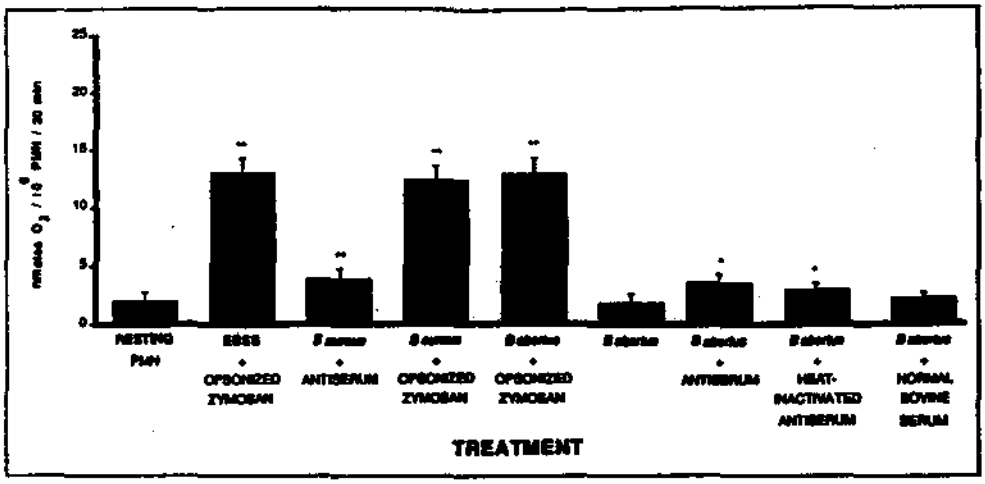

Fig 1—Superoxide anion production by PMN stimulated with opsonized zymosan or various bacterial preparations (mean ± SEM, n = 8). Asterisks denote a significant different response from resting cells. * $P < 0.05$; ** $P < 0.01$.

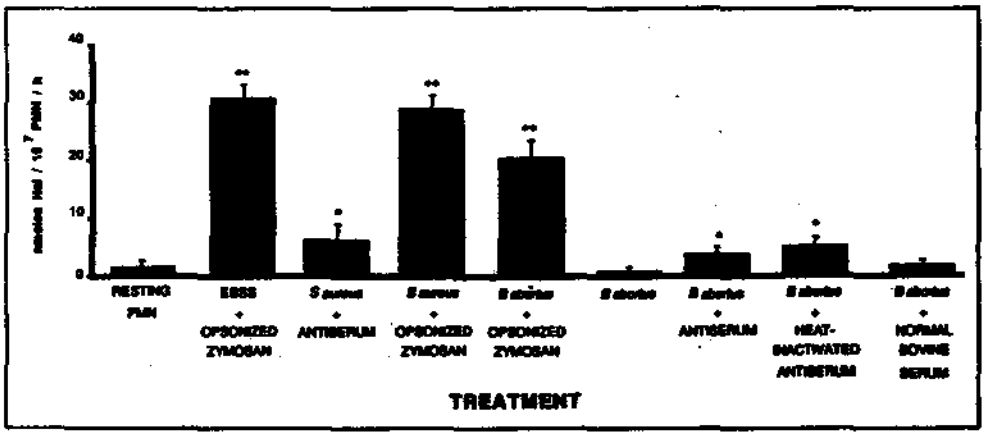

Fig 2—Iodination responses of PMN stimulated with opsonized zymosan or various bacterial preparations (mean ± SEM, n = 8). Asterisks denote a significant different response from resting cells. * $P < 0.05$; ** $P < 0.01$.

stimulated iodination was not affected in the presence of *S aureus*.

Significant ($P < 0.05$) amounts of iodination activity were stimulated by *B abortus* opsonized with fresh or heat-inactivated antiserum and *S aureus* opsonized with fresh antiserum. Iodination did not occur in PMN stimulated with nonserum-treated *B abortus* or bacteria treated with normal bovine serum (Fig 2). *Brucella abortus* opsonized with fresh antiserum appeared to stimulate less iodination than did similarly treated *S aureus* (3.5 nmol of NaI/10^7 PMN/h vs 6.5 nmol of NaI/10^7 PMN/h, respectively); however, this difference was not significant ($P > 0.05$). The amount of iodination activity stimulated by *B abortus* opsonized with fresh antiserum was lower than, but not significantly different from, the amount stimulated by bacteria opsonized with heat-inactivated antiserum (3.5 ± 0.9 nmol of NaI/10^7 PMN/h vs 5.0 ± 1.0 nmol of NaI/10^7 PMN/h, respectively).

Discussion

Ingestion of an opsonized particle by PMN generally stimulates an oxidative metabolic burst, resulting in the formation of toxic oxygen radicals capable of destroying microorganisms. Failure of stimulation of an oxidative metabolic burst response by PMN on ingestion of bacteria would induce a decreased amount of anti-bacterial activity resulting in increased survival of the ingested microorganism. The primary opsonins in bovine sera include antibody against specific bacterial surface antigens and the C3b component of the complement system. Ingestion of nonopsonized bacteria generally results in a diminished oxidative metabolic response by phagocytic cells when compared with that stimulated by opsonized bacteria. Ingestion of *B abortus* does not appear to stimulate an oxidative metabolic response by PMN.[5,6] Those studies indicated that a lack of stimulation of the oxidative metabolic burst compromises the bactericidal capability of the PMN, resulting in enhanced intracellular survival of *B abortus*. Organisms used in the previous studies[5,6] were not exposed to serum or were treated with heat-inactivated normal serum. Heat-inactivated normal bovine serum would possess marginal opsonic activity because of a lack of specific antibody and active complement components. Neither study used serum containing specific anti-*B abortus* antibody as an opsonin. Those studies also indicated that although the organisms were not opsonized, they were ingested by the PMN.

In the present study, *B abortus* exposed to fresh serum containing anti-*B abortus* antibody, heat-inactivated anti-*B abortus* serum (presumably free of complement activity), or normal bovine serum (free of anti-*B abortus* antibody), were evaluated for their ability to stimulate an oxidative response. Seemingly, *B abortus* opsonized with fresh antiserum or heat-inactivated antiserum was capable of stimulating production of O_2^-. These results confirm those of a previous study,[4] which indicated opsonized *B abortus* is capable of stimulating an oxidative meta-

bolic burst by mammary macrophages on ingestion. Amounts of O_2^- formed in response to ingestion of organisms opsonized with fresh antiserum or heat-inactivated antiserum were similar, indicating that exposure of antibody-coated *B abortus* to complement does not enhance its ability to stimulate O_2^- production by PMN above the amount stimulated by bacteria coated with antibody alone. Nonopsonized bacteria and bacteria treated with normal bovine serum did not appear to stimulate formation of O_2^-, confirming the findings of previous investigators.[5,6] Collectively, these results indicate that C3b does not serve as an efficient opsonin of *B abortus*. One explanation of this phenomenon is that *B abortus* does not fix complement on its surface. However, the results of complement-fixation assays indicate that *B abortus* fixes complement in the presence and absence of specific antibody. It is not known if the bound C3b is exposed for interaction with PMN C3b receptors. *Brucella abortus* treated with normal bovine serum (C3b opsonization) stimulated an increase in luminol-assisted chemiluminescence activity of mammary macrophages.[4] The discrepancy between our results, and those of the study by Harmon and Adams[4] may be a result of differences in techniques used to measure the oxidative metabolic response or a difference between the oxidative metabolic responses of PMN and macrophages.

Neither *B abortus* nor *S aureus* inhibited the ability of opsonized zymosan-stimulated PMN to produce O_2^-, indicating that neither organism inhibits the ability of PMN to produce an oxidative metabolic burst. Amounts of O_2^- produced in response to ingestion of *B abortus* opsonized with fresh antiserum were comparable with those obtained with similarly treated *S aureus*. Both bacteria stimulated significantly less O_2^- production by PMN than did opsonized zymosan. Although the amount of O_2^- production stimulated by the bacterial preparations was relatively small, results of control experiments involving SOD inhibition of cytochrome C reduction and the direct effects of *B abortus* and *S aureus* on cytochrome C indicated that the reduction seen in the presence of the bacteria was a result of O_2^- production by the PMN, rather than a technical artifact. The difference between the amounts of O_2^- produced could be attributed to a quantitative difference in the relative number of particles ingested or the amounts of opsonin exposed on the zymosan vs the bacteria. The similarity of the oxidative metabolic response of PMN to *B abortus* and the extracellular organism, *S aureus*, indicates that a lack of stimulation of an oxidative metabolic burst does not appear to contribute to the ability of *B abortus* to survive within PMN.

The iodination assay evaluates the ability of PMN to bind inorganic iodide to proteins via the action of the MPO-H_2O_2-halide antibacterial system.[10] The iodination reaction is dependent on the generation of H_2O_2 through dismutation of O_2^-, degranulation of primary granules to release MPO, the presence of iodine, and the unimpaired activity of the MPO to catalyze the reaction. Results of the PMN iodination studies indicated that opsonized zymosan-stimulated iodination was suppressed in the presence of *B abortus*, whereas *S aureus* had no effect (Fig 2). These results agree with those of a previous study[7] that indicated that *B abortus* inhibits the ability of PMN to perform iodination by suppressing primary granule release.

Iodination activity was stimulated in the presence of *B abortus* opsonized with fresh or heat-inactivated antiserum; however, there was no difference between the amounts stimulated by either preparation. Neither nonopsonized bacteria nor organisms treated with normal bovine serum stimulated iodination activity. These results indicate that the requirement of antibody as an opsonin for stimulation of iodination activity by PMN is similar to that for O_2^- production. This finding might be expected because of the relationship between O_2^- production and iodination activity.

Brucella abortus opsonized with specific antibody is capable of stimulating an oxidative metabolic burst by bovine PMN. Nonopsonized organisms and bacteria opsonized with complement only do not appear to stimulate O_2^- production or iodination activity of PMN. In vivo, invading bacteria would most likely be opsonized with antibody and complement before ingestion. Therefore, because opsonized *B abortus* is capable of stimulating an oxidative metabolic response by PMN, a lack of stimulation of an oxidative metabolic response on ingestion of *B abortus* would not appear to contribute to the intracellular survival of the bacteria as hypothesized.[5,6]

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