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Bovine Neutrophils Ingest but Do Not Kill *Haemophilus somnus* In Vitro

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Phagocytosis of *Haemophilus somnus* by bovine blood neutrophils required opsonization of the bacteria with antibodies against *H. somnus*. Few bacteria were phagocytosed in the absence of serum; in addition, absorption of immune serum with Formalin-killed *H. somnus* significantly reduced ingestion of *H. somnus*. Heat inactivation of antiserum (56°C for 30 min) to eliminate complement activity had little effect on its ability to opsonize *H. somnus* for uptake by bovine neutrophils. Antiserum from an *H. somnus*-immunized calf and autologous sera from adult cattle supported equivalent phagocytosis of *H. somnus* by bovine neutrophils, suggesting that normal, healthy cattle may contain sufficient antibodies in their sera to facilitate phagocytosis of *H. somnus*. Although bovine neutrophils readily ingested *H. somnus*, they were unable to kill the bacterium in vitro. These same neutrophils readily killed opsonized *Escherichia coli* and *Staphylococcus epidermidis*, suggesting that *H. somnus* is able to survive and perhaps multiply within bovine neutrophils. Because bovine neutrophils stimulated with opsonized *H. somnus* demonstrated a reduced oxidative burst (as measured by chemiluminescence) compared with neutrophils stimulated by opsonized *E. coli*, we suggest that reduced production of reactive oxygen intermediates during the phagocytosis of *H. somnus* may account, in part, for the enhanced survival of *H. somnus* in bovine neutrophils.

Haemophilus somnus is a bovine pathogen of worldwide distribution that is a significant cause of economic loss in feedlot cattle (5, 20). *H. somnus* infections can occur as a wide range of clinical syndromes, including respiratory disease, arthritis, septicemia, abortion, and a severe and often fatal thromboembolic meningoencephalitis (10, 20). Although *H. somnus* can be isolated from the nasal and oral mucosa of normal cattle (4, 5), its prevalence and number are increased in animals that have been stressed or that have an underlying infection (5). Regardless of the target organ that is primarily affected, the pathological lesions that occur in *H. somnus* infections are characterized by vasculitis with diffuse thrombi that contain fibrin, inflammatory cells (especially neutrophils), and large numbers of bacteria (10, 20). *H. somnus* appears to be quite virulent for cattle; experimental infections have been reproduced by intravenous inoculation of cattle with less than 10^3 viable bacteria (22). Lesions that occur in experimental *H. somnus* infections histopathologically resemble the thrombotic, necrotic lesions characteristic of natural infections (10, 19, 20). The virulence mechanisms of *H. somnus* have not yet been well defined. *H. somnus* does not produce a large capsule (18), nor does it release potent extracellular toxins.

Because very little information currently exists as to how well bovine phagocytic cells can ingest and eliminate *H. somnus*, we decided to examine directly the interaction of *H. somnus* with bovine neutrophils, the cell type that predominates in the lesions that occur during natural infections. In this study, we found that bovine neutrophils will ingest but not kill *H. somnus* in the presence of antibodies. We also present evidence suggesting that the survival of internalized *H. somnus* may be the result of an inadequate oxidative response by bovine neutrophils.

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MATERIALS AND METHODS

Bacteria. *H. somnus* was obtained from the Wisconsin State Animal Health Laboratory (Madison, Wis.). *H. somnus* 8025 was obtained from Ronald Schultz of the University of Wisconsin School of Veterinary Medicine. *Escherichia coli* was obtained from Josie Yang of the Veterinary Medical Teaching Hospital, University of Wisconsin-Madison. All strains of *H. somnus* were maintained at −70°C and grown in brain heart infusion broth that contained 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) at 37°C with 5% CO_2 for 18 to 22 h before each experiment. *E. coli* was maintained at −70°C and grown in brain heart infusion broth at 37°C for 18 to 22 h before each experiment. All bacteria were washed twice in cold Hanks balanced salt solution with 0.25% bovine serum albumin (HBSA) before being tested in vitro.

Animals. Adult Holsteins from the University of Wisconsin Department of Veterinary Sciences dairy herd were used as blood donors for this study.

Antiserum to *H. somnus*. Antiserum to *H. somnus* was raised in 4- to 6-month-old Holstein calves by subcutaneous injection of 10^9 Formalin-killed *H. somnus* emulsified in incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.) at multiple sites on the neck. Calves were immunized three times at 2-week intervals and were bled from the jugular vein at 2 weeks after the final immunization. After allowing the blood to clot at room temperature, we separated the serum by centrifugation (1,000 × *g* for 10 min at 4°C) and stored it as small samples at −70°C. Serum samples were thawed immediately before being used in an experiment and were not refrozen. The antiserum had a titer of 1:2,560 as determined by an enzyme-linked immunosorbent assay that used intact, Formalin-killed *H. somnus* as the antigen. In contrast, preimmune serum absorbed three times with For-

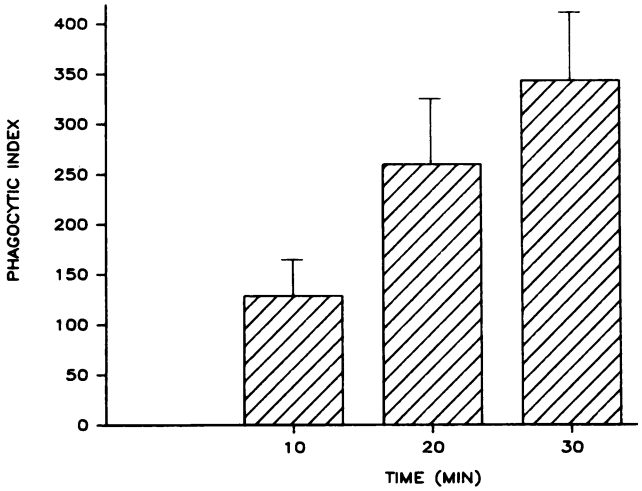

FIG. 1. Phagocytosis of *H. somnus* by bovine neutrophils increases with time. Bovine neutrophils (2.5×10^6) and *H. somnus* (25×10^6) in 20% immune serum were rotated (8 rpm) at 37°C for 10 to 30 min. Results are expressed as the mean PI (± the standard error of the mean; see Materials and Methods) for four experiments (2 to 3 determinations per experiment).

malin-killed *H. somnus* had a titer of 1:20 when measured on the same day in this assay.

Preparation of bovine neutrophils. Blood was collected from the tail vein with sodium citrate (0.38% final concentration) as anticoagulant. The citrated blood was centrifuged ($300 \times g$ for 20 min at 22°C), and the upper layer of platelet-rich plasma was removed and subsequently recalcified (0.02 M final concentration of sterile $CaCl_2$) to obtain autologous plasma-derived serum. The blood cells were centrifuged again ($1{,}000 \times g$ for 20 min at 22°C), and the buffy coat cells were removed. The neutrophils were obtained from the erythrocyte layer by rapid hypotonic lysis in phosphate buffer as described previously (14). The neutrophils were washed three times and suspended in cold HBSA at 4×10^6 cells per ml. Neutrophil viability routinely was >95% as determined by exclusion of trypan blue. The purity of the neutrophil suspensions was assessed by microscopic examination of Diff-Quik-stained cytospin smears (usually >92% neutrophils).

Phagocytosis assay. Phagocytosis of *H. somnus* by bovine neutrophils was determined with duplicate plastic tubes (12 by 75 mm) (Falcon; Becton Dickinson Labware, Oxnard, Calif.) that contained 2.5×10^6 neutrophils, 2.5×10^7 bacteria, and 0.1 to 20% (final concentration) immune serum in a total volume of 1.0 ml of HBSA. The tubes were capped tightly and rotated (Labquake; Labindustries, Berkley, Calif.) for 10 to 60 min at 37°C. The phagocytes were recovered by centrifugation ($100 \times g$ for 5 min at 4°C), washed twice with cold HBSA, and suspended in 1.0 ml of cold HBSA. Cytospin smears were prepared from each reaction tube, stained with Diff-Quik, and examined by light microscopy with an oil immersion objective. At least 100 phagocytes on each slide were scored for the percentage of neutrophils that contained bacteria and for the number of bacteria associated with each ingesting phagocyte. The results were expressed as the phagocytic index (PI), where PI = the percentage of neutrophils that contain bacteria × the average number of bacteria per ingesting neutrophil × 100.

The ingestion of *H. somnus* by bovine neutrophils was confirmed by electron micoscopy. Reaction mixtures treated identically to those described above (10% antiserum) were pelleted, fixed with modified Karnovsky fixative, stained with osmium tetraoxide and uranyl acetate, embedded in epoxy, and examined in a Philips 410 electron microscope. The prepared grids were examined thoroughly, and representative areas were photographed.

Bactericidal assay. The bactericidal activity of bovine neutrophils was determined by a previously described bactericidal assay (6). *H. somnus* (2.5×10^6) were added to duplicate plastic tubes (12 by 75 mm) that contained an equal number of neutrophils and 10% immune serum in a total volume of 1.0 ml of HBSA. The tubes were capped tightly and rotated (8 rpm) at 37°C for 1 to 4 h. At the beginning of the assay and at hourly intervals, 0.1-ml samples were removed from each tube, diluted in sterile distilled water, and plated on chocolate agar (GIBCO) at 37°C with 5% CO_2. In experiments in which neutrophil killing of *E. coli* or *S. epidermidis* was also determined, those reaction tubes contained 10% autologous bovine serum, and the dilutions were plated on blood agar that was incubated in the absence of CO_2.

Chemiluminescence assay. The oxidative response of bovine neutrophils was determined by luminol-enhanced chemiluminescence with a Pico-Lite Luminometer (Packard Instrument Co., Inc., Rockville, Md.) as described previously (3). Neutrophils (5×10^5 in 0.1 ml), luminol (0.1 ml at 10^{-5} M) (Sigma Chemical Co., St. Louis, Mo.), and various stimuli (in 0.1 ml) were all suspended in phenol red-free Hanks balanced salt solution with 10% fetal bovine serum (GIBCO) and added to triplicate tubes. *H. somnus* and *E. coli* were opsonized with 50% immune serum for 30 min in a 37°C water bath and washed three times in cold Hanks balanced salt solution with 10% fetal bovine serum. Graded doses of opsonized bacteria were used to obtain various ratios of bacteria per neutrophil (25:1 to 100:1). The reaction tubes were counted for seven cycles with a counting time of 10 s for each tube. Positive controls included neutrophils stimulated by opsonized zymosan (5 mg) or phorbol myristic

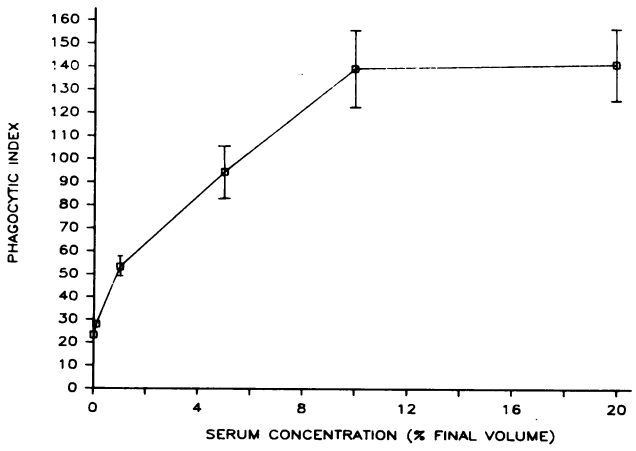

FIG. 2. Phagocytosis of *H. somnus* by bovine neutrophils is dependent on the concentration of immune serum. The phagocytosis of *H. somnus* (25×10^6) by bovine neutrophils (2.5×10^6) was determined at immune serum concentrations of 0.1 to 20%. Results are expressed as the mean PI (± the standard error of the mean; see Materials and Methods) for four experiments (2 to 3 determinations per experiment).

acetate (5 μg; Sigma); negative controls included unstimulated neutrophils and neutrophils stimulated by 5 mg of opsonized zymosan in the presence of sodium azide (10^{-3} M).

Data analysis. Data were analyzed for statistical significance by a one-way analysis of variance followed by the Fisher least-significant-difference test (17). The level of significance was set at $P < 0.05$.

RESULTS

Time course of phagocytosis of *H. somnus* by bovine neutrophils. The ability of bovine neutrophils to phagocytize *H. somnus* in the presence of 20% immune serum was monitored for 10 to 30 min. Uptake of *H. somnus* by bovine neutrophils was a time-dependent process, maximal ingestion of bacteria being observed after a 30- to 60-min incubation period (Fig. 1). We chose 30 min as a convenient incubation period for subsequent experiments that evaluated the mechanism of phagocytosis of *H. somnus* by bovine neutrophils.

Effects of serum concentration on the ingestion of *H. somnus* by bovine neutrophils. Phagocytosis of *H. somnus* by bovine neutrophils was dose dependent at serum concentrations of 0.1 to 10%; increasing the serum concentration to 20% did not significantly enhance the uptake of *H. somnus* by bovine neutrophils (Fig.2). Ten percent immune serum, therefore, was chosen as the serum concentration for all subsequent experiments. Electron microscopy excluded the possibility that *H. somnus* were merely adherent to the neutrophil surface; after 30 min of incubation in the presence of 10% immune serum, most *H. somnus* were in the process of being ingested or were already contained within phagosomes (Fig. 3). Few extracellular bacteria were seen, suggesting that the washing procedure used in the phagocytosis assay was effective.

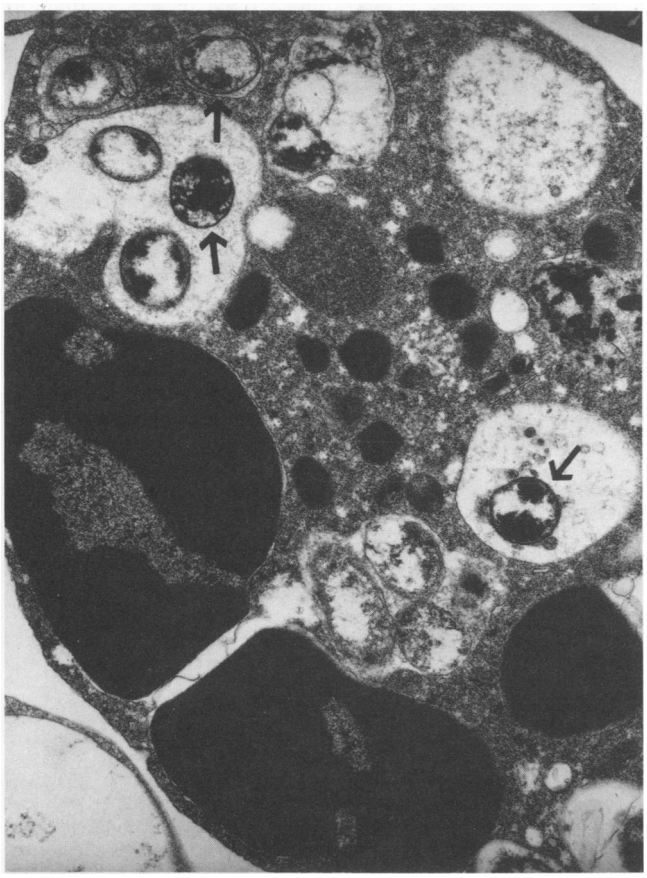

FIG. 3. Electron micrograph showing intracellular *H. somnus* (arrows; magnification, ×11,500) after incubation with bovine neutrophils and 10% immune serum for 30 min under conditions identical to those described in the legends to Fig. 1, 2, and 4. Examination of several fields on the grids showed few extracellular bacteria to be present.

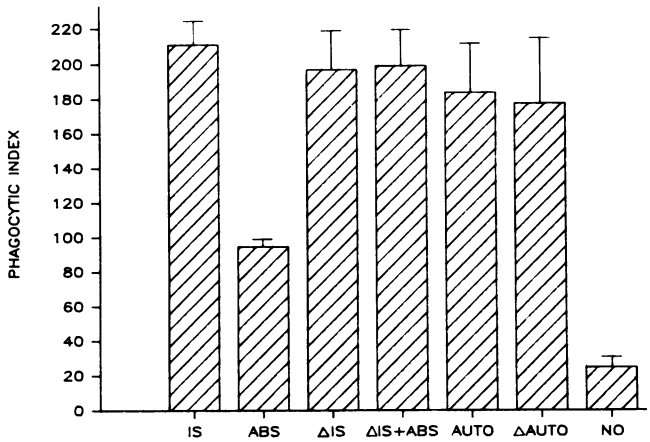

FIG. 4. Phagocytosis of *H. somnus* by bovine neutrophils requires opsonization by antibody but not complement. Uptake of *H. somnus* (25×10^6) by bovine neutrophils (2.5×10^6) was determined in the presence of 10% untreated immune serum (IS), *H. somnus*-absorbed immune serum (ABS), heat-inactivated (56°C for 30 min) immune serum (ΔIS), combined absorbed and heat-inactivated sera (ΔIS + ABS), normal autologous serum (AUTO), heat-inactivated normal autologous serum (ΔAUTO), or in the complete absence of serum (NO). Results are expressed as the mean PI (± the standard error of the mean; see Materials and Methods) for three experiments (2 to 3 determinations per experiment).

Effects of serum heating and absorption on its ability to opsonize *H. somnus*. Phagocytosis of *H. somnus* by bovine neutrophils required the presence of heat-stable opsonins, presumably antibodies directed against *H. somnus*. In the absence of serum, very little uptake of *H. somnus* occurred ($P < 0.05$). In the presence of antiserum whose antibody titer to *H. somnus* had been reduced by repeated absorption with Formalin-killed *H. somnus* (titers by enzyme-linked immunosorbent assay, 1:2,560 and 1:20, respectively), we observed a significant reduction in the uptake of viable *H. somnus* by bovine neutrophils (Fig. 4; $P < 0.05$). Heat inactivation of serum (56°C for 30 min) only slightly reduced the uptake of *H. somnus* by bovine neutrophils ($P > 0.05$), suggesting that complement plays a minor role in opsonization and phagocytosis of this bacterium. Autologous sera obtained from adult cattle also had sufficient amounts of antibody against *H. somnus* to support phagocytosis of *H. somnus* by bovine neutrophils that was equivalent ($P > 0.05$) to what was observed in the presence of *H. somnus* antiserum.

Outcome of the interaction of *H. somnus* with bovine neutrophils. Bovine neutrophils were incubated for 1 to 4 h with an equal number of *H. somnus* in the presence of 10% immune serum as described above. Bovine neutrophils, under conditions that we have shown allowed optimal phagocytosis of *H. somnus*, were unable to kill *H. somnus* in

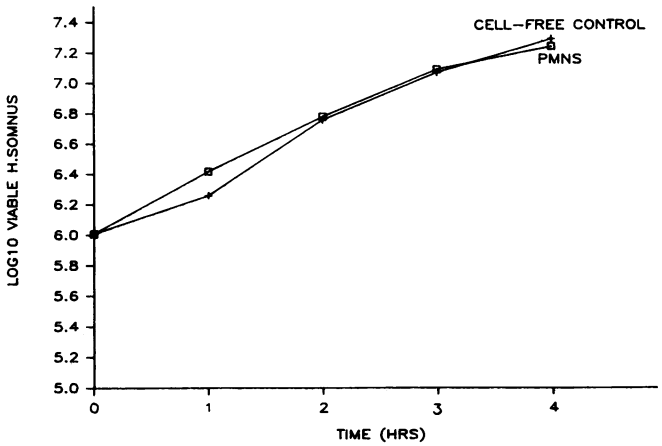

FIG. 5. Failure of bovine neutrophils to kill *H. somnus*. Equal numbers of *H. somnus* and neutrophils (PMNS) in 10% immune serum were rotated (8 rpm) at 37°C for 1 to 4 h; control tubes contained *H. somnus* in the absence of neutrophils (cell-free control). Results are expressed as the mean $\log_{10}$ *H. somnus* present initially (zero time point) and at hourly intervals for four experiments (2 to 3 determinations per experiment).

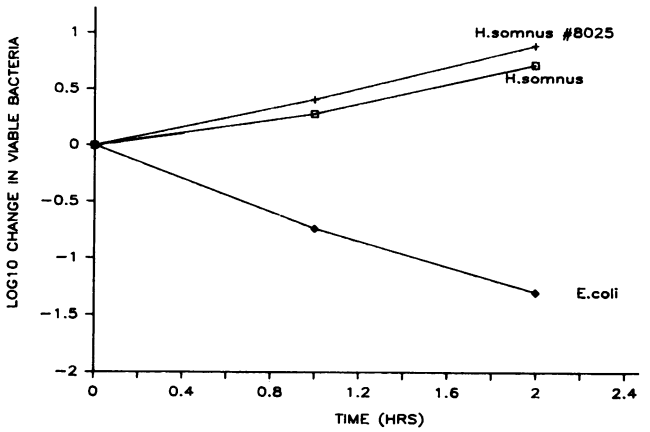

FIG. 6. Killing of *E. coli* but not *H. somnus* by bovine neutrophils. Equal numbers of bacteria and neutrophils in 10% serum were rotated at 37°C for 2 h. Results are expressed as the mean $\log_{10}$ increase (both strains of *H. somnus*) or decrease (*E. coli*) in the number of viable bacteria for five experiments (2 to 3 determinations per experiment).

vitro (Fig. 5). The number of viable *H. somnus* increased similarly in both the neutrophil and cell-free control tubes during the 4-h incubation period. It has been suggested that *H. somnus* may produce inhibitory or cytotoxic factors specific for bovine leukocytes (D. Liggitt, L. Huston, and L. Corbeil, 65th Annu. Meet. Conf. Res. Workers Anim. Dis., abstr. no. 174, 1984; Y. W. Chiang, M. L. Kaeberle, and J. A. Roth, 65th Annu. Meet. Conf. Res. Workers Anim. Dis., abstr. no. 176, 1984). However, we did not observe reduced viability of bovine neutrophils under the conditions of our bactericidal assay.

Although the preceding experiment suggested that bovine neutrophils are unable to kill ingested *H. somnus*, we wanted to exclude the possibility that these neutrophils were generally deficient in bactericidal activity. Bovine neutrophils, which failed to kill *H. somnus*, readily killed *E. coli* in vitro (Fig. 6). Similar killing of *S. epidermidis* was observed in other experiments (data not shown). Bovine neutrophils failed to kill or significantly restrict the growth of both the strain of *H. somnus* used in the preceding experiments and a different strain of *H. somnus* (8025). A recent field isolate of *H. somnus* that was obtained from the Wisconsin State Animal Health Laboratory also was not killed by bovine neutrophils (data not shown).

Bacteria have adopted various strategies for avoiding destruction by phagocytic cells (7). Some bacteria have the ability to provoke a reduced oxidative burst while being ingested by phagocytic cells, thus decreasing their exposure to the potent microbicidal activity of the various reactive oxygen intermediates that are released by phagocytes during the oxidative burst that usually accompanies phagocytosis (1). To test the possible role of this mechanism in the survival of *H. somnus* within bovine neutrophils, we measured the oxidative response of bovine neutrophils stimulated by opsonized *H. somnus* by luminol-enhanced chemiluminescence and compared it with the response of neutrophils stimulated by opsonized *E. coli*. The results indicated (Fig. 7) that opsonized *H. somnus* at a bacteria-to-neutrophil ratio of 100:1 elicited less of an oxidative burst by bovine neutrophils than did opsonized *E. coli* at a bacteria-to-neutrophil ratio of 25:1 ($P < 0.05$). This suggests that the relatively lower levels of reactive oxygen intermediates that are produced by bovine neutrophils after they ingest *H. somnus* may account, at least in part, for the enhanced intracellular survival of *H. somnus*. Examination of the time course of neutrophil chemiluminescence indicated that, in addition to the reduced peak response shown in Fig. 7, the duration of the neutrophil response to *H. somnus* was markedly reduced compared with the response to *E.coli* (Fig. 8).

DISCUSSION

These experiments provide valuable new information on the contribution of neutrophils to the pathogenesis of *H. somnus* infections in cattle. We have shown that opsoniza-

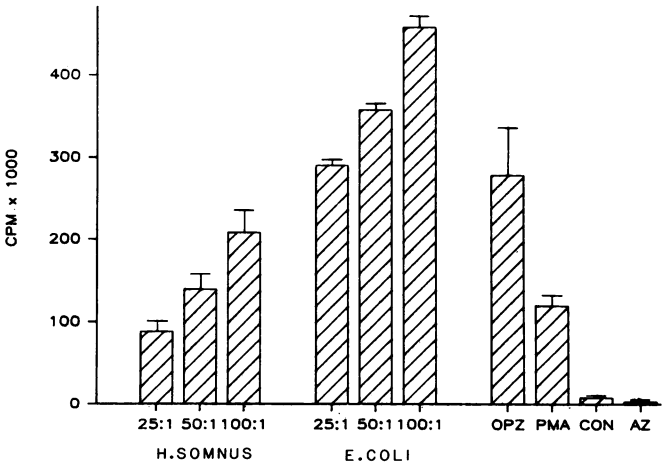

FIG. 7. Reduced chemiluminescence response of bovine neutrophils to opsonized *H. somnus* compared with opsonized *E. coli* at bacteria-to-neutrophil ratios of 25:1, 50:1, and 100:1. Control groups include neutrophils stimulated by 5 mg of opsonized zymosan (OPZ) or 5 μg of phorbol myristic acetate (PMA), unstimulated neutrophils (CON), and neutrophils stimulated by OPZ in the presence of 10^{-3} M azide (AZ). Results are expressed as the mean (± the standard error of the mean) maximal response (counts per minute [CPM] × 1,000) for four experiments (2 to 3 determinations per experiment).

tion of *H. somnus* for phagocytosis by bovine neutrophils requires the presence of heat-stable opsonins, presumably antibodies against *H. somnus*; complement appears to play a relatively minor role in the opsonization of *H. somnus*. Our results also suggest that healthy adult cattle may have sufficient levels of antibody in their sera to facilitate efficient phagocytosis of *H. somnus* by their neutrophils. This observation is supported by a previous report that a high percentage of nonvaccinated, healthy adult cattle have circulating antibodies to *H. somnus* (19). It is not clear whether the presence in cattle of anti-*H. somnus* antibodies indicates prior infection by *H. somnus* or, instead, results from stimulation by cross-reacting antigens of other bacterial species that are present in the bovine normal flora. It has been reported that various strains of *H. somnus* are quite similar antigenically (2, 9); therefore, prior colonization or infection of cattle by one strain of *H. somnus* may result in production of antibodies that will bind to other strains of *H. somnus* that the animal subsequently encounters. Although it has been reported that certain strains of *H. somnus* are susceptible to killing by antibody and complement in bovine serum (16), under the conditions in which the experiments in this study were performed (10% immune bovine serum) serum killing of *H. somnus* did not occur, and the bacteria actually grew during in vitro incubation.

Although bovine neutrophils readily ingested opsonized *H. somnus*, they were unable to reduce the number of viable bacteria significantly. The failure of bovine neutrophils to kill *H. somnus* was not restricted to the strain that we used initially in these experiments; a similar lack of bacterial killing was observed when bovine neutrophils were incubated with *H. somnus* 8025 and a recent field isolate of *H. somnus*. Under similar in vitro conditions, these same neutrophils readily killed *E. coli* and *S. epidermidis*, indicating that the survival of *H. somnus* was not the result of a generalized antibacterial defect of the neutrophils used in this study. It had been reported previously that bovine neutrophils can kill *H. somnus* in the presence of certain lots of antiserum obtained at certain times after immunization (13). In that study, killing also occurred in the presence of antiserum alone, suggesting that serum killing of the *H. somnus* strain used may have confounded the evaluation of neutrophil antibacterial activity. The data presented here suggest that killing of *H. somnus* by bovine neutrophils does not occur under conditions in which serum bactericidal activity is not a factor.

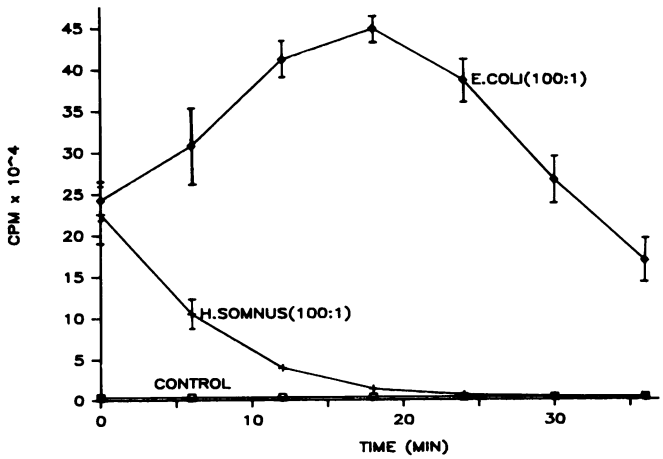

FIG. 8. Reduced duration of the chemiluminescence response of bovine neutrophils to opsonized *H. somnus* compared with opsonized *E. coli* (both at a 100:1 bacteria-to-neutrophil ratio). Results are expressed as the mean (± the standard error of the mean) counts per minute (CPM) × 10^4 for three experiments (2 to 3 determinations at each time point per experiment).

The pathogenesis of the various disease syndromes caused by *H. somnus* are still not well defined. Despite the various locations at which infection occurs, the lesions caused by *H. somnus* are similar pathologically and are characterized by thrombus formation and vasculitis. A previous study suggested that thrombus formation may be initiated by the preferential adherence of *H. somnus* to bovine endothelial cells (21). Because our results suggest that the large numbers of neutrophils that accumulate at infective foci are unable to kill *H. somnus*, the accumulation and activation of large numbers of inflammatory neutrophils in *H. somnus*-induced thrombotic lesions may actually amplify local tissue damage via neutrophil release of reactive oxygen intermediates, lysosomal enzymes, and various inflammatory mediators.

The results of this study indicate that *H. somnus* should be added to the list of bacteria that are not killed by neutrophils. Various strategies for survival have been devised by these bacteria that allow them to circumvent neutrophil antimicrobial mechanisms (7). We present here evidence that the reduced production of reactive oxygen intermediates by bovine neutrophils that have ingested *H. somnus*, compared with neutrophils that have ingested *E. coli*, may account in part for the inability of neutrophils to kill intracellular *H. somnus*. Other pathogenic microbes also use this strategy to survive within neutrophils and macrophages (8, 15, 23). It should be noted, however, that other investigators have reported a dissociation between the ability of bacteria to stimulate neutrophil chemiluminescence (11, 12) and resist intracellular killing (11). We cannot exclude the possibility that the resistance of *H. somnus* to nonoxidative killing mechanisms might also contribute to its survival within bovine neutrophils. Our observation that *H. somnus* can survive within bovine neutrophils suggests that the ability of a vaccine to protect against *H. somnus* infection may require more than just stimulation of an antibody response. Agents that activate bovine neutrophil function might prove to be valuable adjuncts to the prophylactic and therapeutic treatment of *H. somnus* infections in cattle.

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