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In Vivo Production of Neuraminidase by *Pasteurella haemolytica* A1 in Goats after Transthoracic Challenge

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Nine goats were injected transthoracically with *Pasteurella haemolytica* A1 to determine if an extracellular bacterial enzyme, neuraminidase, was produced in vivo during infection with this organism. The principal group of goats ($n = 9$) each received 1 ml of 7.25×10^5 live *P. haemolytica* A1 cells in polyacrylate beads transthoracically in the left lung on days 0 and 21. Six goats were used as negative controls and received 0.3 g of polyacrylate beads subcutaneously in the right flank on days 0 and 21. Serum was obtained from all animals on days −4, 3, 7, 14, 21, 24, and 32. Preimmune serum from all animals showed no detectable antibody to *P. haemolytica* A1 neuraminidase in an enzyme neutralization assay. None of the sera from the negative control animals possessed a significant antibody concentration in response to the *P. haemolytica* A1 neuraminidase. On day 32, serum samples from the nine infected animals possessed enzyme neutralizing activity that ranged from 62% to 100%. Anti-neuraminidase antibody could be detected as early as day 14 by the enzyme neutralization assay. These data demonstrate that the enzyme neuraminidase is produced in vivo during an active *P. haemolytica* A1 lobar infection.

Pasteurella haemolytica serotype A1 causes most acute fibrinohemorrhagic pneumonias that develop after market stress in feeder and stocker calves (8). The same organism can cause a similar pneumonia in goats (12). It is our belief that extracellular products produced by *P. haemolytica* A1 may play a role in this pneumonic condition. We have recently focused on extracellular neuraminidase of *P. haemolytica* A1 as potentially important in the pathogenesis of this disease (14, 15). We have demonstrated that neuraminidase is a heat-labile, extracellular enzyme that is produced maximally in the stationary phase of growth when the organisms are grown in 1 liter of brain heart infusion broth in an incubator shaker at 37°C rotating at 180 rpm and has a molecular weight of approximately 160,000 (15). We have also shown that *P. haemolytica* A1 neuraminidase is similar to the neuraminidases produced by other *P. haemolytica* serotypes in terms of substrate specificity, molecular weight, and antigenic identity (14).

Neuraminidase (or sialidase) production by a *Pasteurella* species was first reported by Scharmann et al. (13). They reported neuraminidase production by 3 of 5 strains of *P. haemolytica* and 102 of 104 *Pasteurella multocida* strains. Later, Frank and Tabatabai (3) observed that *P. haemolytica* serovars 1, 4, 5, 6, 7, 9, and 12 produced a sialidase, and serovar strains 2, 3, 8, 10, and 11 did not. In another study, Tabatabai and Frank (16) examined the relationship between the *P. haemolytica* neuraminidase production by ovine field isolates and that by bovine field isolates. They suggested that the sialidase activity levels from the field isolates examined were serovar associated.

In vivo production of this enzyme by other bacteria has been thought to play a role in the disease process. Examples of these organisms include *Vibrio cholerae* (11), *Corynebacterium diphtheriae* (9), and *Streptococcus pneumoniae* (7). Little is known about how bacterial neuraminidase could act as a virulence factor. One possible mechanism was proposed by Gottschalk (5). He demonstrated that removing sialic acid from salivary glycoproteins reduced their protective effect against potential pathogens. Thus, bacterial neuraminidase could greatly facilitate bacterial survival in an in vivo situation. The purpose of this study was to determine if *P. haemolytica* A1 neuraminidase is produced in vivo during a pneumonic infection.

Bacterium. *P. haemolytica* A1 was isolated from a confirmed fatal case of acute bovine respiratory disease. Cultures were stored at −70°C in reconstituted double-strength powdered milk on filter paper. For routine use, frozen cultures were thawed and incubated for 24 h at 37°C on nutrient agar (Difco, Detroit, Mich.) plus 5% bovine erythrocytes. The frozen stock cultures were transferred only once before use in experiments. Colonies from the blood agar were used to initiate cultures in brain heart infusion broth (Difco). Live *P. haemolytica* A1 cells were obtained from an early-stationary-phase culture (24 h) grown in 1 liter of brain heart infusion broth at 37°C and 180 rpm. Cells were harvested by centrifugation at 17,700 × *g* for 30 min at 4°C. The live *P. haemolytica* A1 cells were washed twice in sterile phosphate-buffered saline (PBS) and then adjusted to an optical density of 1.0 at 650 nm. This suspension was then diluted 1:500 and used to rehydrate the polyacrylate beads. The polyacrylate beads rapidly swell and absorb PBS and bacteria when placed in a beaker containing them.

Goats. Fifteen goats (Spanish weanling males) were purchased from one owner. The goats were treated for internal and external helminth parasites with Ivomec MSD AGVET (Merck and Co., Inc., Rahway, N.J.) and were treated for coccidia with Amprolium MSD AGVET (Merck). The goats were fed alfalfa hay ad libitum for 3 days, and then their diet was changed to a commercial pelleted sheep and goat ration (50% grain concentrate) at the rate of 0.23 kg per goat per day. Fresh water was provided ad libitum. Goats were conditioned for 2 weeks by periodic handling, taking of rectal temperatures, taking of blood specimens from the jugular vein, and weighing. They were then divided into principal (experimental) and

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control groups. The two groups were housed in different barns to prevent cross-infection of the controls.

Experimental design. Goats were randomly assigned to either the negative control group ($n = 6$) or the principal group ($n = 9$). All goats in the negative control group were given 0.3 g of polyacrylate beads (Dow Chemical Company, Midland, Mich.) subcutaneously in the right flank to avoid unnecessary trauma to the animals. These beads were prepared by rehydrating dry beads in sterile saline. Each goat in the principal group received an injection of 1 ml containing 7.25×10^5 *P. haemolytica* A1 cells in 0.3 g of polyacrylate beads in the left lung. An injection of live *P. haemolytica* A1 cells in beads has been previously shown to lead to an active lobar pneumonia in goats (12). To prepare the inoculum, the dry beads were rehydrated with the diluted suspension of freshly prepared *P. haemolytica* A1 cells. Both groups of goats were injected on days 0 and 21 in the same manner. The negative control group was treated before the principal group to avoid cross-contamination by *P. haemolytica* A1. The goats were examined each morning when rectal temperatures or blood samples were obtained. We also observed them every afternoon for signs of clinical disease by walking among them and observing their reactions. The goats were always treated in accordance with the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Testing guidelines (2).

Neutralization of homologous *P. haemolytica* A1 neuraminidase with serum from negative control and principal goats. Blood (30 ml) was drawn from the jugular vein of each of the 15 goats on days −4, 3, 7, 14, 21, 24, and 32. The presence of serum neutralizing antibody to *P. haemolytica* A1 neuraminidase was determined with a neuraminidase assay (15). Stage 1 *P. haemolytica* A1 neuraminidase (100 μl, produced as previously described [15]) was incubated with 100 μl of the appropriate serum in the presence of 100 μg of fetuin, 10 mM $CaCl_2$, and 25 mM sodium acetate (pH 6.5) in a final volume of 0.4 ml. Each set of assays included a substrate blank, and reactions were initiated by the addition of the enzyme to the remaining components. The enzyme and serum preparations were incubated together for 60 min at 4°C before the assay was begun, to allow the antibody to react with the enzyme. After incubation, the percentage of reduction in neuraminidase activity was determined by performing the neuraminidase assay (15) for 60 min at 37°C and comparing the differences in activity between the day −4 serum and the day 32 serum for each animal.

Statistical evaluation. Data were computer analyzed by SAS analysis of variance. Duncan's multiple range test was used to determine significant differences ($P \leq 0.05$) between means.

Neutralization of *P. haemolytica* A1 neuraminidase by sera from a negative control and a principal goat. In the presence of serum from uninfected goats, there was essentially no reduction in activity of the homologous stage 1 *P. haemolytica* A1 enzyme preparation. Figure 1A shows the absorption spectra of complexes obtained from the *P. haemolytica* A1 neuraminidase reaction product with the thiobarbituric acid reagent after exposure to the day −4 and day 32 serum samples of negative control goat 6308, which had not been infected with live *P. haemolytica* A1 cells. This figure clearly shows that there was no anti-neuraminidase antibody produced in this animal during the 32 days of the experiment. The amounts of neuraminidase activity remaining after exposure to serum from goat 6308 were essentially the same on days −4 and 32. However, Fig. 1B shows the absorption spectra of complexes obtained from the *P. haemolytica* A1 neuraminidase reaction product with the thiobarbituric acid reagent after exposure to day −4 and day 32 serum samples from *P. haemolytica*

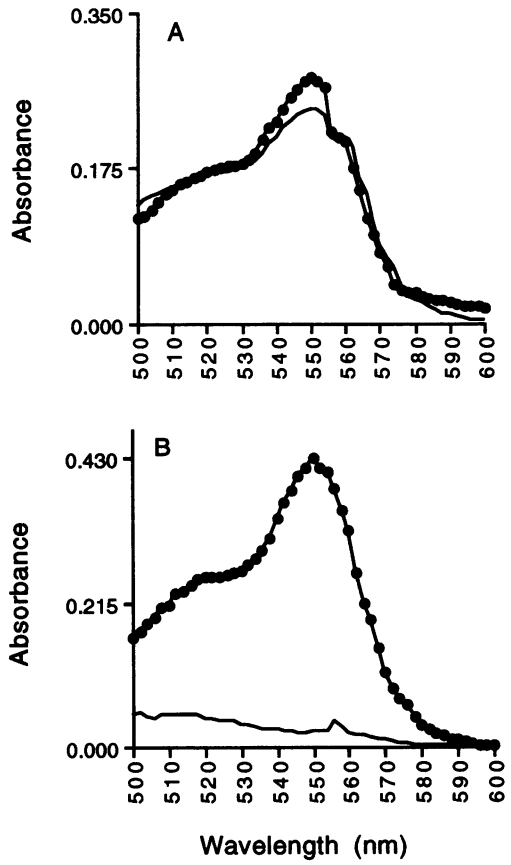

FIG. 1. Absorption spectra of complexes obtained from interaction of *P. haemolytica* neuraminidase with day −4 serum (•) and day 32 serum (——) of negative control goat 6308 (A) and *P. haemolytica* A1-infected goat 6159 (B).

A1-infected goat 6159. This drop in the optical density reading indicates that anti-neuraminidase antibody was produced in this animal during the course of the 32-day experiment. Figure 1B shows that the neuraminidase activity level (optical density at 549 nm) dropped from 0.430 (preimmune serum) to an extremely low level after exposure to day 32 serum from goat 6159.

Neutralization of *P. haemolytica* A1 neuraminidase by sera from negative controls and *P. haemolytica* A1-infected goats. When the sera of the group of negative control goats ($n = 6$) were examined, it was clear that this population did not make anti-*P. haemolytica* A1 neuraminidase antibodies during the 32-day experiment (Fig. 2). One animal (goat 6315) did produce anti-neuraminidase antibody on day 32. This serum specimen showed a 15.6% reduction in *P. haemolytica* A1 neuraminidase activity. When mean values for days −4 and 32 from this group were examined, there was no reduction in enzyme activity over the course of the experiment. When the sera of the group of *P. haemolytica* A1-infected goats ($n = 9$) were examined, this population clearly produced anti-*P. haemolytica* A1 neuraminidase antibodies (Fig. 2). The reduction in enzyme activity ranged from 62.0% (one animal) to 100% (four animals). When the mean values (days −4 and 32) for the *P. haemolytica* A1-infected animals were examined, there was an 86.3% mean reduction in enzyme activity. The mean values for the negative controls and *P. haemolytica* A1-infected animals were significantly different on days 14, 21, 24, and 32.

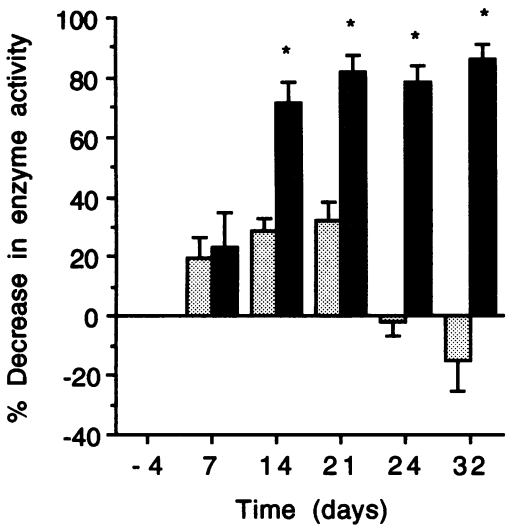

FIG. 2. Antibody neutralization of *P. haemolytica* A1 neuraminidase by sera from negative control (lightly shaded bars) and *P. haemolytica* A1-infected (hatched bars) goats. Means (asterisks) were determined as significantly different from the same-day negative control values at $P \leq 0.05$.

We believe that part of the pneumonic tissue damage produced by *P. haemolytica* A1 is due to the in vivo production of extracellular bacterial products. One of these bacterial exoproducts, *P. haemolytica* A1 neuraminidase, has been the subject of several studies. We recently characterized the production of this enzyme (15). In a later study (14), we demonstrated that all *P. haemolytica* serovars (except serovar 11) produce a neuraminidase. We also observed (14) that all *P. haemolytica* serovar neuraminidases are neutralized by specific anti-*P. haemolytica* A1 neuraminidase antibody and that they are all similar in substrate specificity and molecular weight. The purpose of this study was to determine if the *P. haemolytica* A1 neuraminidase was produced in vivo during an active lobar infection. We found that anti-neuraminidase antibody was produced in *P. haemolytica* A1-infected goats but not in noninfected goats.

We demonstrated that normal goats do not usually possess large quantities of anti-*P. haemolytica* A1 neuraminidase antibody (Fig. 1A and Fig. 2). However, when goats were infected transthoracically with live *P. haemolytica* A1 cells, they responded by producing significant amounts of anti-neuraminidase antibody (Fig. 1B and Fig. 2). This antibody was first detectable on day 14 after the initial live *P. haemolytica* A1 injection.

The enzyme neuraminidase has been implicated as a virulence factor in several bacterial species, particularly those with the ability to thrive on mucosal surfaces (10). *P. haemolytica*, which is found on the mucosal surfaces of cattle and goat turbinates, produces this enzyme (3, 11, 14–16). *S. pneumoniae* is another example of a bacterium that produces neuraminidase and can survive on mucosal surfaces. Kelly et al. (7) demonstrated that fewer than 50% of laboratory strains of *S. pneumoniae* were able to produce this enzyme, whereas 100% of 77 clinical isolates examined produced neuraminidase. These data suggested that pneumococcal neuraminidase was required for survival of the organism in the human host.

Other studies have suggested a more direct role for this enzyme in the disease process. For example, Kelly and Greiff (6) demonstrated that intracerebral injection of purified pneumococcal neuraminidase was lethal for weanling mice.

Other roles for bacterial neuraminidases in mammalian disease have been recently postulated. Histological examination of mice dying after intraperitoneal injection of pneumococcal neuraminidase demonstrated marked decreases in sialic acid content of the liver and kidneys compared with negative controls (6). In addition, it has been demonstrated that both bacteremia and coma occur more frequently among patients with *S. pneumoniae* meningitis when there are elevated sialic acid concentrations in the cerebrospinal fluid (10). Neuraminidase has been hypothesized to play a role in the pathogenesis of bacterial vaginitis induced by the *Bacteroides* and *Prevotella* species associated with upper genital tract infection, prematurity, and preterm birth. Briselden et al. (1) reported elevated neuraminidase activity in 84% of vaginal fluid specimens from women with bacterial vaginitis but no activity in 19 vaginal fluid specimens from women with no bacterial vaginitis. Their data suggested that the presence of vaginal fluid neuraminidase is highly correlated with bacterial vaginitis and that the source of sialidase was probably from the *Prevotella* and *Bacteroides* species found in the vagina. In another study, two *Bacteroides fragilis* neuraminidase-deficient mutants were used to determine the role of neuraminidase activity in the growth of these bacteria in vivo (4). Godoy et al. suggested that neuraminidase activity may be required for the organisms to grow maximally by liberating other carbon sources after available glucose levels are exhausted.

It is clear from our work and the work of others that bacterial neuraminidases are produced in vivo and probably play a role in the pathogenesis of bacterial diseases. We are currently examining how *P. haemolytica* A1 neuraminidase contributes to the acute *P. haemolytica* A1-induced fibrinohemorrhagic pneumonias that develop in market-stressed feeder and stocker calves after shipment.

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