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Proteolysis of Sialoglycoprotein by *Pasteurella haemolytica* Cytotoxic Culture Supernatant

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Proteolytic enzyme activity releasing sialo glycopeptides from ^{3}H-labeled human erythrocyte ghosts was detected in cytotoxic (leukotoxic) culture supernatants from 9 of 12 *Pasteurella haemolytica* serotypes. Microcrystalline cellulose thin-layer chromatograms of radioactive water-soluble products showed the following two radioactive peaks: a high-mobility minor peak (R_f, 0.54 to 0.74), identified as sialic acid, and a low-mobility major peak (R_f, 0.18 to 0.21), partially characterized as a trichloroacetic acid-soluble, sialic acid-rich fragment with a molecular weight of greater than 3,500, not extractable by chloroform. The sialic acid content of this fragment after treatment with *Clostridium perfringens* neuraminidase was estimated to be 7.2×10^{-2} μmol mg^{-1}. The presence of neuraminidase as a separate activity in some culture supernatants was confirmed. It is considered to be responsible for the observed release of free sialic acid. Preliminary studies with the crude enzyme showed that it has a broad pH optimum around pH 7.0 and that activity is not affected by inhibitors of trypsin, chymotrypsin, thermolysin, thio and serine enzymes, nor by an inhibitor of neuraminidase, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid. Activity was, however, inhibited by *o*-phenanthroline at a high concentration after prolonged treatment. The enzyme hydrolyzed glycophorin at a rate four times higher than the rate for casein. Free glycophorin inhibited the enzyme-induced release of radioactive products from ^{3}H-labeled ghosts. It is speculated that the novel enzyme is a neutral protease, probably metal-dependent, with specificity for sialoglycopeptides. The possible relationship of this protease to the previously reported host species-specific leukotoxicity of *P. haemolytica* and its potential role in virulence is discussed.

Pneumonic pasteurellosis associated with *Pasteurella haemolytica* serotype 1 is a major cause of economic loss in the feedlot cattle industry. Both the bacterium and its sterile culture supernatant have been shown to be cytocidal for bovine leukocytes (1, 16). Cytotoxicity may participate in the pathogenesis of pneumonic pasteurellosis by impairing the primary lung defense and subsequent immune response or by the induction of an inflammatory reaction as a consequence of leukocyte lysis. Little is known about the biochemical nature of the soluble cytotoxin, and therefore, several enzyme activities present in the cytotoxic *P. haemolytica* culture supernatant were examined. This report describes a novel enzyme active against glycoproteins of mammalian erythrocyte ghosts.

MATERIALS AND METHODS

***P. haemolytica* culture supernatant (crude cytotoxin).** The *P. haemolytica* cytotoxic culture supernatant was prepared as previously described (16). Briefly, a 4.5-h brain heart infusion broth culture of *P. haemolytica* serotype 1 (originally obtained from E. L. Biberstein, University of California, Davis) was used to inoculate RPMI medium 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 7% fetal calf serum. After 1 h of incubation at 37°C on a rocking platform, bacteria were pelleted by centrifugation, and the supernatant was filtered through a 0.22-μm Millipore filter (Millipore Corp., Bedford, Mass.), dialyzed against two changes of distilled water, and lyophilized. Cytotoxicity was confirmed by the induction of ^{51}Cr release from labeled bovine pulmonary macrophages by the lyophilized culture supernatant reconstituted at 3 mg ml^{-1} in RPMI 1640 (16).

Preparation of ^{3}H-sialyl glycoproteins of erythrocyte membranes. Human erythrocyte ghosts were prepared by the method of Dodge et al. (4). Sialoglycoproteins of the erythrocyte ghosts were labeled in situ in the membrane by periodate oxidation of the terminal sialyl residues essentially by the method of Blumenfeld et al. (3), but with sodium periodate in a 2:1 molar ratio to sialic acid residues, followed by reduction with $NaB^{3}H_4$ (5.92 mCi $mmol^{-1}$; Amersham Corp., Oakville, Ontario, Canada). Total membrane protein was

measured by the method of Lowry as modified by Peterson (14). The molar concentration of sialic acid in membrane materials was estimated by assuming that glycophorin constitutes 10% of total membrane proteins (10) and 75% of membrane sialoglycoproteins (12) and that 10,000 g of sialoglycoproteins was equivalent to 12.1 mol of sialic acid (24). The specific radioactivity of the sialyl residues was determined by radiometric and colorimetric analysis of the sialic acid released from the labeled erythrocyte ghosts by *Clostridium perfringens* *N*-acetylneuraminidase (EC 3.2.1.18; Sigma Chemical Co., St. Louis, Mo.). The labeled erythrocyte ghosts, 0.20 mg of protein, were incubated with a range of acetylneuraminidase concentrations from 5.5×10^{-5} to 8.77×10^{-4} U per 0.2 ml (1 U = 1 μmol of *N*-acetylneuraminic acid (NANA) released per min from sialyllactose [Sigma Chemical Co.] at 37°C in 0.15 M acetate buffer, pH 5.0). Membranous materials were removed by centrifugation at 13,000 × *g* for 2 min, and the amount of sialic acid released into the supernatant was determined colorimetrically (23). The ^3H radioactivity of the supernatant was shown by thin layer chromatography (TLC) on microcrystalline cellulose located in a band which had the same R^f as neuraminic acid. The specific radioactivity of released [^3H]NANA was determined to be 29 μCi μmol^{-1}. Over 40% of the total ^3H radioactivity was released by *N*-acetylneuraminidase (8.77×10^{-5} U ml^{-1}) during 1 h of incubation at 37°C.

Detection of [^3H]NANA released from labeled erythrocyte ghosts by *P. haemolytica* cytoxin. The assay mixture contained ^3H-labeled erythrocyte ghosts (0.2 mg of protein), lyophilized cytotoxin (0.6 mg), 87.5 U of penicillin, and 35 μg of streptomycin in 0.35 ml of 0.10 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) buffer (pH 7.4) containing 0.13 M NaCl. Cytotoxin inactivated by heating at 100°C for 5 min was used as a control. All assays were performed in duplicate and incubated at 37°C for up to 2 h. Undigested ghost cell membrane was pelleted by agglutination with phytohemagglutinin, followed by centrifugation at 13,000 × *g* for 2 min, or by centrifugation only, at 13,000 × *g* for 3 min. Radioactivity released into the supernatant medium was determined by liquid scintillation counting of sample of the supernatant. Another sample was treated with trichloracetic acid (TCA) at a 5% final concentration. The exclusion of the antibiotics in later experiments did not appear to affect the enzyme activity of the cytotoxin in any way.

TLC of water-soluble radioactive products. Soluble radioactive products were prepared by incubating ^3H-labeled erythrocyte ghosts (0.25 mg of protein) with crude cytotoxin (1.4 mg) or *C. perfringens* neuraminidase (0.086 U) in 0.2 ml of 0.1 M HEPES, pH 7.4. Ghost membranes were sedimented after 2 h of incubation at 37°C by centrifugation at 13,000 × *g* for 3 min. Supernatant (150 μl) from each sample was applied to a microcrystalline cellulose TLC plate and chromatographed in propanol-acetone-concentrated HCl (50:50:0.3 [vol/vol]). Radioactive products were located by using a Panax TLC radiometric scanner.

Assay for neuraminidase activity. Crude cytotoxin was assayed for neuraminidase activity by using the substrate sialyllactose as described by Taha and Carubelli (18). Sialic acid release after 4 h of incubation was assayed colorimetrically by the method of Warren (23).

Inhibition of enzyme activity. A number of specific inhibitors were tested for their effect on the release of soluble radioactive products by crude cytotoxin. The concentration of the inhibitor used was as follows: tolylsulfonyl phenylalanyl chloromethyl ketone (chymotrypsin inhibitor), 5 mM; soybean trypsin inhibitor, 0.01 mg ml^{-1}; pepstatin (cathepsin D inhibitor), 0.01 mg ml^{-1}; *p*-chloromercuribenzoate (thio enzyme inhibitor), 1 mM; tetraisopropylpyrophosphoramide (serine enzyme inhibitor), 1 mM; 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (neuraminidase inhibitor), 1 mM; EDTA (metal chelator), 5 mM; and *o*-phenanthroline (metal chelator), 2 or 13.5 mM.

Crude cytotoxin (0.3 mg) was preincubated with each inhibitor at 22°C for 30 min to 1 h, with the exception of *o*-phenanthroline which was incubated for 24 h. After incubation, 10 μl of ^3H-labeled erythrocyte ghosts (400 μg of protein) was added, and the release of radioactivity was measured as described above.

The effect of proteins on the enzyme activity of the cytotoxin was investigated. ^3H-labeled ghosts were digested with cytotoxin in the presence of 0.96 to 9.7 mg of insulin ml^{-1} (in crystalline form of bovine origin; Sigma Chemical Co.) and 0.5 to 1.5 mg of glycophorin ml^{-1}

Assay of the hydrolytic enzyme activity of crude cytotoxin. Crude cytotoxin was tested for the presence of trypsin, chymotrypsin, and thermolysin, three proteolytic enzymes which release radioactive products from ^3H-labeled erythrocytes. Standard procedures involving the use of the specific substrates benzyl-L-arginine ethyl ester (8), benzyl-L-tyrosine ethyl ester (8), and furylacrylolylglycl-L-leucinamide (5), respectively, were employed. The possible presence of phospholipases was also investigated by using [*N*-methyl ^{14}C]phosphatidylcholine (7) as the substrate.

Casein and glycophorin hydrolysis. Hydrolysis of casein (BDH Chemicals, Toronto, Ontario, Canada) was performed by the method Reimerdes and Klostermeyer (15). The assay mixture containing 4.7 mg of crude cytotoxin was incubated at 37°C for 2 h. The reaction was stopped by the addition of TCA.

Glycophorin was prepared from lyophilized erythrocyte ghosts by extraction with lithium diiodosalicylate as described by Marchesi and Andrews (11), except that chromatography on phosphocellulose was omitted. After dialysis against 0.01 M HEPES buffer, pH 7.4, glycophorin was subjected to a hydrolysis procedure similar to that described previously for casein (15). The reaction mixture contained 0.05 mg of glycophorin and 3.6 mg of crude cytotoxin in a final volume of 0.3 ml. Enzyme action was stopped by using TCA at intervals of 30 min, 1, 2, and 4 h.

For both substrates, TCA-soluble products of proteolysis were quantitated by the ninhydrin technique (15). Results were expressed as aspartate equivalents.

Screening of 12 serotypes of *P. haemolytica* for enzyme activity. A sterile culture supernatant was prepared from each of the 12 recognized serotypes of *P. haemolytica* (type strains 1 through 12, originally obtained from G. H. Frank, National Animal Disease Center, Ames, Iowa) and screened for proteolytic enzyme, neuraminidase, and cytotoxic activity as described above. TLC analysis and radiometric scanning were used to estimate the soluble radioactive products of each *P. haemolytica* serotype.

RESULTS

Hydrolytic enzyme activity of *P. haemolytica* culture supernatant. Incubation of crude cytotoxin with ^{3}H-labeled erythrocyte ghosts at 37°C resulted in the release of soluble radioactive products (Fig. 1). Released radioactivity increased linearly with time for over 4 h. The net release after 4 h of incubation at 37°C amounted to 6.68 nmol of NANA mg^{-1} of toxin. Heat-inactivated toxin controls showed a constant low background release over this time period. The rate of release by the cytotoxin was 2.78 ± 0.10 × 10^{-2} nmol of NANA min^{-1} mg^{-1} of cytotoxin. Virtually all of the radioactive products released by the cytotoxin were soluble in 5% TCA. Very little radioactivity was detected in the TCA-insoluble fraction or the chloroform-soluble fraction of the products when these were subjected to lipid extraction (2).

TLC analysis of products. TLC on microcrystalline cellulose of the soluble radioactive products released by crude cytotoxin showed two peaks of radioactivity (Fig. 2A). The major product was more polar than sialic acid and chromatographed close to the origin (R_f, 0.18 to 0.21). Its migration coincided with that of radioactive sialoglycopeptides released from the substrate by trypsin (Fig. 2C). The second and minor peak had greater mobility (R_f, 0.59 to 0.74) and chromatographed with an R_f equal to that of NANA released by *C. perfringens* neuraminidase (Fig. 2B). The double peak for this product, shown in Fig. 2B, could result from differences in the degree of oxidation of the sialic acid during the periodate oxidation step of the titration procedure, which may yield seven and eight carbon ^{3}H-labeled sialic acid residues (3). The neuraminidase preparation also released low levels of a low-mobility product similar to that released by the cytotoxin, probably due to contaminating enzyme activity. It is apparent from these results that the crude cytotoxin preparation possesses neuraminidase activity in addition to proteolytic enzyme activity which induces the release of sialic acid-containing peptides from ^{3}H-labeled erythrocyte membranes.

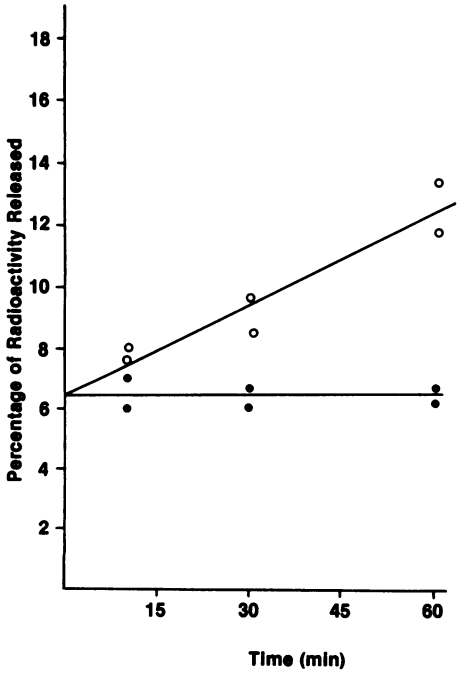

FIG. 1. Release of soluble radioactive products from [^{3}H]sialoglycoprotein of human erythrocyte ghosts by culture supernate (○) and heat-treated culture supernate (●) of *P. haemolytica* serotype I.

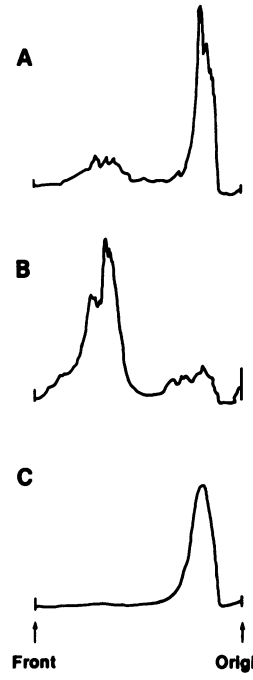

FIG. 2. The distribution of ^{3}H radioactivity in thin-layer chromatograms of the soluble products released by (A) the *P. haemolytica* culture supernatant (B) *C. perfringens* neuraminidase, and (C) bovine pancreatic trypsin from [^{3}H]sialoglycoproteins of erythrocyte ghosts.

Dialysis of TCA supernatants. The TCA-soluble products of cytotoxin digestion were found to be retained by dialysis membranes having a molecular weight retention limit of 3,500. These nondialyzable products contained no free sialic acids; however, treatment with neuraminidase

resulted in the release of large amounts of sialic acid. Treatment of 4.8 mg of the product with 0.086 U of neuraminidase yielded 7.2×10^{-2} μmol of NANA mg^{-1} of product after 2 h of incubation.

Effects of inhibitors. No significant inhibition was detected with any of the specific enzyme inhibitors used, with the exception of *o*-phenanthroline which reduced the hydrolytic activity of the cytotoxin to 29% after prolonged treatment (24 h) at a high concentration (13.5 mM).

Both insulin and insulin chain A showed relatively weak inhibition, with 50% inhibitory concentrations of 3.5 and 2.5 mg ml^{-1}, respectively. Glycophorin showed much greater inhibition. The hydrolytic activity of crude cytotoxin, measured by the release of soluble radioactive products was reduced to 20% at a glycophorin concentration of 1.1 mg ml^{-1}. Only 0.8 mg of glycophorin ml^{-1} was required for 50% inhibition.

The effects of various concentrations of 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid on both the crude toxin and *C. perfringens* neuraminidase activities were compared. Although this NANA analogue completely inhibited *C. perfringens* neuraminidase action on the ^{3}H-labeled erythrocyte ghosts at 10^{-3} M and showed some inhibition at 10^{-6} M, it had no effect on the activity of the cytotoxin even at 10^{-2} M.

Hydrolytic enzyme activity of crude cytotoxin. No activity was detected in assays for trypsin, chymotrypsin, thermolysin, or phospholipase, using the crude cytotoxin. To ensure that the release of radioactive products from ^{3}H-labeled erythrocyte ghosts was not due to the presence of subdetectable levels of proteolytic enzymes, ghosts were incubated with these three enzymes at concentrations equal to the lower limits of detection in the assays used. The release of radioactive products was minimal and far below the level of activity for the cytotoxin in each case. No activities for phospholipase A_1, A_2, B, or C were detected, using phosphatidylcholine labeled with ^{14}C in the *N*-methyl residue as the substrate.

Hydrolysis of casein and glycophorin. After 2 h of incubation with cytotoxin, 0.139 μmol of aspartic acid equivalents was released from glycophorin, whereas only 0.036 μmol of aspartic acid equivalents was released from casein. The time-dependent hydrolysis of glycophorin over a 4-h period is shown in Fig. 3.

Enzyme activity in culture supernatant from 12 serotypes of *P. haemolytica*. Culture supernatant from 12 *P. haemolytica* serotypes which had been previously assayed for cytotoxicity (17) were screened for the presence of proteolytic and neuraminidase activity. The results obtained

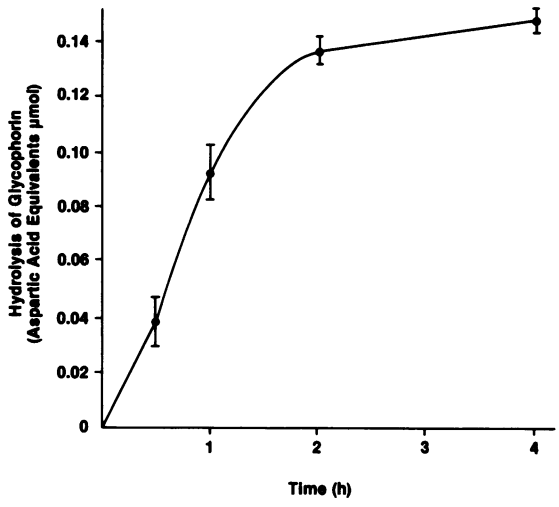

FIG. 3. The hydrolysis of glycophorin by the *P. haemolytica* culture supernatant.

are given in Table 1. Cytotoxicity varied among the 12 serotypes, with the supernatant from type 4 showing the least toxicity. Culture supernatants from serotypes 1, 2, 3, 6, 7, 8, 9, and 12 were able to induce the release of radioactive products from ^{3}H-labeled erythrocyte ghosts. TLC analysis of the products of hydrolysis showed similar patterns for these serotypes. The major products were sialoglycopeptides with minor amounts of free sialic acid derivatives. There was little hydrolysis of the sialoglycoprotein substrate by serotypes 4, 10, and 11, either by neuraminidase or proteolytic activity. Serotype 5 showed very little neuraminidase activity but did show a level of proteolytic enzyme similar to that seen in the majority of serotypes. The highest amount of neuraminidase activity was associated with serotype 2 in which activity was double that observed for any other serotype.

DISCUSSION

The cytotoxic culture supernatant from *P. haemolytica* serotype 1 was shown to contain hydrolytic enzyme activities capable of releasing soluble radioactive products from ^{3}H-labeled erythrocyte ghosts. Although detailed studies were carried out only on serotype 1, subsequent screening revealed that these activities were also present in culture supernatants from several other recognized serotypes (Table 1).

Two components of the soluble radioactive products have been identified. The major component is a sialic acid-containing fragment which

TABLE 1. Protease and neuraminidase activities and cytotoxicity of culture supernants from 12 serotypes of *P. haemolytica*

Serotype	Protease activity[a] (nmol of NANA mg^{-1} toxin h^{-1})	Neuraminidase activity[b] (nmol NANA mg^{-1} toxin h^{-1})	Ratio of protease/neuraminidase activity[c]	Cytotoxicity[d] (% ^{51}Cr release)
1	1.67	20.00	14.0	93.5 ± 30.9
2	1.75	47.00	5.6	93.4 ± 11.1
3	1.58	15.70	8.6	74.1 ± 24.3
4	0	0.70		33.7 ± 17.6
5	1.37	2.10	58.8	53.0 ± 25.0
6	1.42	10.00	24.7	71.6 ± 27.9
7	1.11	8.60	31.0	60.5 ± 30.8
8	1.84	17.90	16.3	73.4 ± 25.6
9	1.77	15.00	14.8	52.4 ± 46.4
10	0	1.40		62.6 ± 23.5
11	0	2.10		53.3 ± 38.1
12	1.76	8.60	49.3	47.7 ± 13.2

[a] Sialoglycopeptides released from [^{3}H]sialoglycoproteins converted to NANA equivalents by using the specific activity of 29 μCi of radioactivity, $nmol^{-1}$ of NANA.
[b] NANA released from sialyllactose.
[c] Measured as the ratio of areas under the peak in the radiometric TLC scan.
[d] Cytotoxicity for ^{51}Cr-labeled bovine pulmonary lavage cells. Mean of three trials ± standard deviation.

chromatographed with an R_f value (0.18 to 0.21) similar to that of trypsin-released sialoglycopeptides. The minor component was identified as sialic acid.

Preliminary characterization of the sialoglycopeptide product shows that it is TCA soluble, is not extractable in organic solvents, has a molecular weight of over 3,500, and contains 7.2×10^{-2} μmol of NANA mg^{-1}. There is thus strong indication that this fragment is a sialoglycopeptide. It is unlikely that any component of the erythrocyte membrane other than sialoglycopeptide would contain a comparable density of sialic acid. Trypsin has been shown to release TCA-soluble sialoglycopeptides with a high content of sialic acids from human erythrocyte membranes (13, 19). However, release of the sialo compound identified in this study was not due to tryptic activity, nor was it attributable to the activity of proteolytic enzymes similar to chymotrypsin and thermolysin. Specific inhibitors of these enzymes did not affect the activity of the cytotoxin enzyme, nor did crude cytotoxin hydrolyze the specific substrates of these enzymes. No phospholipase activity was detected in the cytotoxin preparation.

The release of sialic acid (NANA) by crude cytotoxin was confirmed as a separate activity.

Only prolonged pretreatment with *o*-phenanthroline significantly reduced the proteolytic enzyme activity. It is therefore probable that this enzyme is metal dependent. The novel enzyme has been shown to have a broad pH optimum around pH 7.0.

The proteolytic activity described in the crude cytotoxin is compatible with the ability of the enzyme to release sialoglycopeptides from erythrocyte membranes. Additionally, the presence of glycophorin was found to inhibit the release of soluble radioactive fragments from ^{3}H-labeled erythrocyte ghosts. Casein, which was only weakly hydrolyzed by the toxin, also showed very weak inhibition. Although the evidence presented is not yet conclusive, it is probable that the enzyme in the crude cytotoxin responsible for the release of the sialoglycopeptide is a neutral protease, metal dependent, and specific for sialoglycoproteins. Although this description is consistent with that of thermolysin, no thermolysin activity was observed.

Like cytotoxicity, both protease and neuraminidase activity were widely distributed among *P. haemolytica* serotypes. Cell-bound neuraminidase activity in several serotypes of *P. haemolytica* has been reported by Frank and Tabatabai (6). As was found in our experiments, these workers reported no neuraminidase activity associated with types 4, 10, and 11, but activity was also absent from types 2 and 3. In contrast, the highest neuraminidase activity detected in the current investigation was in the culture supernatant from serotype 2. The apparent discrepancy in these two studies may be accounted for by the different methods of preparation used or it could reflect bacterial strain differences, unrelated to serotype.

During growth, bacteria may release a variety of extracellular enzymes or products, some or all of which may be important in pathogenesis. Culture supernatant from *P. haemolytica* poten-

tially contains a complex mixture of bacterial components. The demonstrated cytotoxic activity of this crude supernatant for bovine leukocytes (16) prompted investigation of the enzyme activity of the preparation with the hope of elucidating possible virulence factors. Both neuraminidase and the novel protease activity described were detected.

Neuraminidase, produced by a wide variety of bacteria, both pathogenic and nonpathogenic, is generally considered to play only a minor role, if any, in virulence. However, the neuraminidase of *Vibrio cholerae* can unmask the receptor for cholera toxin on cells (22), and a similar role for the enzyme might be proposed here.

Of special interest was the detection of the novel protease. Proteases have been implicated as virulence factors for a number of bacteria, particularly in the pathogenesis of bacterial pneumonias, such as those associated with *Legionella pneumophila* and *Pseudomonas aeruginosa* (9, 20). Both of these bacteria also exhibit cytotoxic activity for alveolar macrophages, although toxicity is apparently not restricted to leukocytes, nor is it host species-specific as is the case with *P. haemolytica*. Purified proteases of *Pseudomonas aeruginosa* were found to cause agglutination and intense vacuolization of rabbit alveolar macrophages but not cell death (9). Electron microscopy indicated damage at the level of the cell membrane. If the *P. haemolytica* protease exerts a similar effect it may render the cell susceptible to the action of the cytolytic principle. Like neuraminidase, the protease may, by removal of specific glycoproteins from the cell membrane, unmask receptors for the cytotoxin. It is possible that the cytotoxin may be released as a prototoxin, such as *Clostridium botulinum* type A or *Escherichia coli* labile toxin (21), requiring cleavage by protease for activation. Any or all of these mechanisms could be important in pathogenesis.

The relationship of protease activity in the supernatant of *P. haemolytica* to the cytotoxic activity is speculative. Studies are now in progress to purify and characterize both the proteolytic enzyme and the cytotoxic principle. Interestingly, both of these factors and neuraminidase activity were found in many serotypes of *P. haemolytica*, even though only type 1 and very rarely type 2 have been associated with bovine pneumonia. Clearly the pathogenesis of pneumonic pasteurellosis is complex. It is possible that factors related to serotype determine the ability of the organism to colonize the respiratory tract. The subsequent release of factors, such as those described above, may contribute to its virulence. The failure of whole bacteria bacterins to protect cattle against pneumonia may well reflect the complexity of virulence factors involved; their elucidation could lead to successful prophylaxis in the future.

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