Time and temperature dependence of granulocyte damage by leucotoxic supernatants from *Pasteurella haemolytica* A1

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Bacterial exotoxins may contribute to the pathogenic potential of micro-organisms through interactions with cells of the host defence system as well as by directly damaging host tissue. The present studies were designed to explore mechanisms of interaction between bovine granulocytes and the leucotoxin produced by *Pasteurella haemolytica*, a major cause of bovine respiratory disease. Leucotoxin-containing supernatant from *P. haemolytica* A1 caused rapid cell death in isolated bovine granulocytes that was close to half-maximal by 5 min and nearly 90% complete after 30 min at 37 °C. Maintaining granulocytes at ice-water temperature markedly attenuated or prevented the toxic effect; furthermore, if exposed to supernatants at ice-water temperature and then washed, most cells remained viable even after rewarming to room temperature. However, even a very brief exposure (about 5 s) at 37 °C led to extensive cell death even after immediate cold dilution and washing. Granule enzymes such as arylsulphatase were released far more slowly than cytosol contents. Leucotoxin purified by column chromatography showed temperature dependence and divergence between cytosol and granule marker release similar to those observed with the crude supernatant preparation. These findings indicate that irreversible interaction between *P. haemolytica* leucotoxin and bovine granulocytes is initiated very rapidly at 37 °C but markedly impeded at low temperature, while granule enzyme release follows cytosol marker release over a much longer period. The results suggest either a requirement for target cell metabolic activity to initiate toxin effects or a temperature-dependent receptor conformation, with granule enzyme release following as a secondary consequence of granulocyte death.

Introduction

Exotoxin release is among the major factors contributing to virulence in bacterial infections. Well-recognized modes of action of bacterial exotoxins include direct interactions such as host-cell destruction by membrane lysis, inhibition of host cell metabolism by interference with protein synthesis, and host cell energy depletion by exhaustion of energy-rich phosphates. Exotoxins may also play an indirect role in pathogenesis by interacting with cells of the host defence system to decrease the microbicidal efficacy of these defensive cells and induce secondary host tissue damage through release of toxic metabolites or enzymes from the cells of primary host defence. Bovine pneumonic pasteurellosis represents an

intriguing model for these latter categories of indirect exotoxin action because the pathogen, *Pasteurella haemolytica*, releases a leucotoxin active against several leucocyte types in susceptible species (Chang *et al.*, 1986; Chang & Renshaw, 1986; Clinkenbeard *et al.*, 1989; Kaehler *et al.*, 1980; O'Brien & Duffus, 1987). Challenge studies have suggested that neutrophils are a major component of the pulmonary response (Walker *et al.*, 1985) and that neutrophil depletion protects calves against at least one phase of the lung damage which is characteristic of this infection (Breider *et al.*, 1988; Slocombe *et al.*, 1985). Modification of neutrophil function may therefore play an important role in pathogenesis. The possible participation of the leucotoxin in this process is not well understood at this time, but a role in virulence may be suspected, as with other bacteria that release toxins active against phagocytes (Densen & Mandell, 1980).

Abbreviations: IEF, isoelectric focusing; LDH, lactate dehydrogenase.

To delineate the contribution of *P. haemolytica* leucotoxin to the pathogenesis of pulmonary infection, it is important to elucidate the mechanisms of interaction between this toxin and susceptible host leucocytes. Understanding these mechanisms may also provide insight into the action of other bacterial exotoxins which modify host defence responses in analogous ways. In the studies reported here, we sought to define requirements for initiation of the leucotoxin effect on bovine granulocytes and the time course of cytotoxic effects.

Methods

Granulocyte isolation. Blood samples were obtained from adult dairy cattle by venipuncture using heparinized syringes. Granulocytes were separated from mononuclear leucocytes by Histopaque (Sigma) density gradient centrifugation as in our previous studies (Styrt *et al.*, 1990). Erythrocytes were lysed using two or three cycles of hypotonic lysis with 0.2% (w/v) NaCl, followed by addition of an equal volume of 1.6% (w/v) NaCl to return the suspension to isotonicity. Granulocyte purification was checked in selected experiments using Wright differential staining. Isolated granulocytes were resuspended in Hanks' balanced salt solution at concentrations approximating 5 to 6×10^6 cells ml^{-1} for assays for cell viability and marker release. Granulocyte preparations obtained by this method typically consist mostly of neutrophils with a smaller population of eosinophils and occasional contaminating mononuclear cells.

Leucotoxin-containing supernatants. *P. haemolytica* (biotype A, serotype 1) was originally isolated on Brain Heart Infusion (BHI) agar (supplemented with 1% (w/v) yeast extract, 1% (v/v) horse serum and 5% (v/v) defibrinated sheep blood) from the pneumonic lung of a calf. To maintain its virulence, this organism was subsequently passaged in calves by intrabronchial inoculation. Isolates from the calves were passaged twice on supplemented BHI agar suspended in sterile skim milk, lyophilized and frozen at -70°C . Lyophilized stock was reconstituted with sterile distilled water and used to inoculate supplemented BHI plates. Following overnight incubation, BHI broth supplemented with 1% heat-inactivated horse serum was inoculated with 5 to 10 isolated colonies. The primary broth was incubated overnight in a 37°C shaking water bath prior to making a 1% transfer to RPMI 1640 medium (Gibco) supplemented with 7% (v/v) heat-inactivated foetal bovine serum. This secondary culture was grown for 5.5 h in a 37°C shaking water bath and then centrifuged for 10 min at 10000 r.p.m. at 4°C . The supernatant was harvested, filter-sterilized ($0.22\ \mu\text{m}$) and stored in aliquots at -70°C . Supernatants which did not kill the great majority of granulocytes at 1:10 dilution were discarded as having insufficient leucotoxin activity for appropriate study.

Purified leucotoxin. An overnight culture of *P. haemolytica* A1 strain B122 (Deneer & Potter, 1989; Potter *et al.*, 1988) was diluted 100-fold into BHI broth (Difco) and shaken at 37°C until the OD_{660} was 1.0. The culture was centrifuged at 5000 r.p.m. in a Sorvall GSA rotor at 4°C for 10 min and the supernatant was filtered through a $0.22\ \mu\text{m}$ membrane. This was then frozen in a dry ice/ethanol bath and lyophilized. This material was rehydrated with 0.1 volume of 10 mM-Tris/HCl, pH 7.5, and dialysed for 48 h against double-distilled water (six changes, 3500 ml each). This crude culture supernatant was fractionated by preparative isoelectric focusing (IEF) along a pH 3 to

10 gradient in a Rotofor IEF apparatus (Bio-Rad) as described by the manufacturer. Each IEF run was for 4 h at 12 W constant power. Twenty fractions were collected and a sample of each was tested for leucotoxin activity by lactate dehydrogenase (LDH) release from bovine neutrophils (Korzeniewski & Callewaert, 1983). Those fractions containing components with pI values between 6.0 and 7.2 were pooled, NaCl was added to 1 M and the fractions were desalted on Sephadex G25 disposable columns (Pharmacia). Endotoxin was removed by affinity chromatography using a Detoxigel column (Pierce) as described by the manufacturer. Because the purified leucotoxin showed substantial variation in activity, comparative experiments were done with leucotoxin concentrations adjusted to give approximately the same degree of cytotoxicity as the culture supernatants used in analogous settings.

Assays of granulocyte death. Granulocyte death was measured in two ways. Release of the cytosol marker LDH was measured spectrophotometrically by standard methods as described previously (Styrt *et al.*, 1985). Trypan blue dye uptake was measured by adding 0.1 ml 0.4% trypan blue in normal saline to 0.1 ml of cell suspension and 0.8 ml phosphate-buffered saline (PBS Dulbecco), and examining microscopically under a coverslip. Cells which stained blue were considered non-viable. Trypan blue dye exclusion was used preferentially to measure cell viability in experiments involving extremely long incubations or extensive dilution of the cell suspension, because the dilution or prolonged incubation tended to result in LDH activity that was too low to be accurately measured.

Release of granule contents. Release of granule contents was measured by splitting the samples into two equal portions, centrifuging one of the aliquots for 10 min at 4°C and 300 g, and measuring enzyme content in the supernatant. This was compared with total cellular enzyme content in the remaining duplicate aliquot of cells lysed with Triton X-100.

Arylsulphatase was measured fluorometrically by release of 4-methylumbelliferone from its sulphate conjugate (Fluharty & Edmond, 1978). Incubation was for 30 min at 37°C and fluorescence was read at 365 nm excitation and 450 nm emission in an Aminco-Bowman spectrophotofluorometer. The fluorescent product 4-methylumbelliferone was used to construct standard curves.

Elastase-like activity was measured fluorometrically as release of 7-amino-4-methylcoumarin from MeOSucc-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin in a modification of methods described by Sklar *et al.* (1982) and Brown & Roth (1990). Incubation was done for 4 h at room temperature and the fluorescence generated was compared against 7-amino-4-methylcoumarin standards.

Temperature effects. To determine whether cytotoxicity could be initiated at low temperatures, duplicate samples of cells were incubated with leucotoxic supernatant at 37°C or in an ice-water bath. In selected experiments, granulocytes were then washed with cold PBS and allowed to return to room temperature for 30 min before viability determinations.

For assessment of the cytotoxicity of very brief leucotoxin exposures, granulocytes were warmed to 37°C for 5 min before addition of leucotoxin. Within 5 to 10 s after leucotoxin addition, the cell suspension was diluted with three volumes of ice-cold PBS, centrifuged and washed a second time with cold PBS. The granulocytes were then resuspended in PBS and rewarmed to 37°C for 5 min before viability determination.

Statistical treatment. Replicate experiments were done at least three times unless otherwise noted in Results. Results are shown as the mean and standard error of the mean for the stated number of experiments (blood drawn on different days or from different animals) unless otherwise indicated. *t*-test analyses (Colton, 1974) were used for evaluations of statistical significance.

Results

Time course of cell damage

Fig. 1 shows cell death measured as failure to exclude trypan blue dye in granulocytes exposed to a 1 in 10 dilution of leucotoxin-containing *P. haemolytica* culture supernatant. The onset of cell damage was very rapid, with half-maximal cytotoxicity in this series of experiments observed after 5 min and nearly 90% cell death after 30 min of incubation.

Measurements of cell death were compared using both trypan blue exclusion and LDH release in selected experiments. The two methods gave generally parallel results at incubation times allowing appropriate comparisons. For example, in three experiments paired at 5, 30 and 60 min time points with or without supernatant, there were no significant differences between percentage cell death determined by failure to exclude trypan blue and LDH release ($72 \pm 5\%$, $88 \pm 1\%$, $85 \pm 4\%$ death by trypan blue at 5, 30 and 60 min with supernatant compared with $73.4 \pm 7.7\%$, $96.2 \pm 1.0\%$, and $95.7 \pm 2.0\%$ LDH release). Attempts at incubations of less than 5 min led to poorly comparable results (data not shown) because of the difficulty of stopping the interaction with toxin at a precise time; the cold-dilution methods described below were developed to approach this issue.

Time course of degranulation

Fig. 2 shows the release of the granule enzyme arylsulphatase. In accordance with our previous findings (Styrt *et al.*, 1990), a much smaller percentage of arylsulphatase was released during the initial incubation period relative to percentage loss of viability. When incubation with leucotoxic supernatant was prolonged, $32.5 \pm 5.3\%$ of arylsulphatase was released after 4 h (Fig. 2a); with overnight incubation (Fig. 2b) this rose to $81.1 \pm 7.0\%$. A modest excess arylsulphatase release relative to cell death was seen in samples without supernatant after overnight incubation, and was thought possibly attributable to a small number of cells entirely lysed and not detectable on the trypan blue slides, to selective destruction of cells with high arylsulphatase content (e.g. eosinophils), or to active secretion due to stimulation of cells by the very prolonged incubation.

In selected experiments, the release of activity associated with another lysosomal enzyme, elastase, was measured. The supernatant preparations used in this series of experiments had somewhat less leucotoxic activity and granule enzyme release was somewhat higher than in the longer-incubation experiments, but the delay in release of both enzymes relative to

Fig. 1. Cytotoxicity of *P. haemolytica* supernatant (1 in 10 dilution) as measured by loss of the ability to exclude trypan blue dye. ●, Cells exposed to leucotoxic supernatants; ○, control cells incubated in buffer for the indicated time periods. Mean $\pm$ SEM of 3 to 5 values are shown for each time point, representing experiments with at least 4 h total incubation and granule enzyme measurements (see Fig. 2) done for comparison.

cytotoxicity was confirmed. Cell death (by trypan blue), arylsulphatase release and release of elastase-like activity were $69 \pm 4\%$, $32.6 \pm 5.8\%$ and $26.9 \pm 6.4\%$, respectively at 30 min, and $84 \pm 2\%$, $60.9 \pm 6.4\%$ and $50.2 \pm 9.7\%$, respectively at 2 h (mean $\pm$ SEM of five experiments; $P < 0.001$ for comparison of elastase release with cell death at each time point; $P < 0.01$ for arylsulphatase release versus cell death at 30 min and $P < 0.05$ at 2 h).

Temperature effects on granulocyte damage

Although cell death occurred rapidly at 37°C , granulocytes maintained at ice-water temperature showed much less evidence of damage even after prolonged exposure to leucotoxic supernatants. Fig. 3 shows loss of cell viability assessed by LDH release following 60 min of exposure to 1 in 10 dilutions of supernatant in an ice-water bath. Very little cell death was apparent at the end of incubation. Furthermore, if cells exposed to toxin at low temperature for 5 min were diluted with cold PBS and returned to room temperature, most of the granulocytes ($97 \pm 1\%$) remained viable although direct exposure to leucotoxic supernatant at room temperature was highly toxic ($68 \pm 13\%$ cell death).

In additional experiments, granulocytes were pre-warmed to 37°C , leucotoxic supernatant was added, and the cell suspension was immediately diluted with ice-cold buffer and washed at 4°C before rewarming and

Fig. 2. (a) Percentage release of arylsulphatase in the same experiments as Fig. 1. ●, Cells exposed to leucotoxic supernatants; ○, control cells incubated in buffer. (b) Cell death (percentage of cells failing to exclude trypan blue) and percentage of arylsulphatase release in cell suspension incubated overnight with leucotoxic supernatant at 1 in 10 dilution (□) or control buffer (▨). Mean $\pm$ SEM of five experiments.

assessment of viability. Thus, cells were exposed to supernatant for only about 5 to 10 s at 37 °C before being chilled and washed. In contrast to the results observed when cells were kept cold throughout the period of supernatant exposure, Fig. 4 shows that even momentary exposure at 37 °C led to cell damage which could not be prevented or reversed by washing.

Effects of purified leucotoxin

Selected experiments were replicated using the small quantities of purified leucotoxin available. As shown in Fig. 5(a), three experiments using a 30 min incubation

Fig. 3. Percentage release of LDH following 60 min of exposure to leucotoxic supernatant (□) or control buffer (▨) at 37 °C or 0 °C. Mean $\pm$ SEM of four experiments. $P < 0.001$ for toxin-induced release at 37 °C versus 0 °C.

Fig. 4. Percentage cell death (failure to exclude trypan blue) after exposure to 1 in 10 dilution of leucotoxic supernatant at 37 °C or 0 °C followed by immediate dilution with 37 °C or 0 °C buffer, washing, and incubation at 37 °C for 5 min. □, Exposure and dilution at 37 °C; ▨, exposure and dilution at 0 °C; ▤, exposure at 37 °C and dilution at 0 °C. Mean $\pm$ SEM of five experiments. $P < 0.001$ for comparison of either right or left bar with centre bar.

with the purified toxin (at concentrations giving cell death comparable to that observed with culture supernatants) produced a similar divergence between cell death and arylsulphatase release. As shown in Fig. 5(b), momentary exposure to the purified toxin at 37 °C

Fig. 5. (a) Percentage release of LDH (□) and arylsulphatase (▨) following 30 min of exposure to purified toxin at 37 °C. Mean $\pm$ SEM of three experiments. $P < 0.001$ for comparison of LDH release with arylsulphatase release. (b) Percentage cell death (failure to exclude trypan blue) after momentary (5 to 10 s) exposure to purified toxin (□) or buffer (▨) followed by immediate cold dilution, washing and rewarming. Mean $\pm$ SEM of four experiments. Excess cell death attributable to toxin was $37.5 \pm 11.6\%$ ($P < 0.05$ by single sample *t*-test).

produced a much higher rate of cell death than was seen in cells not exposed to toxin. A control experiment using toxin at 0 °C for 30 min showed only 5.4% LDH release, suggesting again that contact with cells at 37 °C was needed for full toxin activity.

Discussion

Bacterial release of exoproteins may contribute to the pathogenesis of many bacterial infections. Direct damage to surrounding tissue is perhaps the most familiar effect of such toxins; systemic effects from specific vascular or neuronal toxicity are also well recognized. Less well studied and more indirect is the potential pathogenic contribution of exotoxins which attack host phagocytic cells. These may exert their pathogenic activity primarily by depressing the ability of the phagocytes to contend with bacterial invaders, and secondarily by eliciting phagocytic release of oxygen metabolites or digestive enzymes which may further exacerbate local host tissue damage.

The leucotoxin produced by *P. haemolytica* has an evident depressive effect on phagocytic cell function through inhibition of the respiratory burst of oxidative metabolism (Chang & Renshaw, 1986). We have previously found this effect to be preventable by the use of antiserum raised against whole *P. haemolytica* (Styrt *et al.*, 1990). In the studies reported here, we found that

keeping cells chilled throughout the period of exposure to leucotoxin-containing culture supernatants prevented most of the cytotoxic effect; however, even a momentary exposure to toxin at 37 °C caused irreversible granulocyte damage. We also found that leakage of granule enzymes such as arylsulphatase (which might be a marker for either neutrophils or eosinophils; Healy, 1982) could continue for many hours after granulocyte death appeared effectively complete. Release of activity associated with elastase, a neutrophil granule enzyme (Sklar *et al.*, 1982), was also slower than the time course of cell death. While most of these studies were done with a crude leucotoxin preparation (culture supernatant), results of selected experiments repeated with the small amount of purified toxin available were generally confirmatory. While this finding does not establish whether the leucotoxin molecule is responsible for all of the effects seen with culture supernatants, it does suggest that the observed divergence between cytosol and granule enzyme release is a feature of the leucotoxin activity.

While investigation of the molecular mechanism of action and pathogenic role of *P. haemolytica* leucotoxin has been hampered by the difficulty of obtaining pure preparations with good biological activity, several tentative conclusions can be drawn regarding its structural and functional relationships to other bacterial exotoxins with cytotoxic activity. Although additional granule marker assays and ultrastructural studies would be needed for confirmation, our findings suggest that this leucotoxin is unlikely to act in a manner similar to that of streptolysin S (Densen & Mandell, 1980; Alouf, 1980; Alouf & Loridan, 1988) or staphylococcal alpha toxin (Densen & Mandell, 1980), which are thought to stimulate massive degranulation or directly lyse granules as part of their activity. Staphylococcal leucocidin is also thought to induce degranulation (Densen & Mandell, 1980) but requires two protein components for activity which is lost when these are separated during purification (Noda *et al.*, 1980; Noda & Kato, 1988); a similar requirement might explain the difficulty of purifying *P. haemolytica* leucotoxin in an active form. Another possible analogy is to the leucocidin recently described as a product of *Fusobacterium necrophorum*, which itself requires further characterization (Emery *et al.*, 1984; Emery & Vaughan, 1986; Kanoe *et al.*, 1988). The reported sequence homology between *P. haemolytica* leucotoxin and *Escherichia coli* alpha haemolysin (Lo *et al.*, 1987; Strathdee & Lo, 1987) would also suggest a common mechanism of action, but we are not aware of any detailed studies of interactions between the *E. coli* haemolysin and granulocytes.

These findings have several implications both for understanding the molecular and cellular basis of

leucotoxin/granulocyte interaction and for elucidating the pathophysiology of *P. haemolytica* infection. On the one hand, the rapid initiation of irreversible cell damage at 37 °C and its attenuation by cold temperatures suggest that leucotoxin activity depends either on a contribution of metabolic activity by the host cell (e.g. endocytic internalization of the toxin) or on properties of the cell membrane or the toxin that might change with temperature (e.g. membrane viscosity or receptor conformation). On the other hand, the differing time courses of cytotoxicity and release of granule contents suggest a sequence of events which could begin with immediate suppression of neutrophil ability to combat the micro-organism and progress to delayed granulocyte enzyme leakage contributing to ongoing tissue damage. While further investigation is needed for determination of *in vivo* relevance, additional studies in these areas may help to elucidate and eventually to manage the effects of interactions between host phagocytes and bacterial exoproducts.

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