EFFECTS OF *ACTINOBACILLUS PLEUROPNEUMONIAE* CYTOTOXINS ON SIZE, GRANULARITY AND VIABILITY OF PORCINE NEUTROPHILS

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ABSTRAK

TARIGAN, S. 1998. Pengaruh sitotoksin *Actinobacillus pleuropneumoniae* terhadap ukuran, granularitas dan viabilitas neutrofil babi. *Jurnal Ilmu Ternak dan Veteriner* 3 (3): 188-196.

Sitotoksin yang dihasilkan oleh *Actinobacillus pleuropneumoniae* diduga mempunyai peranan yang sangat penting dalam patogenisitas dan virulensi bakteri. Untuk mengungkapkan lebih mendalam tentang mekanisme patogenisitas bakteri tersebut, aktivitas sitotoksik dari toksin yang dihasilkannya diteliti secara *in vitro* pada neutrofil babi. Perubahan ukuran, granularitas dan viabilitas sel diamati dengan sebuah sitometer alir *(flow cytometer)*. Ukuran sel dan granulasi berkorelasi erat masing-masing dengan *forward light scatter* dan *right angle light scatter*, sedangkan viabilitas sel ditunjukkan oleh intensitas *fluorescent* yang dipancarkan oleh sel yang diwarnai dengan propidium jodida. Pada konsentrasi rendah (pengenceran supernatan kultur bakteri antara 1/10 dan 1/100), sitotoksin mengakibatkan pembengkakan dan degranulasi neutrofil, sedangkan pada konsentrasi yang lebih tinggi (pengenceran supernatan kultur bakteri $\leq$1/10), sitotoksin mengakibatkan kematian sel dengan cepat. Tidak terdeteksi adanya perbedaan yang nyata dalam aktivitas sitotoksik dari sitotoksin yang dihasilkan oleh berbagai serotipe (serotipe 1, 2, 3, 5 dan 7) *A. pleuropneumoniae*. Di bawah mikroskop, neutrofil yang diperlakukan dengan sitotoksin dan diwarnai dengan Giemsa memperlihatkan perubahan patologis yang sangat jelas. Neutrofil yang diperlakukan dengan toksin dosis rendah membengkak dengan hebat disertai inti yang membulat. Sitotoksin dengan konsentrasi yang lebih tinggi mengakibatkan vakuolisasi sitoplasma dan pembesaran atau fragmentasi inti sel. Penelitian *in vitro* ini mengindikasikan dengan kuat bahwa intoksikasi neutrofil oleh sitotoksin yang dihasilkan oleh *A. pleuropneumoniae* merupakan salah satu mekanisme penting dalam patogenisitas bakteri tersebut.

Kata kunci: *Actinobacillus pleuropneumoniae*, sitotoksin, neutrofil, babi, sitometri alir

ABSTRACT

TARIGAN, S. 1998. Effects of *Actinobacillus pleuropneumoniae* cytotoxins on size, granularity and viability of porcine neutrophils. *Jurnal Ilmu Ternak dan Veteriner* 3 (3): 188-196.

Cytotoxins produced by *Actinobacillus pleuropneumoniae* are supposed to play major roles in bacterial pathogenicity and virulence. To gain better understanding in the mechanism of the pathogenicity, cytotoxic activities of the toxins on porcine neutrophils were investigated *in vitro*. Changes in cell size, granularity and viability were examined with a flow cytometer. Cell size and granularity correlate with forward light scatter and right angle light scatter, respectively; whereas, cell viability corresponds with fluorescent intensity of cells stained with propidium iodide. At low concentrations (dilutions between 1/10 and 1/100 of bacterial culture supernatants), the cytotoxins induced severe swelling and degranulation of neutrophils; whereas, at higher concentrations (dilutions of $\leq$1/10 bacterial culture supernatants), the cytotoxins caused rapid cell death. There was no significant difference in cytotoxic activities of cytotoxins derived from various serotypes (serotypes 1, 2, 3, 5 and 7) of *A. pleuropneumoniae*. Morphologically, the cytotoxin-treated neutrophils stained with Giemsa showed profound changes. Neutrophils treated with low dosages of cytotoxins became swollen with spherical nuclei. Higher concentration of cytotoxins caused vacuolation of cytoplasm, enlargement or disintegration of nuclei. This *in vitro* study indicates strongly that intoxication of neutrophils by cytotoxins produced by *A. pleuropneumoniae* comprises an important mechanism in the pathogenicity of the bacteria.

Key words : *Actinobacillus pleuropneumoniae*, cytotoxin, neutrophils, pig, flow cytometry

INTRODUCTION

Actinobacillus pleuropneumoniae (App), formerly called *Haemophillus parahaemolyticus* or *H. pleuro-* pneumoniae, is a Gram-negative bacterium, causing necrotizing haemorrhagic pleuropneumonia in pigs (SHOPE, 1964; SHOPE *et al.*, 1964). This disease which is found in many parts of the world causes significant

economic losses to swine industry. Based on antigenic properties of their capsular polysaccharide, App devided into 12 distinct serotypes (serotypes 1 to 12). The capsule, lipopolysaccharide and cytotoxins produced by App constitute the virulent and pathogenic factors for the bacteria (ROSENDAL *et al.*, 1980; FENWICK and OSBURN, 1986; JENSEN and BERTRAM, 1986). Cytotoxins consider to be the most important virulent and pathogenic factors (ROSENDAL *et al.*, 1980). The cytotoxins have been called Apx (*A. . pleuropneumoniae* Rtx) because the toxins belong to Rtx (repeat structure toxin) toxin family (FREY *et al.*, 1993).

Although molecular structure of Apxs is relatively well characterised, the mechanisms of cytotoxicity of these toxins are still poorly understood. Alveolar macrophages and recruited neutrophils constitute the first line of defence against microbial invasions in the lungs. These cells phagocytize invading bacteria that slip into the lungs. Our previous studies indicate that cytotoxin produced by App are very toxic to porcine alveolar macrophages (TARIGAN *et al.*, 1994). The present study investigate the effects of the toxin on porcine neutrophils and concludes that the toxin also very toxic to porcine neutrophils.

MATERIALS AND METHODS

Bacterial isolates

Twenty-seven isolates of *Actinobacillus pleuropneumoniae* (App) used in this study were a kind gift from Dr. P.J. Blackall of the Animal Research Institute, Moorooka, Queensland, Australia. The isolates consisted of serotype 1 (13 isolates), serotype 2 (4 isolates), serotype 3 (2 isolates), serotype 5 (3 isolates) and serotype 7 (5 isolates).

Production of cytotoxin

Actinobacillus pleuropneumoniae was grown on chocolate blood agar (CBA) plates at 37°C for about 18 hours in a candle jar. Bacteria from the CBA plates were suspended in RPMI-1640 (Gibco Laboratories, Grand Island, USA) buffered with 10 mM (n-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid) (HEPES) (Sigma Chemical, St. Louis, USA), approximately one plate per 15 ml of liquid media. After incubation at 37°C for 2 hours, the bacteria were pelleted by centrifugation at 12,000 x g for 10 minutes, and the supernatant was collected and then either concentrated, or its activity was directly assayed.

Culture supernatants from isolates HS25, HS35, HS77, HS238, HS30 representing serotypes 1, 2, 3, 5

and 7, respectively were concentrated to 1/300 of their original volumes. Proteins contained in the culture supernatants were precipitated by slowly adding ammonium sulphate to 80% saturation, followed by centrifugation at 15,000 x g for 30 minutes. Before addition of the ammonium sulphate, bovine serum albumin (Sigma Chemical, St. Louis, USA) was added to the supernatant to a final concentration of 0.5 mg/ml in order to stabilise Apxs and facilitate their concentration. The precipitate was dissolved in RPMI and undissolved materials were removed by centrifugation at 15,000 x g for 30 minutes. Desalting and further concentration were achieved by ultra filtration using Centriprep-10 concentrators (Amicon Co., Denvers, USA). The concentrated Apxs were aliquoted and stored at -70°C. These concentrated culture supernatants are referring to as (concentrated) Apx preparations.

Purification of neutrophils

Porcine neutrophils were purified by use of discontinuous Percoll gradient according to a previously described technique (ROBERTS *et al.*, 1987) with some modification. First, isotonic Percoll was prepared by mixing one part of 10 x PBS with nine parts of Percoll solution (Pharmacia, Uppsala, Sweden). For the use in cell separation, the isotonic Percoll was diluted with PBS to obtain 75% Percoll (75% isotonic Percoll plus 25% PBS) and 62.5% Percoll (62.5% isotonic Percoll plus 37.5% PBS). A 3 ml volume of the 75% Percoll was put into a 15 ml-polypropillene centrifuge tube and on top of the 75% Percoll, 3 ml of the 62.5% Percoll was gently layered using a Pasteur pipette of which the tip had been hooked in a flame.

Porcine blood which was obtained by jugular venipuncture and anticoagulated with Ethylene-diaminetetraacetic acid (EDTA) (15 mM), was diluted with an equal volume of PBS. Diluted blood (4 ml) was gently layered on the top of the 62.5% Percoll using a hooked Pasteur pipette, and then the tube was spun at 400 x g for 25 minutes at room temperature.

After spinning, mononuclear cells which formed a band on the top of the 62.5% Percoll were descarded. Granulocytes which formed a band on top of the 75% Percoll were usually contaminated by red blood cells (RBC). These contaminating RBC were lysed by mixing the cell suspension with equal volume of cold lysing reagent (154 mM NH$_4$Cl, 13 mM NaHCO$_3$, 0.1 mM EDTA) for 5 minutes at 4°C, then the granulocytes were washed thrice with fresh RPMI containing 2.2 mM Ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) (Sigma

Chemical, St. Louis, USA), and finally suspended in RPMI plus 10% heated fetal calf serum (FCS, Commonwealth Serum Laboratory, Melbourne, Australia) or other medium when indicated, at 2.5 x 10^6 cells/ml.

Cytotoxic assay

Culture supernatant serially diluted (ten fold) in RPMI was added to an equal volume (250 µl) of a neutrophil suspension in RPMI and incubated at 37°C for 2 hours. Parallel samples in which culture supernatant had been heated at 60°C for 60 minutes were also run, and these samples served to ascertain the heat-lability of the culture supernatants. Cytotoxic assays for the concentrated Apx preparations were similar to that for culture supernatant except that the concentrated Apx preparations were serially diluted in five-fold dilutions, and incubation was shorter, only 30 or 60 minutes.

Semi-quantification of Apx-induced toxic changes

Changes in the volume and granularity of neutrophils and lymphocytes exposed to the culture supernatants were monitored using a FACscan flow cytometer (Becton Dickinson, Sunnyvale, USA). Mean channel number of forward light scatter (FSC) and right angle light or side scatter (SSC) of 10,000 cells from each sample were collected. Percent changes in mean FSC or relative cell volume of Apx-treated cells over untreated (control) cells were calculated as :

$$\frac{\text{(Mean FSC Apx-treated cells) - (Mean FSC control cells)}}{\text{(Mean FSC control cells)}} \times 100\%$$

Using a similar formula, percent changes in SSC (indication of granularity) of cells treated with the toxins were also obtained.

Cell death or cell with membrane damage caused by the toxins was identified by uptake of propidium iodide (PI, Sigma Chemical, St. Louis, USA) by the cells. Percent increase in proportion cell death (PI-positive cells) in Apx-treated cells over untreated (control) cells was calculated as:

$$\frac{\text{(% PI-positive cells in Apx-treated cells) - (% PI-positive cells in control cells)}}{\text{(100%) - (% PI-positive cells in control cells)}} \times 100\%$$

Changes in cell structure

Equal volume cell suspensions (4×10^6 cells/ml in RPMI-FCS) were mixed and incubated with Apx

preparation from serotype 1 to final dilution of 1/1,000, 1/5,000, 1/10,000, 1/20,000 or 1/40,000. Samples were removed at 30, 60 and 120 minutes incubations and stained with Giemsa.

Statistical analysis

Differences in toxic activities between culture supernatants from serotypes producing ApxI and ApxII (serotypes 1 and 5), serotypes producing ApxII and ApxIII (serotypes 2 and 3), and serotypes producing only ApxII (serotype 7) were analysed using one way analysis of variance (ANOVA).

RESULTS

Culture supernatants from all Australian isolates of App examined caused substantial changes in neutrophils (Figure 1). These changes, the magnitude of which depended on the dilution of culture supernatant, consisted of cell swelling, decreased in cell granularity, and cell death, as determined by an increased in FSC, decreased SSC, and positive staining for propidium iodide, respectively.

No toxic change was observed when the neutrophils were exposed to a 1/1,000 dilution of culture supernatant from any serotype. At higher concentrations (between 1/100 and 1/10 dilution) the supernatants of all serotypes induced severe cell swelling and decreased cell granularity. The increases in FSC coincided with similar decreases in SSC. Although profound changes in size and granularity were observed at 1/100 and 1/10 dilutions of Apx, the majority of the neutrophils (above 75%) were still impermeable for propidium iodide (PI).

Cell swelling (increased FSC) in neutrophils treated with undiluted culture supernatants was not apparent (Figure 1). Because the majority of the cells treated with undiluted culture supernatant were lysed (dead), as confirmed by uptake of PI, it was suspected that undiluted culture supernatants caused the cell to rapidly swell, and subsequently undergo shrinkage. The cell shrinkage concurred with uptake of PI (Figure 2).

There was no difference in cytotoxicity of culture supernatant between serotypes (Figure 1). Culture supernatants of serotypes 1 and 5 (which produce ApxI and ApxII) were not significantly different in cytotoxicity (P > 0.05) from either culture supernatant of serotypes 2 and 3 (which produce ApxII and ApxIII) or to culture supernatants of serotype 7 (which produces only ApxII). Nor was the cytotoxicity of culture supernatant of serotype 7 significantly different from those of serotypes 2 and 3.
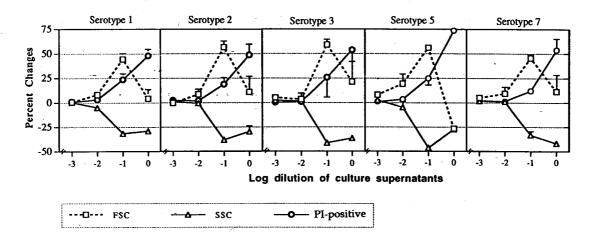
Figure 1. Toxicity of *Actinobacillus pleuropneumoniae* culture supernatant on porcine neutrophils. Data represent mean ± SE of 13 isolates (serotype 1), 4 isolates (serotype 2), 2 isolates (serotype 3), 3 isolates (serotype 5) and 5 isolates (serotype 7). The toxicity of the supernatants were not significantly different between serotypes (P>0.05)
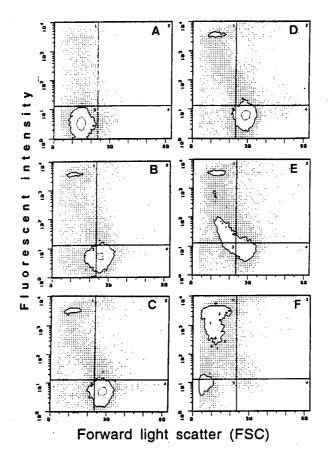

Figure 2. Contour graphs showing changes in FSC (size) and fluorescene (cell viability) of neutrophils treated with Apx preparation for 2 hours. A : untreated control, B : treated with Apx preparation at 1/25,000, C : at 1/5,000, D : at 1/1,000, E : at 1/200 and F : at 1/40. Note that treatment at low concentrations (B, C, D) causes marked swelling, without emission of fluorescene. Treatment at high concentrations (E, F) results in emission of strong fluorescence and cell shrinkage (E)

Cytological changes in Apx-treated neutrophils were obvious, even at a very high dilutions of the Apx preparations (1/40,000) and for short periods (30 minutes) of incubation. The most obvious changes were observed in the nuclei; changes in the cytoplasm were difficult to see, because of faint staining. The nuclei of about half the cells became round or fusiform, with the remaining cells still having characteristic segmented nuclei.

At a higher toxin concentration (1/20,000 dilution) only a small proportion (about 5%) of the neutrophils had typical segmented nuclei; the majority of the cells had rounded, elongated or irregular nuclei. These cells were swollen with a vacuolated cytoplasm (Figure 3B). At a 1/10,000 dilution or lower, practically no neutrophils displayed segmented nuclei. At 1/5,000 and 1/1,000 dilutions, many of the neutrophils had enlarged, vacuolated, irregularly shaped nuclei and lacked obvious cytoplasm (Figure 3C).

More severe morphologic changes were observed when the Apx exposure was prolonged. At 60 minutes incubation, practically no cells had characteristic segmented nuclei even at the lowest toxin concentration (1/40,000 dilution). The nuclei were marginated with rounded, elongated, crescent or irregular in shape (Figure 3D, E).

DISCUSSION

This study characterised various responses of porcine neutrophils and lymphocytes to Apx preparations derived from Australian isolates of App. To monitor these responses, flow cytometry techniques were developed. These techniques were very convenient and facilitated the rapid and simultaneous measurement of three different type of responses; cell size, granularity and cell viability. In addition, the techniques allowed us to quantify responses of large number (10,000) of individual cells, rather than measure the mean responses of the total cell population. The capability to simultaneously measure several types of response on a single cell basis is unique, and has not been previously applied to the study of Apx-induced cytotoxicity. Conventional techniques that have been used include the dye-exclusion test (KAMP et al., 1991), lactate dehydrogenase (LDH) release (ROSENDAL et al., 1988), [51]Chromium release (BENDIXEN et al., 1981), reduction of a tetrazolium (MTT) dye (UDEZE and KADIS, 1992) and chemiluminescence techniques (DOM et al., 1992).

Cytotoxins of Rtx family injure mammalian target cells by creating transmembrane pores due to partial insertion of the toxins into the lipid bilayers (BHAKDI et al., 1986). The pore size is sufficiently small that only allow passage of small osmolytes, especially cations, but not macromolecules such intracellular proteins. The resulting higher intracellular osmotic pressure causes influx of water, which results in cell swelling.

The swelling cells Rtx toxins treated cells is well recognised (BHAKDI et al., 1986). Probably because of the lack of simple, accurate techniques to monitor the toxin-induced swelling, this cell response has received little attention. Swelling in bovine lymphoma cells (BL3 cells) induced by P. haemolytica leukotoxin was demonstrated by bright field microscopy, scanning electron microscopy, and increased packed cell volume (CLINKENBEARD et al., 1989b). Although the bright field and scanning electron microscopy techniques gave an absolute value of cell size, these techniques were very time-consuming. In the present study, changes in the cell volume induced by the toxins were assessed by changes in the FSC as measured with a FACScan flow cytometer. Although this technique does not give an absolute value for the cell volume, it is very sensitive, rapid, and results obtained are reproducible. Because the relative change rather than absolute cell volume was more important in this study, flow cytometry was sufficient. Cell swelling, as indicated by the increase in FSC of Apx-treated cells was confirmed by Giemsa-stained cytological preparations.

Treatment of bovine neutrophils, or BL3 cells, with P. haemolytica leukotoxin resulted in cell swelling followed by formation of large membrane defects which allow the release of large cytoplasmic components such LDH (CLINKENBEARD et al., 1989a, b, c). The formation of the large membrane defects and rate of LDH release were proportional to the leukotoxin concentration (CLINKENBEARD et al., 1989a, b, c). In agreement with these previous studies, we noted that neutrophils treated with high concentration of Apx preparations developed rapid (within 30 minutes) formation of membrane defects as indicated by uptake of PI. Neutrophils treated with a high concentration of Apx preparation for 30 minutes or longer did not display cell swelling. It is suspected that swelling might have occurred, but because of the rapid formation of large membrane defects, it lasted for only a short time. This suggestion is supported by our study in alveolar macrophages that the duration swelling was inversely correlated with the Apx dosages (TARIGAN et al., 1994). Perhaps cells lose volume later as further membrane leakage and damage occurs.
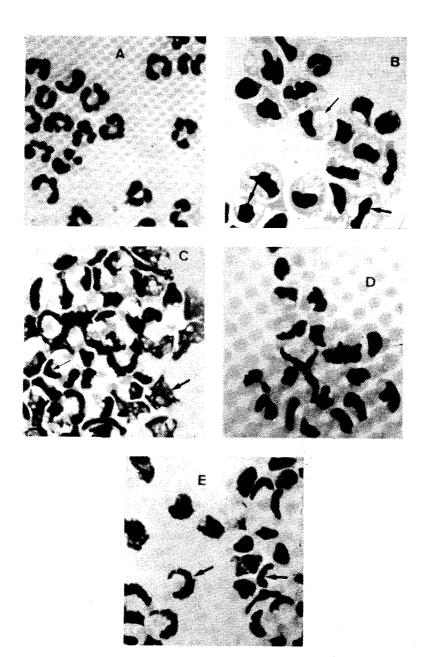
Figure 3. Cytological changes in neutrophils treated with Apx preparation at different dilutions and time of incubation. Giemsa, 768 X

A : Untreated (control) neutrophils

B : Neutrophils treated with Apx preparation at 1/20,000 dilution for 30 minutes. The cells are swollen, cytoplasms are vacuolated (small arrow), and the nuclei have become elongated (large arrow) or rounded (arrow head)

C : Neutrophils were treated with Apx preparation at 1/5,000 dilution for 30 minutes. The nuclei have become marginated (small arrow), or enlarged, irregular, vacuolated and "smudged" with indistinc borders (large arrow)

D : Neutrophils were treated with Apx preparation at 1/40,000 dilution for 60 minutes. The nuclei of most cells are marginated with rounded or elongated shape

E : Neutrophils treated with Apx preparation at 1/20,000 dilution for 60 minutes. The changes are more severe with prolonged incubation (compare with B). Many cells have crescentic nuclei (arrows), and others are "smudged"

Increases in the relative cell volume (FSC) of neutrophils treated with Apx preparations as observed in this study, were always accompanied by a parallel decrease in cell granularity (SSC). However, the two type of light scattering were independent (FLETCHER and SELIGMANN, 1984; SKLAR et al., 1984). In other words, changes in SSC are not directly the result of FSC changes and the relationship between FSC and SSC are not consistent. The SSC value of cells is determined largely by their cytoplasmic contents (FLETCHER and SELIGMANN, 1984; SKLAR et al., 1984; SKLAR et al., 1985). Because granules and F-actin represent the major constituent in neutrophil cytoplasm, each comprising between 10 to 20% of the cell protein, the value of SSC is closely correlated with the quantity these cell components (FLETCHER and SELIGMANN, 1984; SKLAR et al., 1984; SKLAR et al., 1985).

In neutrophils, the SSC value is also determined by the release of granules. When the cells degranulate or discharging their granule contents, the SSC value of the cells decreases (FLETCHER and SELIGMANN, 1984; SKLAR et al., 1984). In fact, FLETCHER and SELIGMANN (1984) reported that decreased SSC of fMLP-stimulated neutrophils treated with Cytochalasin B was closely correlated (r = 0.9899) with degranulation, as determined by b-glucuronidase release.

Degranulation in Rtx-treated neutrophils is associated with the formation of large membrane defects. Release of secondary granules (vitamin B_{12}-binding protein) by E. coli a-haemolysin treated human neutrophils occurred at doses similar to that causing ATP leakage and PI influx; whereas, release of primary granules (myeloperoxidase and elastase) and tertiary granules (glucosaminidase) required higher doses of toxin (BHAKDI et al., 1989). Similarly, P. haemolytica leukotoxin caused the release of secondary granules (myeloperoxidase and arylsulphatase B) in bovine neutrophils only at doses similar to those required to induce the release of LDH (MAHESWARAN et al., 1992). If degranulation in neutrophils were only induced by lytic doses of Apxs and subsequently LDH release, as described for other members of Rtx toxin family, the decreased SSC of neutrophils found in this study could not be related to degranulation, because it decrease was induced by sublytic doses of Apx preparations.

Neutrophils treated with the Apx preparations swell as a result of the transmembrane pores created by the toxins (BHAKDI et al., 1986). These swollen cells, as shown in this study, are not permeable for PI. This suggests that the fluorochrome could not pass through the Apx-created pores, possibly because the effective diameter of the pores was smaller than the diameter of PI molecules.

Subsequent to cell swelling, formation of large membrane defects has been demonstrated by the use of scanning electron microscopy in bovine neutrophils and BL_3 lymphoma cells treated with P. haemolytica leukotoxin (CLINKENBEARD et al., 1989a, b). Release of intracellular macromolecules such LDH is thought to be the consequence of large membrane defects (CLINKENBEARD et al., 1989a, b). Influx of PI is likely also to be the consequence of the large membrane defects because influx of this fluorochrome occurs simultaneously with efflux of intracellular proteins in neutrophils treated with E. coli a-haemolysin (BHAKDI et al., 1989). In studies reported here, permeability for PI was related to cell shrinkage and reduction in FSC values for neutrophils treated with relatively high doses of Apx preparations. This is consistent with the development of large membrane defects as a late, severe toxic change in Apx-treated neutrophils.

One of the characteristics of acute lesion in the lung of pigs infected with App, and in the lungs of cattle infected with P. haemolytica, is the appearance of unusual cells characterised by rounded, elongated or fusiform nuclei; often in streaming or swirling orientation (SANFORD and JOSEPHSON, 1981; SLOCOMBE et al., 1985; LIGGETT et al., 1987). The origin of these cells was unknown, but they were thought to be mononuclear cells (SANFORD and JOSEPHSON, 1981). Histochemically, the granule of these cells, unlike neutrophils, were only weakly positive for chloroacetate esterase and PAS (periodic acid-Schiff reaction) (SLOCOMBE et al., 1985; LIGGETT et al., 1987). Ultrastructurally, the round or elongated cells displayed severe signs of degeneration that preclude identification (LIGGETT et al., 1987).

The results of this study suggest that the round or elongated cells found in the acute lung lesions are most likely to be degenerated neutrophils induced by the Apxs produced by the bacteria during infection. In this in vitro study, the nuclei of neutrophils invariably became round, elongated or fusiform, similar to those found in the lung of acutely infected pigs, when treated with Apx preparations. Purified blood mononuclear leucocytes or alveolar macrophages did not display similar changes when exposed to Apx in vitro (TARIGAN et al., 1994). The degenerative changes in the neutrophils were caused by Apxs and not by endotoxin because heated Apxs preparation and purified App lipopolysaccharide failed to induced similar changes (Unpublished data).

Actinobacillus pleuropneumoniae serotypes producing ApxI are more virulent than other serotypes (FREY and NICOLET, 1990; KOMAL and MITTAL, 1990). This was thought to be associated with the fact that ApxI was strongly haemolytic and strongly cytolytic,

unlike ApxII, reported to be weakly haemolytic and moderately cytolytic, and ApxIII which is non haemolytic but strongly cytolytic (KAMP *et al.,* 1991). Results reported here, however, indicates that culture supernatants or Apx preparations derived from all (Australian) serotypes studied were equally cytotoxic. The differences between results obtained in this study and those reported previously by KAMP *et al.* (1991) could result from differences in techniques employed. The techniques used in the present studies are considered to be more sensitive than the dye (nigrosin) exclusion test employed by KAMP *et al.* (1991). In addition, the techniques employed in this study were capable of analysing various cell responses to the toxins. Because culture supernatants from all serotypes were equally toxic designation of ApxI and ApxIII as strongly cytolytic and ApxII as moderately cytolytic is not appropriate.

CONCLUSIONS

Cytotoxins produced by App are very toxic for porcine neutrophils. The toxins create pores in the cell membrane which in turn result in cell swelling, degranulation and ultimately cell death. Although serotypes of App produce different cytotoxins, all the cytotoxins are equally toxic for porcine neutrophils. This *in vitro* study indicates that intoxication of neutrophils, and other defensive cells, comprises an important mechanism in the pathogenicity of App. Cytotoxins produced by App in the lungs paralise or kill the defensive cells allowing rapid growth of the bacteria, which in turn causes severe pathological changes.

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