EFFECTS OF *ACTINOBACILLUS PLEUROPNEUMONIAE* CYTOTOXINS ON GENERATION OF OXYGEN RADICALS BY PORCINE NEUTROPHILS

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ABSTRAK

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Sitotoksin yang dihasilkan oleh *Actinobacillus pleuropneumoniae* (App) diyakini sebagai faktor patogenik dan virulen yang paling utama. Akan tetapi, peranan sitotoksin tersebut dalam proses terjadinya dan perkembangan penyakit belum jelas. Tujuan dari penelitian ini adalah untuk menyelidiki pengaruh dari sitotoksin App (Apx) terhadap *oxidative burst* atau pembentukan radikal oksigen oleh sel neutrofil babi. Dalam penelitian ini, neutrofil babi dilabel dengan suatu probe oksidatif, dichlorofluorescin diacetate (DCFDA), kemudian didedah (di*expose*) dengan preparat sitotoksin. Sel yang menghasilkan radikal oksigen memancarkan *fluorescence* yang intensitasnya diukur dengan sebuah *FACScan flow cytometer*. Semua sitotoksin, yang dihasilkan oleh serotipe App penghasil ApxI dan ApxII, serotipe App penghasil ApxII saja, ataupun serotipe penghasil ApxII dan ApxIII, mampu merangsang neutrofil untuk menghasilkan radikal oksigen. Akan tetapi, dibandingkan dengan *phorbol myrstate acetate (PMA)*, sitotoksin App merupakan stimulan *oxidative burst* yang jauh lebih lemah. Sebagai tambahan, preparat sitotoksin merangsang *oksidative burst* neutrofil pada dosis yang rendah dan kisaran yang sempit. Dengan dosis yang lebih tinggi, sitotoksin bukannya merangsang tetapi malah menghambat *oxidative burst*. Pengaruh Apx yang dihasilkan oleh App sewaktu infeksi pada neutrofil yang direkrut ke dalam paru-paru diperkirakan sama dengan yang diamati pada penelitian ini. Neutrofil dan sel lainnya yang berdekatan dengan bakteri App menjadi lisis akibat Apx konsentrasi tinggi, sedangkan sel fagosit yang lebih jauh terhadap bakteri tersebut terangsang untuk menghasilkan radikal oksigen yang selanjutnya merusak jaringan yang manifestasinya terlihat sebagai nekrosis.

Kata kunci: *Actinobacillus pleuropneumoniae*, sitotoksin, Apx, neutrofil, babi, radikal oksigen, *flow cytometer*

ABSTRACT

TARIGAN, S. 1999. Effects of *Actinobacillus pleuropneumoniae* cytotoxins on generation of oxygen radicals by porcine neutrophils. *Jurnal Ilmu Ternak dan Veteriner* 4(1): 48-54.

Cytotoxins produced by *Actinobacillus pleuropneumoniae* (App) suggested to be the most important pathogenic and virulent factors for this organism. However, the mechanisms on how the cytotoxins contribute to the disease process remain unclear. The purpose of this study is to investigate the effect of the cytotoxins on the oxidative-burst metabolism of porcine neutrophils. In this study, neutrophils were firstly loaded with an oxidative probe *dichlorofluorescin diacetate* (DCFHDA) then expose to cytotoxins. Cells producing oxygen radicals emitted fluorescence and its intensity was measured with a FACScan flow cytometer. All cytotoxins derived from either App serotypes producing ApxI and ApxII, App serotypes producing ApxII only, or App serotypes producing ApxII and ApxIII were capable of stimulating neutrophils for oxygen-radical generation. However, compared with *phorbol myristate acetate* (PMA), App cytotoxins were much weaker as stimulants for oxygen radicals. In addition, Apx preparation stimulated an oxidative-burst metabolism of neutrophils at a low, narrow range of Apx doses. At higher doses, the toxins inhibit the oxidative burst metabolism. The effects of cytotoxins produced by App during infection on recruited neutrophils into the lungs are assumed to be comparable to those observed in this *in vitro* study. Neutrophils, and other host cells, adjacent to the bacteria become lysis due to high toxin concentration, whereas those at some distance to the bacteria produce oxygen radicals which in turn cause tissue damage or necrosis.

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

INTRODUCTION

Contagious pleuropneumonia due to *Actinobacillus pleuropneumoniae* (App) causes significant economic losses to swine industries worldwide. The causal agent produced cytotoxins which consider to play important roles in the bacterial pathogenicity and virulence (ROSENDAL *et al*., 1980; INZANA, 1991). The cytotoxins belong to the Rtx (repeat structure toxin) toxin family and therefore have been suggested to be called Apx (*A. pleuropneumoniae* Rtx toxin) (FREY *et al*., 1993). So far, three types of Apxs have been identified: ApxI (produced by App serotypes 1, 5, 9, 10 and 11), ApxII (produced by all App serotypes except serotype 7) and ApxIII (produced by App serotype 2, 3, 4, 6, 8 and 12). The gene encoding, activating and secretion for all the three Apxs have been sequenced (CHANG *et al*., 1989; FREY *et al*., 1991; JANSEN *et al*., 1993).

Although molecular structures of Apxs have been relatively well characterised, the toxic mechanism of the Apxs are sill poorly understood. Our previous studies, and others, indicated that the toxins are very toxic to porcine alveolar macrophages and neutrophils. The toxins even at very low concentrations cause swelling, degranulation and loss of chemotactic and phagocytic functions of alveolar macrophages (TARIGAN *et al*., 1994; TARIGAN, 1996). The present study focused on the effects of the Apxs on production of oxygen radicals by porcine neutrophils. Oxygen radicals produced by phagocytic cells are very toxic for invading microorganisms but also for the surrounding host tissue.

MATERIALS AND METHODS

Bacterial isolates and production of cytotoxin

All 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).

Cytotoxins (Apxs) were produced according to the methods previously described (TARIGAN *et al*., 1994), briefly, App previously grown on chocolate blood agar were suspended in RPMI-1640. After 2 hours incubation, the bacterial suspension was spun and the supernatant was collected and then either directly used or concentrated.

Measurement of oxygen radical generation

Generation of oxygen radicals by neutrophils was monitored by used of an oxidative probe, *dichlorofluorescin diacetate* (DCFHDA) using a technique previously described by BASS *et al.* (1983). Isolation of porcine neutrophils and cytotoxic assay have been described previously (TARIGAN, 1998). The magnitude of oxygen radical generation of toxin-treated neutrophils was expressed directly as the mean channel number of fluorescent intensity (MCNFI) emitted by the cells, or expressed as the percent increase or decrease over untreated (control) cells which was calculated as:

$$\frac{\text{(MCNFI of treated cells) - (MCNFI of control cells)}}{\text{(MCNFI of control cells)}} \times 100\%$$

Identification of oxygen radicals

In order to determine the type of oxygen radicals responsible for the fluorescence (whether the fluorescence emitted by the DCFHDA-loaded neutrophils was related to the production of superoxide anion $[O_2^-]$ or to hydrogen peroxide $[H_2O_2]$), superoxide dismutase (SOD, 25 μg/ml) or catalase (500 units/ml) were added to the suspension of DCFHDA-loaded neutrophils prior stimulation with PMA (0.1 to 100 ng/ml) or five-fold dilutions of concentrated Apx preparation from serotype 1 for 30 minutes at 37°C.

Serotype differences

To determine whether culture supernatants derived from different serotypes were equally strong in stimulating the metabolism of oxygen radicals, Apx preparations derived from each serotype was serially diluted and then coincubated with DCFHDA-loaded neutrophils.

Heat lability and LPS

To examine the possibility that heat-stable substances were responsible for the activity of the culture supernatant, concentrated Apx of serotype 1 were serially diluted, heated (60°C, 1 hour) and then coincubated with suspension of DCFHDA-loaded neutrophils. The cytotoxic activity of those heated toxin preparations were compared to that of native toxin preparation.

It was also probable that culture supernatants used in this study were contaminated with endotoxin. To examine whether purified App LPS, purified according to the method of FENWICK *et al.* (1986), was capable of inducing cytotoxic activity similar to that caused by

culture supernatant, suspensions of DCFHDA-loaded neutrophils were exposed to a purified App LPS at concentrations ranging from 0.64 ng/ml to 10 µg/ml for 30 minutes at 37°C. Changes in cell volume, granularity and generation of oxygen radicals were examined.

Priming effect of culture supernatant and LPS

To examine the possibility of Apx preparations having the capacity to prime neutrophils for augmented production of oxygen radical upon second stimulus, DCFHDA-loaded neutrophils were firstly exposed to Apx preparation for 30 minutes at 37°C, a second stimulus, either PMA (100 ng/ml), App LPS (1 µg/ml) or RPMI (as control) was added, and then incubated for another 30 minutes. In a parallel experiment, the possibility of App LPS (1 µg/ml) having the capacity to prime neutrophils for a higher oxygen radical production upon second stimulation with Apxs was also examined.

RESULTS

Unstimulated DCFHDA-preloaded neutrophils emitted only weak fluorescence. However, when DCFHDA- preloaded cells were stimulated with PMA or Apxs, cells emitted strong fluorescence, indicating that the cells generated oxygen radicals. The intensity of the fluorescence was positively correlated with PMA concentration, in the range of PMA concentrations used (0.1 to 100 ng/ml) (Figure 1). Production of oxygen radicals by neutrophils induced by PMA was much higher than that induced by Apxs. The means fluorescence of cells stimulated with PMA (100 ng/ml) were on average 10 times higher than those of unstimulated cells, whereas, the maximum means fluorescence of neutrophils stimulated by Apxs was only about 3 times higher than those of unstimulated cells (Figure 1).

Identification of oxygen radicals

Addition of superoxide dismutase (25 µg/ml) into DCFHDA-loaded neutrophils before stimulation with PMA or Apx preparation resulted in only slight or practically no reduction in the intensity of fluorescence. On the other hand, addition of catalase (500 units/ml), a hydrogen peroxide consuming enzyme, before stimulation with PMA or Apx preparation resulted in a marked reduction in the fluorescence intensity (Figure 1). This inhibition experiment suggested that the fluorescence was mainly associated with the production of hydrogen peroxide (H_2O_2) by the stimulated cells but not, or only slightly, attributed to the production of superoxide.

Figure 1. The inhibition by catalase (500 units/ml) or superoxide dismutase (SOD, 25 µg/ml) of fluorescence emitted by DCFHDA-loaded neutrophils stimulated by PMA (A) or Apx preparation derived from serotype 1 (B). Log dilution of Apx preparations of -6.5, -5.8, -5.1, -4.4, -3.7, -3, -2.3 (horizontal axis of graph B) are equal to 1/3,125,000, 1/625,000, 1/125,000, 1/25,000, 1/5,000, 1/1,000 and 1/200 dilutions, respectively. Noted that scale in A is twice of that in B

Serotype differences

The capacity and pattern of stimulation of neutrophils for generation of oxygen radicals by Apx preparations derived from all serotypes were similar (Figure 2). The highest stimulatory effect of the Apx preparation was found at 1/25,000 dilution (serotypes 1, 2, 3 and 5) or 1/5,000 dilution (serotype 7). Stimulation of neutrophils with the toxin preparations at dilutions higher or lower than those dilutions resulted in a sharp decline in the intensity of fluorescence, indicating that the concentration range of the toxins that was stimulating for the oxygen radical production was very narrow. Apx preparations at concentration that had the highest stimulatory effect for oxygen radical production also caused cell swelling (increased FSC) and reduction in granularity (decreased SSC).

Figure 2. Effect of Apx preparations derived from serotypes 1, 2, 3, 5 and 7 on the production of oxygen radicals, indicated by intensity of fluorescence (F1) emitted by DCFHD-loaded neutrophils. Note that oxygen radical production was caused by the supernatant at concentrations below those required to cause maximum increase in forward light scatter (FSC) or maximum decrease in right angle light scatter (SSC). Log dilution of Apx preparations of -5.8, -5.1, -4.4, -3.7, -3, -2.3, -1.6 (horizontal axis) are equal to 1/625,000, 1/125,000, 1/25,000, 1/5,000, 1/1,000, 1/200 and 1/40, respectively

Figure 3. Heat-lability of Apx preparation. The Apx preparation derived serotype 1 were heated at 60°C for 60 minutes then, its activities to induce change in size (FSC) and granularity (SSC) (A), and to stimulate the production of oxygen radicals of neutrophils (B) were compared to those of unheated (native) toxin

Heat-lability of culture supernatant and LPS

To determine whether the changes in the neutrophils were caused by the heat labile toxins (Apxs) or contaminating endotoxins, the concentrated Apx preparation was serially diluted, heated at 60°C for 60 minutes then, coincubated with DCFHDA-loaded neutrophils. As in unconcentrated culture supernatants, heating of the concentrated Apx destroyed most of its toxicity. In fact, the heat lability of the toxins was more obvious in the Apx preparations. Treatment of neutrophils with native Apx preparations at 1/5000 resulted in maximum changes in FSC and SSC, whereas treatment with heated concentrated Apx at similar or even higher concentration did not cause changes in FSC and FSC (Figure 3A). This heated toxin preparation was only caused changes in the light scatters of neutrophils when used at very high concentrations (≤1/200 dilution). Likewise, the capacity of the toxin to stimulate the production of oxygen radicals was practically abolished by heat treatment (Figure 3B).

To further substantiate the contention that the toxic changes were not caused by contaminating endotoxin in the culture supernatants, DCFHDA-loaded neutrophils were exposed to a wide concentration range of purified App LPS. The LPS preparation failed to cause any change in FSC or SSC even at the highest concentration (10 μg/ml) used in this study and only at this highest concentration did a weak generation of oxygen radicals occur.

Priming capacity of culture supernatant and purified LPS

To examine the possibility of Apx preparations having the capacity to prime neutrophils for augmented production of oxygen radicals upon second stimuli, DCFHDA-loaded neutrophils were firstly exposed to Apx preparations for 30 minutes at 37°C, and then stimulated with second stimulus, either PMA (100 ng/ml), App LPS (1 μg/ml) or RPMI (as control), for 30 minutes at 37°C. There was no indication that Apx preparations at any concentration have the capacity to prime neutrophils. In fact, the toxins markedly inhibit the oxygen radical production by stimulation with the second stimulus (PMA) in a dose dependent manner. However, when the pre-exposed neutrophils were stimulated with LPS, the cells produced slightly higher oxygen radicals than control (stimulated with RPMI) (data not presented).

In a parallel experiment, the priming capacity of App LPS for neutrophils was also examined. This experiment indicated that pre-exposure of neutrophils with App LPS (1 μg/ml) did not enhance the production of oxygen radicals when cells were stimulated with Apx preparations as the second stimulus (data not presented).

DISCUSSION

Oxygen radicals produced during oxidative burst of stimulated phagocytes play an important role in an infection because these reactive compounds have the capacity to kill invading organisms and to injure host tissues. In this study the effects of Apx preparations on the oxidative burst of neutrophils were monitored by a flow cytometric procedure. The principle of this procedure, as explained by BASS *et al.* (1983) who introduced this technique for the first time, can be summarised as follows: dichlorofluorescin diacetate (DCFHDA) diffuses into phagocytes in which it is hydrolysed to 2',7'-dichlorofluorescin (DCFH), and hence trapped inside the cells. When stimulated, the phagocytes produced oxygen radicals which oxidise the trapped DCFH into highly fluorescent compound, 2',7'-dichlorofluorescein (DCF). The oxidative burst product responsible for the DCFH oxidation has been shown to be largely the hydrogen peroxide (H_2O_2), therefore, the intensity of the fluorescence measured with flow cytometry reflects the quantity of H_2O_2 produced by the cells (BASS *et al.*, 1983). In agreement with BASS *et al.* (1983), in this study H_2O_2 is also considered to be the major oxygen radical product responsible for the development of the fluorescence because, the intensity of fluorescence emitted by DCFH-DA loaded neutrophils stimulated with Apx preparations or PMA was greatly inhibited by catalase, a H_2O_2-consuming enzyme, but not superoxide dismutase, an O_2^--consuming enzyme. The incomplete inhibition of the fluorescence by the catalase, as found in this study and also by BASS *et al.* (1983), is explained by either the enzyme not diffusing into cells sufficiently, or other oxygen radicals are involved in the oxidizing the probe (DCFH).

Recently DOM *et al.* (1992) demonstrated that undiluted or low dilutions of culture supernatant of App serotype 2 inhibited the oxidative burst of porcine neutrophils, as monitored by a luminol-dependent chemiluminescence assay. At higher dilutions, the supernatant stimulated the oxidative burst of cells. The inhibition and stimulation of oxidative burst were ascribed to the action of Apxs because recombinant ApxII and ApxIII produced similar effects, and heat-treated culture supernatant and killed bacteria neither inhibit nor stimulate the oxidative burst. Effects of *E. coli* α-haemolysin and *P. haemolytica* leukotoxin on the oxidative burst of human and bovine neutrophils respectively, as monitored by chemiluminescence assays, were similar to that of Apxs (BHAKDI and

MARTIN, 1991; CZUPRYNSKI *et al.*, 1991). In this study, the effect of Apx preparations on the oxidative burst of neutrophils were assessed with a completely different technique, nevertheless, the results obtained are principally similar. Preparations of Apxs stimulate an oxidative burst metabolism of neutrophils at a low and narrow range of Apx doses, but at higher doses, the toxins inhibits the oxidative burst metabolism.

Using SOD-inhibitable reduction of ferri-cytochrome c, and horse-radish peroxidase-mediated oxidation of phenol red to measure the release of O_2^- and H_2O_2, respectively, UDEZE and KADIS (1992) failed to demonstrate stimulation effect of Apx preparations derived from serotype 1. However, using similar methods, MAHESWARAN *et al.* (1992) demonstrated stimulating effects of *P. haemolytica* leukotoxin on bovine neutrophils. The results obtained seem inconsistent between assays. This suggests that some assays may be unsuitable for assessing the oxidative burst of neutrophils stimulated with Rtx-toxins.

The inhibition of neutrophil oxidative burst by Rtx-toxins is considered to be associated with cytolytic activity or formation of large membrane defects, which occurs very rapidly when the cells treated with a relatively high concentration of the toxins (BHAKDI and MARTIN, 1991; MAHESWARAN *et al.*, 1992; UDEZE and KADIS, 1992). While the cause of the inhibition is known, the mechanism by which the Rtx-toxins stimulate the oxidative burst is unclear. Working with *E. coli* haemolysin, BHAKDI and MARTIN (1991) speculated that the triggering of the oxidative burst is the consequence of binding the toxin to a specific site in the cell membrane which in turn activates a signal transduction. Direct evidence to prove this suggestion, however, is yet unavailable.

Leucocyte priming has been documented for *E. coli* haemolysin, which at very low concentrations has the capacity to prime human neutrophils for an enhanced oxidative burst, when these primed cells are then exposed to a second stimulus (BHAKDI and MARTIN, 1991). In this study, however, there was no evidence to suggest that Apxs posses similar priming activity. The failure to demonstrate the priming activity might have been due to differences in technique used to monitor the oxidative burst. However, CZUPRYNSKI *et al.* (1991) also failed to demonstrate priming activity of *P. haemolytica* leukotoxin even though this group used a similar technique to that of BHAKDI and MARTIN (1991).

CONCLUSIONS

Cytotoxins produced by App (Apx) were capable of stimulating porcine neutrophil for generation of oxygen radicals. The generation of these radicals is conveniently monitored by a FACScan flow cytometer and an oxidative probe, dichlorofluorescin diacetate. This probe detect the generation of hydrogen peroxide and not superoxide anion. The capacity and pattern of stimulation of neutrophils for generation of oxygen radicals by cytotoxin derived from all serotypes are similar. Lipopolysaccharide and other heat-stable substance produced by App fail to stimulate the oxidative burst metabolism. Cytotoxins do not have the capacity to prime neutrophils for augmented production of oxygen radicals upon second stimulation with LPS, and vice versa.

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