Tilmicosin Induces Apoptosis in Bovine Peripheral Neutrophils in the Presence or in the Absence of Pasteurella haemolytica and Promotes Neutrophil Phagocytosis by Macrophages

ALEX C. CHIN,1 WILSON D. LEE,1 KATHERINE A. MURRIN,1 DOUGLAS W. MORCK,1 JOHN K. MERRILL,2 PAUL DICK,1,3 AND ANDRE G. BURET1,3,*

Department of Biological Sciences1 and Mucosal Inflammation Research Group,3 University of Calgary, Calgary, Alberta T2N 1N4, and Provel Division Eli Lilly Canada Inc., Guelph, Ontario N1G 4T2,2 Canada

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Pathogen virulence factors and inflammation are responsible for tissue injury associated with respiratory failure in bacterial pneumonia, as seen in the bovine lung infected with Pasteurella haemolytica. Tilmicosin is a macrolide antibiotic used for the treatment of bovine bacterial pneumonia. Recent evidence suggests that tilmicosin-induced neutrophil apoptosis may have anti-inflammatory effects. Using bovine leukocytes, we sought to define whether live P. haemolytica affected tilmicosin-induced neutrophil apoptosis, assessed the proapoptotic effects of tilmicosin in comparison with other drugs, and characterized its impact on phagocytic uptake of neutrophils by macrophages. Induction of apoptosis in the presence or absence of P. haemolytica was assessed by using an enzyme-linked immunosorbent assay for apoptotic nucleosomes. In addition, fluorescent annexin-V staining identified externalized phosphatidylserine in neutrophils treated with tilmicosin, penicillin, cefotaxime, oxytetracycline, or dexamethasone. Neutrophil membrane integrity was assessed by using propidium iodide and trypan blue exclusion. As phagocytic clearance of apoptotic neutrophils by macrophages contributes to the resolution of inflammation, phagocytosis of tilmicosin-treated neutrophils by esterase-positive cultured bovine macrophages was assessed with light microscopy and transmission electron microscopy. Unlike bovine neutrophils treated with penicillin, cefotaxime, oxytetracycline, or dexamethasone, neutrophils exposed to tilmicosin became apoptotic, regardless of the presence or absence of P. haemolytica. Tilmicosin-treated apoptotic neutrophils were phagocytosed at a significantly greater rate by bovine macrophages than were control neutrophils. In conclusion, tilmicosin-induced neutrophil apoptosis occurs regardless of the presence or absence of live P. haemolytica, exhibits at least some degree of drug specificity, and promotes phagocytic clearance of the dying inflammatory cells.

Pneumonic pasteurellosis is a pulmonary disease of domestic animals and is particularly prominent in calves placed in feedlots (4, 11, 40). Pasteurella haemolytica A1 is the most common etiologic agent of this acute fibrinous pneumonia (15, 27). The pathogenesis of pneumonic pasteurellosis involves both host inflammation and bacterial virulence factors which, together, lead to respiratory failure and death. Bacterial factors include endotoxins (44) and leukotoxins (34). P. haemolytica leukotoxins are known to lyse macrophages and polymorphonuclear neutrophils (PMNs), which in turn impairs the host’s antibacterial defense and promotes further infiltration of PMNs (11, 34, 36). Local accumulation of PMNs and leukotriene B4 at the site of inflammation plays a central role in the pathogenesis of bovine pneumonic pasteurellosis (6, 11, 17, 24, 42). PMNs release large amounts of reactive oxygen species and proteolytic enzymes that target the invading bacteria but concurrently damage the bronchial epithelium. These host products, compounded with the effects of leukotoxins, contribute to delayed elimination of P. haemolytica and subsequent uncontrolled self-perpetuating inflammation.

Eukaryotic cells, such as PMNs, may die via two distinct processes: apoptosis or necrosis (7, 12, 18, 35, 43). When PMNs die via necrosis, the cells swell and burst, spilling proteolytic compounds into surrounding tissues and amplifying local inflammatory injury. Throughout the apoptotic process, the cytoplasmic organelles remain intact, and nuclear chromatin condenses and is cleaved into mono- and oligonucleosomes (43). The detection of these fragments is a reliable marker of apoptosis. Preservation of membrane integrity in apoptotic PMNs helps minimize self-perpetuation of inflammation and subsequent tissue injury (35, 43). Moreover, apoptotic cells are quickly phagocytosed by neighboring cells such as macrophages (29, 43), a phenomenon known to be mediated by phosphatidylserine which is translocated onto the outer cell membrane leaflet during apoptosis. Indeed, apoptotic cells lose normal membrane asymmetry, leading to the externalization of phosphatidylserine (10, 21, 30, 31). One mechanism by which macrophages recognize apoptotic PMNs, prior to phagocytosis, is through the phosphatidylserine receptor (15, 44). This process ensures that the contents of these cells and organelles are not released into the extracellular space (16, 43). Therefore, the phagocytosis of apoptotic PMNs by macrophages plays a key role in the resolution of inflammation in a number of systems, including the respiratory tract (8, 16, 29, 31, 32).

The clinical efficacy of tilmicosin in the treatment of pneumonic pasteurellosis has been attributed to its pharmacodynamics in appropriate tissues (13, 23, 24, 33) and low inhibitory concentrations (15). A recent study suggested that tilmicosin may reduce pulmonary inflammation in calves with pneumonia (24). Previous studies have indicated that some macrolides may have anti-inflammatory properties by reducing accumulation of inflammatory cells such as PMNs, mononuclear leuko-
cytes, and lymphocytes; decreasing the secretory functions of airway secretory cells; increasing epithelial airway miliary tility; and decreasing epithelial synthesis of proinflammatory cytokines such as interleukin-6 (14, 25, 28, 37–39, 41). Recent evidence suggests that erythromycin and other macrolides may induce PMN apoptosis in vitro (1), but the physiological significance of this observation needs to be further assessed. Recently, using calves infected with live P. haemolytica, we demonstrated that bronchoaveolar PMNs recovered from tilmicosin-treated animals exhibit elevated levels of apoptosis and phagocytosis and the levels of proinflammatory leukotriene B4 in infected lungs of these animals is reduced (5). As P. haemolytica alone has been shown to induce PMN apoptosis (36), additional experiments are warranted to clearly distinguish the apparent anti-inflammatory benefits of tilmicosin from its antibacterial properties. Further, this model represents a useful system in which to investigate whether induction of PMN apoptosis confers anti-inflammatory benefits to an antibiotic. In an attempt to improve our understanding of the physiological significance of tilmicosin-induced PMN apoptosis, the aims of the present study were as follows: (i) to determine the effects of tilmicosin on PMN apoptosis in the presence or in the absence of live P. haemolytica, (ii) to compare the proapoptotic effects of tilmicosin in PMNs with those of other drugs, and (iii) to assess the effects of tilmicosin-induced apoptosis on phosphatidylserine translocation and subsequent macrophage phagocytosis of PMNs.

MATERIALS AND METHODS

Bacteria. P. haemolytica A serotype 1 (strain B122) isolated from a steer that died from pneumonia was cultured overnight on Columbia agar (Difco Laboratories, Detroit, Mich.) containing 5% sterile defibrinated sheep blood at 35°C in a microaerophilic environment (5% CO2). Cells were harvested and suspended in pyrogen-free phosphate-buffered saline (PBS) (pH 7.2, 0.15 M NaCl). A bacterial concentration of 5 × 108 cells/ml was prepared for use in the experimental protocol. The bacterial concentrations were estimated by using McFarland nephelometry and were confirmed by CFU enumerations on Columbia blood agar.

PMN purification. Peripheral blood was drawn into ACD vacutainers (ACD solution from Becton Dickinson Vacutainer Systems, Franklin Lakes, N.J.) from the jugular veins of healthy Holstein calves. The blood was pooled in two 20-ml polypropylene centrifuge tubes and spun at 1,171 × g in an IEC Centra-7R swinging bucket centrifuge (IEC 210 rotor; International Equipment Company, Needham Heights, Mass.) for 20 min at room temperature without braking. The plasma, buffy coat, and the top one-half of the erythrocyte pack were removed down to the 10-ml level. Then 20 ml of cold hypotonic lysis solution (10.6 mM NaH2PO4, 2.7 mM Na2HPO4) was added to the cell pack and gently mixed for 5 min. Isotonicity was restored through the addition of 10 ml of 3 times hypertonic solution (10.6 mM NaH2PO4, 2.7 mM Na2HPO4, 462 mM NaCl). The mixture was centrifuged at 650 × g for 10 min, and the supernatant was discarded. The lysing procedure was repeated once again. After the final lysing step, the leukocyte cell pellet was resuspended in 10 ml of PBS (8.1 mM Na2HPO4, 1.47 mM KH2PO4, 2.68 mM KCl, 136.9 mM NaCl). This cell solution was poured into a 15-ml centrifuge tube and then centrifuged at 650 × g for 10 min, and the supernatant was discarded. The pellet was resuspended in 10 ml of PBS containing 2% fetal bovine serum (FBS) (Hyclone, Logan, Utah) for use in the experimental protocol.

PMN apoptosis. Peripheral blood was drawn into ACD vacutainers (ACD solution from Becton Dickinson Vacutainer Systems, Franklin Lakes, N.J.) from the jugular veins of healthy Holstein calves. The blood was pooled in two 20-ml polypropylene centrifuge tubes and spun at 1,171 × g in an IEC Centra-7R swinging bucket centrifuge (IEC 210 rotor; International Equipment Company, Needham Heights, Mass.) for 20 min at room temperature without braking. The plasma, buffy coat, and the top one-half of the erythrocyte pack were removed down to the 10-ml level. Then 20 ml of cold hypotonic lysis solution (10.6 mM NaH2PO4, 2.7 mM Na2HPO4) was added to the cell pack and gently mixed for 5 min. Isotonicity was restored through the addition of 10 ml of 3 times hypertonic solution (10.6 mM NaH2PO4, 2.7 mM Na2HPO4, 462 mM NaCl). The mixture was centrifuged at 650 × g for 10 min, and the supernatant was discarded. The lysing procedure was repeated once again. After the final lysing step, the leukocyte cell pellet was resuspended in 10 ml of PBS (8.1 mM Na2HPO4, 1.47 mM KH2PO4, 2.68 mM KCl, 136.9 mM NaCl). The cell solution was poured into a 15-ml centrifuge tube and then centrifuged at 650 × g for 10 min, and the supernatant was discarded. The pellet was resuspended in 10 ml of PBS containing 2% fetal bovine serum (Hyclone, Logan, Utah) for use in the experimental protocol.

PMN apoptosis was measured by using a cell death detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals, Laval, Quebec, Canada) according to the manufacturer’s instructions. This quantitation kit specifically measures phosphatidylserine which is translocated from the inner side of the plasma membrane to the external surface of the cell during apoptosis and necrosis. Apoptotic cells can be differentiated from necrotic cells by using annexin-V labeling. Annexin-V has high affinity for phosphatidylserine, and propidium iodide, which binds DNA. Briefly, the incubated cells were pelleted at 200 × g for 5 min, were washed with 1 ml of pyrogen-free 1× PBS, and were resuspended again at 200 × g for 5 min (Baxter Canlab Biofuge A; Heraeus Sepatech GmbH, Hanau, Germany). The supernatant was discarded, and the pellet was resuspended with 0.1 ml of a staining solution containing 20 μl of annexin-V fluorescein, 20 μl of propidium iodide, and 1 ml of 1% HEPES buffer. The cells were incubated in the dark for at least 15 min at room temperature. After incubation, the cells were washed with 0.1 ml of HEPES buffer warmed to room temperature and were centrifuged at 200 × g for 5 min. A wet mount of 15 μl of the stained cells was prepared and was visualized under fluorescent microscopy. The same experiment was performed by using a 1μl Philco inverted microscope where apoptotic cells appeared solid green (fluorescin isothiocyanate filter, excitation = 450 nm, emission = 490 nm) and necrotic cells appeared green (fluorescin isothiocyanate filter) with red nuclei (Cy-3 filter, excitation = 535 nm, emission = 550 nm). The percentages of apoptotic and necrotic cells in each sample were obtained from 10 randomly selected fields under 400× magnification.

Measures integrity. The effects of the various drugs on PMN membrane integrity were investigated by using the diesterase assay or absence of annexin-V positive celldetected by FITC inverted microscope where apoptotic cells appeared solid green (fluorescin isothiocyanate filter, excitation = 450 nm, emission = 490 nm) and necrotic cells appeared green (fluorescin isothiocyanate filter) with red nuclei (Cy-3 filter, excitation = 535 nm, emission = 550 nm). The percentages of apoptotic and necrotic cells in each sample were obtained from 10 randomly selected fields under 400× magnification.

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with large and vacuolated cytoplasm were counted as macrophages while small cells with undifferentiated cytoplasm were counted as undifferentiated monocytes. Nonspecific esterase staining was performed as previously described by Partridge and Dravet (26) to confirm macrophage differentiation. Briefly, fixed cells were incubated for 30 min at 37°C in a buffered esterase substrate solution containing hexazotized pararosaniline (Sigma) and α-naphthyl acetate (Sigma). Cells were counterstained with 1% methyl green acetate (Sigma) for 2 min. The percentage of esterase-positive cells was determined with light microscopy.

PMN phagocytosis by macrophages. PMNs (2.5 × 10^7 cells/ml) were incubated (37°C, 5% CO_2) for 2 h in either PBS (pH 7.1, control) or 0.5 μg of tilmicosin (Micotil; Provet) per ml and then centrifuged at minimum speed for 5 min (Hermle Z180M; National Labnet Co., Woodbridge, N.J.). The pellet was washed twice in 1× PBS and was resuspended to a volume of 300 μl. PMNs were added to wells containing mature macrophages from the same steer at an approximately 50:1 PMN-macrophage ratio in keeping with the high PMN-macrophage ratio found in the bronchoalveolar space of the inflamed lung (5, 42). Cells were coincubated (37°C, 5% CO_2) for either 0.5, 1, or 2 h. The wells were washed three times with 37°C 1× PBS, were fixed, and were stained with Diff Quik (Baxter). The percentage of macrophages that had completely engulfed at least one PMN was determined under light microscopy from 300 macrophages in each preparation.

Transmission electron microscopy. Phagocytosis of PMNs by macrophages was confirmed with transmission electron microscopy. Purified PMNs (1.46 × 10^7 cells/ml) were incubated (37°C, 5% CO_2) for 2 h with 0.5 μg of tilmicosin per ml in PBS. After incubation, PMNs were centrifuged at 1,500 × g for 10 min. The pellet was washed once with PBS and then resuspended to a final concentration of 1.17 × 10^7 cells/ml in PBS. PMNs (4.0 ml) were added to a flask of 7-day-old macrophages and were incubated (37°C, 5% CO_2) for 1 h. The medium was aspirated to remove nonadherent cells, and attached macrophages were washed three times with PBS prewarmed to 37°C. Cells were fixed in 5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) in the flasks, were scraped into 15-ml centrifuge tubes, were transferred into BEEM capsules (JBS Supplies, JB EM Services Inc., Dorval, Quebec, Canada), were postfixed in 1% osmium tetroxide (JBS Supplies), were dehydrated in acetone, and were embedded in Spurr low-viscosity medium (Electron Microscopy Sciences). Thin sections (90 nm) were stained with saturated uranyl acetate in 50% aqueous ethanol and 0.04% (wt/vol) lead citrate. Photomicrographs were obtained with a Hitachi H-7000 transmission electron microscope at an acceleration voltage of 75 kV. Macrophages with phagocytosed PMNs and PMNs with signs of apoptosis (i.e., intact blebbled membranes, condensed nuclear chromatin, nuclear delamination, condensed perinuclear cytoplasm, and intact organelles) were identified.

Statistical analysis. Results were expressed as means ± standard errors of the means and compared by one-way analysis of variance, followed by Tukey’s test for multiple-comparison analysis where applicable. When results were expressed as percentages, all values underwent an arcsine transformation prior to statistical comparison and were transformed back to percentages for the expression of the means ± standard errors of the means. For the comparison of two lines, linear regression and one-way analysis of variance were performed, followed by Tukey’s test for pairwise multiple-comparison analysis. P values of less than 0.05 were considered significant.

RESULTS

PMN apoptosis and phosphatidylserine translocation. The first experiments assessed the effects of live *P. haemolytica* on tilmicosin-induced PMN apoptosis. The production of apoptotic nucleosomes by peripheral PMNs incubated with 5 or 0.5 μg of tilmicosin per ml, concentrations which are consistent with the pharmacokinetic profile of this compound (24), was measured in the presence or absence of *P. haemolytica* using quantitative ELISA. After 2 h of incubation, tilmicosin at either concentration, in the presence or absence of *P. haemolytica*, significantly increased the induction of PMN apoptosis compared to controls (Fig. 1). There was no significant difference between the degree of induction of PMN apoptosis by tilmicosin alone and that of tilmicosin plus *P. haemolytica* at either drug concentration.

Another series of experiments determined whether tilmicosin-induced PMN apoptosis was associated with phosphatidylserine translocation and whether this effect was drug specific. Tilmicosin (17.7 ± 3.4), but not penicillin (7.6 ± 1.3), ceftiofur (6.8 ± 1.6), oxytetracycline (6.1 ± 1.3), or dexamethasone (4.8 ± 0.6), significantly increased phosphatidylserine translocation in PMNs when compared to controls (7.1 ± 1.5). The deduction of necrotic cells from these numbers confirmed that tilmicosin-induced phosphatidylserine translocation reflected a significantly (*P < 0.01*) increased incidence of apoptotic PMNs (Fig. 2). Neither penicillin, ceftiofur, oxytetracycline, nor dexamethasone affected apoptosis in PMNs (Fig. 2).

The percentages of necrotic cells positive for both phosphatidylserine and propidium iodide were not significantly different between any groups (control, 4.0 ± 1.0; tilmicosin, 3.4 ± 0.7; penicillin, 3.6 ± 0.6; ceftiofur, 3.7 ± 0.5; oxytetracycline, 2.7 ± 0.7; dexamethasone, 2.6 ± 0.7). Similarly, trypan blue exclusion was not significantly different between any groups (data not shown).

Macrophage differentiation. Phagocytic clearance of proinflammatory products in the bronchoalveolar spaces of the lung is carried out by fully differentiated alveolar macrophages. Therefore, circulating bovine monocytes used in this study had to be allowed to mature in vitro prior to use in the phagocytic...
assays. Based on morphological appearance (overall cell size, cytoplasm-to-nucleus volume ratio, presence of differentiated cytoplasmic vacuoles), the percentage of monocytes that had differentiated into mature macrophages significantly increased over the course of 7 days of incubation and reached 77.5% on day 2, 89.7% on day 5, and 96.5% on day 7. Nonspecific esterase staining confirmed this increase in macrophage maturity over the 7-day period (76.6% on day 2, 88.5% on day 5, and 97.7% on day 7) (Fig. 3).

**Macrophase phagocytosis assay.** As phagocytosis of apoptotic PMNs by macrophages is an important contributor to the resolution of inflammation, additional experiments assessed the effects of tilmicosin-induced apoptosis on PMN phagocytosis by macrophages. Rates of macrophase phagocytosis were significantly enhanced if PMNs had been exposed to tilmicosin for 2 h when compared to controls (Fig. 4). Macrophage phagocytic indices for PMNs were increased over threefold after 1 h of coincubation, and this increase was detectable after 0.5, 1, or 2 h of coincubation (Fig. 4). Coincubated cells were prepared for transmission electron microscopy to verify that our observations truly reflected complete phagocytic ingestion of apoptotic PMNs. Tilmicosin-treated PMNs exhibited ultrastructural evidence of apoptosis at various stages of cell death (Fig. 5). Apoptotic cells were characterized by the presence of a blebbed but intact plasma membrane, nuclear membrane delamination, chromatin condensation, cytoplasmic vacuolation, and maintenance of organelle integrity. Apoptotic bodies were also observed. A large number of apoptotic PMNs were either found within phagocytic vacuoles of macrophages (Fig. 5A and C) or had their plasma membranes in close apposition to that of an adjacent macrophage (Fig. 5A and B).

**DISCUSSION**

Results from this study confirm our previous observation that tilmicosin induces apoptosis in bovine PMNs. The results also indicate that tilmicosin-induced PMN apoptosis is independent of the presence or absence of live *P. haemolytica*. Furthermore, this effect appears to be antibiotic specific, at least to some degree, as neither penicillin, ceftiofur, nor oxytetracycline increased PMN apoptosis in sham conditions. Finally, tilmicosin-induced apoptosis in bovine PMNs is associated with increased translocation of phosphatidylserine and enhanced phagocytic uptake of these cells by macrophages.

Recruitment of PMNs serves as a protective mechanism against *P. haemolytica* (42). However, pathologial accumulation of PMNs associated with high levels of local leukotriene B₄ has been implicated in the propagation of tissue injury during pneumoniae pasteurellosis (6, 17, 24, 42). During a *P. haemolytica* infection, a soluble heat-labile leukotoxin released by the bacteria destroys PMNs, promoting the release of proinflammatory products in surrounding tissues and further amplifying inflammation (2, 3, 34). We recently demonstrated that tilmicosin treatment of calves infected with live *P. haemolytica* significantly increases apoptosis in bactereal alveolar PMNs and reduces the synthesis of proinflammatory leukotriene B₄ in the lung (5). We speculated that induction of PMN apoptosis by tilmicosin may confer anti-inflammatory properties on this
macrolide and hence contribute to its clinical efficacy. Consistent with this observation, pulmonary inflammation is significantly reduced in tilmicosin-treated calves experimentally infected with *P. haemolytica* when compared with sham-treated animals (24). Experiments described in the present study further assessed the proapoptotic effects of tilmicosin on bovine peripheral PMNs. The findings indicate that tilmicosin equally induces apoptosis in bovine PMNs in the presence or absence of live *P. haemolytica*. This observation suggests that tilmicosin may exert its proapoptotic property regardless of the variable bacterial loads of an infected lung.

In addition, experiments measuring the externalization of phosphatidylserine on the cell membrane of bovine PMNs were used to further characterize tilmicosin-induced PMN apoptosis. Translocation of phosphatidylserine occurs early in apoptotic cells (21) and signals macrophages for phagocytic elimination of these cells via ligand binding to the phosphatidylserine receptor (10, 32). This in turn prevents the release of proinflammatory contents in situ by the dying cells and hence significantly contributes to the resolution of inflammation (10, 29, 32). PMN apoptosis and exteriorization of phosphatidylserine were examined in bovine PMNs and compared with the effects of exposure to an anti-inflammatory corticosteroid (dexamethasone) or other antibiotics (penicillin, ceftiofur, oxytetracycline) commonly used for the treatment of bovine pneumonic pasteurellosis. Results show that tilmicosin, but not the other drugs, induces PMN apoptosis. As opposed to findings from other studies on human PMNs (9, 20, 22), dexamethasone did not inhibit spontaneous apoptosis in bovine PMNs. Apart from species differences, a possible explanation for this observation is that inhibition of apoptosis by dexamethasone was observed after 24 h in previous experiments (9, 20, 22), whereas the 2-h incubation used in this study may not have been sufficient to significantly inhibit apoptosis. Indeed, recent findings have shown similar results with human PMNs exposed to another macrolide azalide, azithromycin (19). Tilmicosin-induced phosphatidylserine externalization was associated with a significant enhancement of PMN phagocytosis by macrophages. With transmission electron microscopy, further observation of tilmicosin-treated PMNs cocultured with macrophages clearly revealed PMNs at various stages of phagocytosis, from tight apposition on the surface of a neighboring macrophage to full enclosure within phagocytic vacuoles. In the light of the role played by phagocytic elimination of apoptotic PMNs by macrophages in the resolution of local inflammation, the findings reported here are consistent with our hypothesis that induction of PMN apoptosis may confer anti-inflammatory properties to tilmicosin.

Additional experiments using propidium iodide or trypan blue exclusion assays confirmed that tilmicosin-induced PMN apoptosis did not compromise membrane integrity with this experimental design. Treatment of bovine PMNs with the other drugs included in this study did not affect cell membrane integrity either.

In summary, the findings from this study show that, unlike penicillin, ceftiofur, oxytetracycline, or dexamethasone, tilmicosin induces PMN apoptosis. Induction of PMN apoptosis by tilmicosin occurs independently of the presence or absence of live *P. haemolytica*. Increases phosphatidylserine translocation, and promotes the phagocytic ingestion of PMNs by macrophages. We postulate that, in addition to its antibacterial properties, induction of PMN apoptosis by an antibiotic such as tilmicosin may provide significant clinical benefits in the treatment of bacterial infections associated with severe inflammation.

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