Influence of *Actinobacillus pleuropneumoniae* Serotype 2 and Its Cytolysins on Porcine Neutrophil Chemiluminescence

P. DOM,[1]* F. HAESEBROUCK,[1] E. M. KAMP,[2] AND M. A. SMITS[3]

*Laboratory of Veterinary Bacteriology, Faculty of Veterinary Medicine, University of Ghent,
Casinoplein 24, B-9000 Ghent, Belgium,[1] and Departments of Bacteriology[2] and
Molecular Biology,[3] DLO-Central Veterinary Institute,
8200 AB Lelystad, The Netherlands*

The effects of *Actinobacillus pleuropneumoniae* serotype 2 and its metabolites on the oxidative activity of porcine neutrophils were studied by using a chemiluminescence technique. Viable *A. pleuropneumoniae* stimulated the production of oxygen radicals by neutrophils. After having reached a peak value, the oxidative activity decreased until a total inhibition of the oxidative activity of the neutrophils was achieved. All effects were neutralized with homologous convalescent-phase pig sera which had been adsorbed by heat-inactivated *A. pleuropneumoniae*. Inactivated bacteria and bacteria in the presence of chloramphenicol each had no influence on the oxidative activity of neutrophils. In contrast, a heat-labile factor in *A. pleuropneumoniae* culture supernatants stimulated and inhibited the oxidative activity of the neutrophils in a dose-dependent manner. Undiluted and low dilutions of culture supernatants were toxic for the phagocytes, while high dilutions stimulated the oxygen radical production of the neutrophils. These effects were neutralized with homologous convalescent-phase pig sera. In order to investigate whether the heat-labile factors in the culture supernatant could be cytolysins, we repeated the experiments with cytolysin II and cytolysin III produced by recombinant *Escherichia coli*. It was demonstrated that stimulation and inhibition could be reproduced by both cytolysins. In conclusion, the observations made in this study showed that *A. pleuropneumoniae* secretes heat-labile metabolites that stimulate neutrophil-oxidative metabolism at relatively low concentrations and kill the neutrophils at higher concentrations. Cytolysins may be responsible, at least in part, for these effects.

Contagious pleuropneumonia, caused by *Actinobacillus pleuropneumoniae*, has important economic implications for the pig industry worldwide (24). The protection of swine against *A. pleuropneumoniae* infection and the development of disease has been an aim of veterinary scientific research for several years. Successfully controlling the disease, however, requires knowledge of the virulence factors of the bacteria.

Several bacterial structures and products have been suggested to be important factors in the virulence of *A. pleuropneumoniae*. The capsular structure (14), the cell wall lipopolysaccharides (8), and the recently described fimbriae (28) are thought to be involved in virulence. The cytolysins, however, are considered to be the most important virulence factors. These proteins, which are secreted by the bacteria, are cytotoxic for porcine erythrocytes, endothelial cells, macrophages, and neutrophils (12, 17, 21, 25). Recently, the following three different proteins (with the following sizes) having cytotoxic and/or hemolytic activities have been distinguished among the 12 *A. pleuropneumoniae* serotypes: cytolysin I (ClyI) (±105 kDa), ClyII (±103 kDa), and ClyIII (±120 kDa) (6, 11, 16). ClyI and ClyII are assumed to be the counterparts of hemolysin I and hemolysin II (26). ClyIII is assumed to be the counterpart of what has been described as a pleurotoxin (22). Each *A. pleuropneumoniae* serotype produces either one or two of these cytolysins. It has been shown previously that *A. pleuropneumoniae* serotype 2 strains produce ClyII and ClyIII (16). The exact meaning of these cytolysins in the pathogenesis of pleuropneumonia, however, is still unclear.

It has been shown previously that neutrophils migrate from the circulation into the lung alveoli as early as 3 h after inoculation with *A. pleuropneumoniae* (2, 18). Neutrophils are professional phagocytes that destroy bacteria and promote inflammation (1). The role of neutrophils in the pathogenesis of *A. pleuropneumoniae* infections is still unclear, however.

It was the purpose of the present report to study the effects of an *A. pleuropneumoniae* serotype 2 strain on the oxidative activity of porcine neutrophils by using a chemiluminescence (CL) technique. With this technique, it is possible to screen the activity of the neutrophils during a period rather than merely assess the inactivation of the cells after a certain time. Whether the observed effects were attributable to bacterial cell surface structures or to metabolites secreted by the bacterium was one question examined in this study. In order to study the role of the cytolysins, ClyII and ClyIII produced by recombinant *Escherichia coli* were used.

MATERIALS AND METHODS

Porcine neutrophils. Blood samples were obtained from the jugular veins of healthy, 7- to 9-week-old pigs and mixed with equal volumes of Alsevers' solution. Processing was performed at 4°C. Erythrocytes were sedimented by incubating 2 parts of the diluted blood in Alsevers' solution with 1 part (vol/vol) of a 6% (g/vol) dextran sulfate (Dextran T500; Pharmacia, Uppsala, Sweden) solution. After 1 h of incubation, the upper layer was collected and cells were washed once in Hanks balanced salt solution (HBSS). After centrifugation (400 × *g* for 15 min), the resulting pellet was suspended in 6 ml of HBSS. Neutrophils were separated from mononuclear cells and contaminating erythrocytes by

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* Corresponding author.

using a discontinuous Percoll (Pharmacia) gradient. A stock Percoll solution was prepared by adding 9 parts of Percoll to 1 part (vol/vol) of 10× concentrated HBSS. The stock Percoll solution was further diluted with HBSS to 70% (vol/vol) and 80% (vol/vol) dilutions. The 70% dilution was layered carefully on top of the 80% dilution in a tube, forming the discontinuous gradient. Then, the cell suspension was layered carefully on top of the gradient. The tube was centrifuged for 30 min at 500 × g. Neutrophils were collected from the interphase between the two Percoll dilutions. The cells were washed twice in HBSS and suspended at 10^6 cells per ml in leukocyte medium (RPMI 1640 [GIBCO Europe, Ghent, Belgium] supplemented with 10% nonessential amino acids–10% glutamine–10% fetal calf serum [FCS]–1% sodium pyruvate). The purity of the neutrophil suspensions was assessed by microscopic examination of May-Grünwald-Giemsa-stained cytospin smears and was approximately 95%. The viability of the neutrophils was determined by the exclusion of trypan blue and exceeded 98%.

Bacterial strain and production of crude toxins. An NAD-dependent serotype 2 strain (266/7656) of *A. pleuropneumoniae*, isolated from a pig with pleuropneumonia in Belgium, was used in these studies. Stock suspensions were stored at −70°C in leukocyte medium. For each experiment, a proper sample was thawed. After being thawed, the bacteria were grown at 37°C for 18 h in an atmosphere of 5% CO_2 on Columbia agar (Columbia Agar Base; Lab M, Bury, Manchester, United Kingdom) supplemented with 5% bovine blood, 0.03% NAD (Sigma Chemical Co., St. Louis, Mo.), and 5% yeast extract. Subsequently, colonies were transferred to Columbia agar supplemented with 3% equine serum, 0.03% NAD, and 5% yeast extract. These plates were incubated for 6 h at 37°C in a 5% CO_2 atmosphere. Bacteria were harvested in phosphate-buffered saline solution (PBS) (pH 7.3), centrifuged at 400 × g for 20 min, and suspended in leukocyte medium. The suspension was checked for purity, and the number of CFU was determined by plating 10-fold dilutions on Columbia agar supplemented with 5% blood, 0.03% NAD, and 5% yeast extract. Bacterial suspensions were stored overnight at 4°C. The next day, the bacteria were washed once in PBS before being used in the experiments.

For crude toxin production, leukocyte medium supplemented with 10^{-2} mol of $CaCl_2$ per liter (10) and 0.03% NAD was inoculated with 3 × 10^2 CFU of *A. pleuropneumoniae* per ml. After being incubated for 9 h, the suspension was centrifuged at 10,000 × g for 3 min. The resulting supernatant was filtered through a 0.2-μm-thick filter (Gelman Sciences, Ann Arbor, Mich.) and tested in the CL or neutral red uptake assay. In order to evaluate its heat sensitivity, supernatant was heated for 20 min at 100°C before being tested.

Cytolysins produced by recombinant *E. coli*. The cloning of the genetic determinants of ClyII and ClyIII has been described elsewhere (15, 26). *E. coli* LE 392 (23) cells that contained the ClyII or ClyIII determinant on pUC8 plasmid DNA were cotransformed with a compatible pACYC 184-base plasmid carrying the *hlyB* and *hlyD* secretion genes of *E. coli* (13). A sterile ClyII and a sterile ClyIII containing supernatants of logarithmically growing cultures of these *E. coli* cells were used, undiluted or diluted in the CL.

CL. Luminol (Sigma Chemical Co.) was used to amplify the CL induced by the respiratory burst of stimulated neutrophils. A luminol stock solution was prepared by dissolving 1.77 mg of luminol in 1 ml of dimethyl sulfoxide

(Sigma Chemical Co.) to obtain a concentration of 10^{-2} mol/liter. The stock was stored at −70°C. Immediately before being used, it was diluted to a concentration of 2 × 10^{-4} mol/liter in HBSS.

Opsonized zymosan was used as a triggering agent. The zymosan stock solution was prepared by suspending 25 mg of zymosan (Sigma Chemical Co.) in 10 ml of PBS. The suspension was heated for 30 min in a water bath at 90°C. After being cooled, the suspension was stored at −20°C until it was used. The zymosan was opsonized by incubating 1 ml of the zymosan suspension with 1 ml of fresh guinea pig complement (Behringwerke AG, Marburg, Germany) for 30 min at 37°C. Following centrifugation (200 × g for 10 min), the precipitate was suspended in 1 ml of HBSS and used the same day.

CL was performed with polystyrene biolumcuvettes (Lumac, Landgraaf, The Netherlands) at 37°C with a six-channel luminometer (Lumicon; Hamilton, Bonaduz, Switzerland). Preparation of the neutrophils was identical for all experiments. Briefly, neutrophils (5 × 10^4 in 400 μl of leukocyte medium) were transferred to the cuvettes. In order to allow adherence of the neutrophils, the cuvettes were incubated in a humid atmosphere of 95% air–5% CO_2 for 80 min at 37°C. Thereafter, the medium was discarded and 100 μl of luminol in HBSS and 200 μl of HBSS with 3% FCS were added. The cuvettes were placed in the six channels of the luminometer, and the CL background values were determined for 5 min. Then the following experiments were performed. Each experiment was performed four times.

Influences of *A. pleuropneumoniae*, crude toxins, and recombinant cytolysins on the oxidative activity of neutrophils. In order to study the influence of *A. pleuropneumoniae* on the oxidative activity of the neutrophils, 100 μl of HBSS with 3% FCS containing 10^4, 10^5, 10^6, or 10^7 CFU of *A. pleuropneumoniae* was added to the neutrophils after the background value was determined for each cuvette. A control sample consisting of 100 μl of HBSS with 3% FCS was included in each run. The CL response was measured for 80 min and expressed as counts per minute. Thereafter, the remaining oxidative activity of the neutrophils was checked by adding 20 μl of opsonized zymosan to each cuvette.

The following experiments were performed to determine whether the effects observed in the first experiment could be attributed to the bacteria or to their metabolites. In the first of the new set of experiments, bacterial suspensions containing 10^5 or 10^6 CFU of *A. pleuropneumoniae* per 100 μl of PBS were inactivated by heating (56°C for 20 min) or by UV irradiation. The inactivated bacteria were washed once in PBS and suspended in the same volume of HBSS with 3% FCS. Neutrophils were incubated in cuvettes as described above. After the CL background value was determined for each cuvette, 100 μl of the inactivated bacterial suspensions was added. Control samples consisted of 100 μl of HBSS with 3% FCS. CL was measured for 50 min, after which opsonized zymosan was added.

In the second experiment, neutrophils were incubated in cuvettes as described above. After the CL background value was determined for each cuvette, 10^5 or 10^6 CFU of the *A. pleuropneumoniae* strain in 100 μl of HBSS with 3% FCS and chloramphenicol (final concentration, 25 μg/ml) was added. Control samples consisted of 100 μl of HBSS with 3% FCS and chloramphenicol. CL was measured for 50 min, after which opsonized zymosan was added.

In the third experiment, neutrophils were incubated in

cuvettes as described above. After the CL background value was determined for each cuvette, 100 μl of HBSS with 3% FCS that had been inoculated with 10^5 or 10^6 CFU of the *A. pleuropneumoniae* strain, incubated at 37°C for 30 min, and filtered through a 0.2-μm-thick filter was added. CL was measured for 20 min, after which heat- or UV-inactivated bacteria were added.

In order to examine the effects of crude toxins on the CL of neutrophils, neutrophils were incubated in cuvettes as described above. After the CL background value was determined for each cuvette, 100 μl of twofold dilutions of crude toxin with or without chloramphenicol (final concentration, 25 μg/ml) was added. Control samples consisted of 100 μl of HBSS with or without chloramphenicol. The CL response was measured for 40 min. Thereafter, 20 μl of opsonized zymosan was added to each cuvette, and the CL response was measured for another 20 min.

In order to examine the effects of ClyII and ClyIII produced by recombinant *E. coli* on the CL of neutrophils, neutrophils were incubated in cuvettes. After the CL background value was determined for each cuvette, 100 μl of serial twofold dilutions (starting with 1:10) of the culture supernatant of ClyII or ClyIII secreting recombinant *E. coli* cells was added. Control samples consisted of 100 μl of twofold dilutions of supernatants of cultures lacking the ClyII or ClyIII genetic determinant. The CL response was measured for 40 min. Thereafter, 20 μl of opsonized zymosan was added to each tube, and the CL response was measured for another 20 min.

Demonstration of crude toxin production by neutral red uptake. In order to determine whether a reduced CL response implies a loss of neutrophil viability, neutral red uptake by neutrophils after the addition of dilutions of a crude toxin preparation was examined. Neutrophils (10^6) in 100 μl of leukocyte medium were added to the cups of a 96-well microtiter plate and were incubated at 37°C in a humid atmosphere of 95% air–5% CO_2. After 80 min, 100 μl of twofold dilutions of culture supernatants in leukocyte medium was added. Controls consisted of 10^6 neutrophils in 200 μl of leukocyte medium. After 30 min, 25 μl of a 0.1% neutral red solution was added to the cups, and then the plates were incubated in the dark for 1 h at 37°C in a humid atmosphere of 95% air–5% CO_2. Thereafter, the plates were washed twice with PBS supplemented with Ca^{2+} (final concentration, 9×10^{-4} mol/liter) and Mg^{2+} (final concentration, 5×10^{-4} mol/liter). Then, 50 μl of 10% sodium dodecyl sulfate and 100 μl of 0.2 mol of HCl per liter were added to the cells. The optical density of the resulting suspension was measured at 492 nm. The cytotoxic titer was defined as the reciprocal of the highest dilution for which the optical density was less than 50% that for the control samples.

Neutralization tests. Neutralization tests were carried out with convalescent-phase sera from four specific-pathogen-free pigs that were infected intranasally with 10^3 CFU of the *A. pleuropneumoniae* serotype 2 strain. Preimmune sera, obtained from these pigs before infection, were used as negative control sera. Sera were inactivated at 60°C for 30 min and diluted 1/4 in PBS. To eliminate opsonins, sera were adsorbed with homologous inactivated or viable *A. pleuropneumoniae*. For adsorption with inactivated bacteria, a suspension containing 10^{10} CFU of the *A. pleuropneumoniae* strain per ml was heated at 56°C for 20 min. One milliliter of this suspension was incubated for 1 h at 37°C with 1 ml of inactivated sera. For adsorption with viable *A. pleuropneumoniae*, a suspension of 10^8 or 10^{10} CFU/ml was incubated

with inactivated sera for 1 h at 4°C. Thereafter, sera were centrifuged at $10,000 \times g$ for 3 min, and the supernatant was used.

In order to study neutralization of the effects of viable *A. pleuropneumoniae* on the oxidative activity of neutrophils, neutrophils were incubated in cuvettes as described earlier. After the CL background value was determined for each cuvette, 50 μl of adsorbed sera and 50 μl of HBSS supplemented with 3% FCS and containing 10^7 or 10^6 CFU of the *A. pleuropneumoniae* strain were added. The CL response was measured as described above. Each experiment was performed three times.

In order to study neutralization of the effects of the crude toxin preparation on the oxidative activity of neutrophils, neutrophils were incubated in cuvettes as described earlier. After the CL background value was determined for each cuvette, 50 μl of adsorbed or nonadsorbed sera and 50 μl of twofold dilutions of the supernatant were added. The CL response was measured as described above. Each experiment was performed three times.

RESULTS

Influence of *A. pleuropneumoniae* on CL of neutrophils. CL background values of neutrophils varied between 1×10^3 and 3×10^3 cpm. *A. pleuropneumoniae* at a dose of 10^5, 10^6, or 10^7 CFU first stimulated the oxidative activity of the cells. After the peak value was reached, light emission declined until, finally, background values were reached (Fig. 1). At that time, the neutrophils were refractory to subsequent stimulation with opsonized zymosan. The peak value and the time to the CL peak value, as well as the time to the drop to the background value, increased with decreasing bacterial titers. When 10^4 CFU of the bacteria was added to the neutrophils, the peak value was reached only at the end of the measurement. The dose-related CL responses for a representative run are illustrated in Fig. 1.

UV- or heat-inactivated bacteria or viable bacteria in the presence of chloramphenicol did not provoke a CL response. The subsequent addition of opsonized zymosan, however, provoked a normal CL response, indicating that neutrophils were not inhibited by inactivated bacteria. Chloramphenicol in a concentration of 25 μg/ml did not influence the CL response of the neutrophils to opsonized zymosan.

Adding 100 μl of HBSS that had been incubated with 10^5 and 10^6 CFU of the *A. pleuropneumoniae* strain and subsequently filtered resulted in an increased CL response which was not influenced by adding inactivated bacteria, indicating that the metabolites formed by *A. pleuropneumoniae* are responsible for the observed effects.

All convalescent-phase sera that were diluted 1:8 and adsorbed with heat-inactivated bacteria totally neutralized the inhibitory and stimulating effects of *A. pleuropneumoniae* on the oxidative activity of neutrophils. Neutralization was found to be dose responsive (data not shown). In contrast, preimmune sera or convalescent-phase sera that were adsorbed with viable bacteria did not neutralize these effects.

Influence of crude toxins on CL of neutrophils. The crude toxin preparation, undiluted or diluted 1:2, did not induce a CL response. The subsequent addition of opsonized zymosan likewise induced no CL response, indicating that the oxidative metabolism of neutrophils was inhibited. Incubating neutrophils with the crude toxin preparation diluted twofold from 1:4 to 1:32 induced stimulation of oxidative
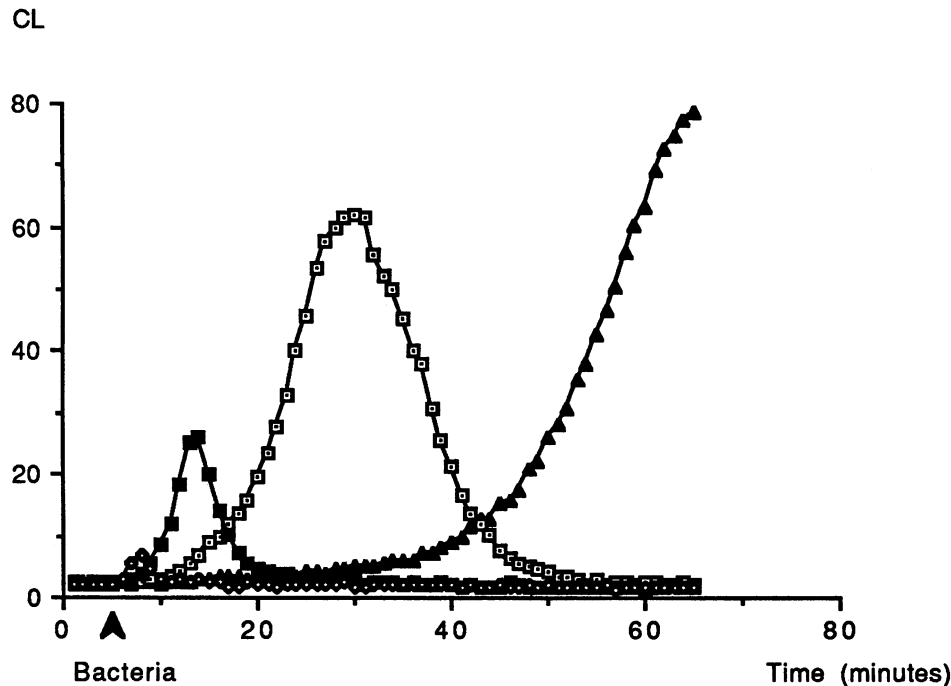
CL

FIG. 1. CL patterns of neutrophils incubated with 10^4 (▲), 10^5 (□), 10^6 (■), and 10^7 (◇) CFU of *A. pleuropneumoniae* serotype 2. CL responses are expressed as counts per minute (in thousands).

activity. CL values reached a peak and subsequently dropped to background values. Peak values increased when neutrophils were incubated with dilutions increasing from 1:4 to 1:16 (Fig. 2). The time to each CL peak value increased with increasing dilutions, but the differences were not significant. After declining to background values, neutrophils could no longer be stimulated by opsonized zymosan. Incubation of the neutrophils with 1:64 and 1:128 dilutions of the crude toxin preparation resulted in increased CL values. The peak value for neutrophils that were incubated with the 1:64 dilution was twice as high as the peak value for neutrophils that were incubated with the 1:128 dilution. Unlike the values obtained after addition of 1:4 to 1:32 dilutions, however, these CL values declined only gradually after having reached a peak. The subsequent addition of opsonized zymosan resulted in CL responses which were similar to those of the controls, indicating that neutrophils were not suppressed. The CL patterns for the different dilutions are illustrated in Fig. 2.

Chloramphenicol at a concentration of 25 μg/ml did not influence the CL response of porcine neutrophils to *A. pleuropneumoniae* culture supernatants.

The cytotoxic titer of the supernatant in the neutral red uptake assay was 128. The optical density values of serial supernatant dilutions revealed that cytotoxicity was dose responsive.

In contrast to nontreated supernatant, heat-treated supernatant dilutions neither inhibited nor stimulated the oxidative activity of the neutrophils.

Convalescent-phase, nonadsorbed sera as well as convalescent-phase sera that were adsorbed with inactivated bacteria in a 1:8 dilution neutralized the inhibition and the stimulation by *A. pleuropneumoniae* dilutions of the crude toxin preparation. Neutralization was found to be dose responsive (data not shown). Preimmune sera and convales-

cent-phase sera that were adsorbed with viable bacteria did not neutralize the effects on CL.

Influence of cytolysins produced by recombinant *E. coli* on CL of neutrophils. Incubating neutrophils with dilutions of supernatants of the *E. coli* cultures without the *A. pleuropneumoniae* cytolysins had no influence on CL. The subsequent addition of opsonized zymosan resulted in a CL response that was comparable with control responses, indicating that the oxidative metabolism of the neutrophils was not inhibited. The addition of dilutions of 1:10 to 1:160 of recombinant ClyII and ClyIII resulted in stimulation of the oxidative activity of the neutrophils (Table 1). After having reached peak values, CL dropped to background values. Then, neutrophils could no longer be stimulated by opsonized zymosan. When the neutrophils were incubated with a 1:320 dilution of recombinant ClyII or with dilutions of 1:320 to 1:640 of recombinant ClyIII, the oxidative activity of the neutrophils was stimulated. Unlike the values obtained after addition of 1:10 to 1:160 dilutions, however, these CL values declined only gradually after having reached a peak. The subsequent addition of opsonized zymosan resulted in CL responses which were comparable to those of controls, indicating that the oxidative metabolism of the neutrophils was not inhibited. The CL patterns for different dilutions of recombinant ClyII are illustrated in Fig. 3.

DISCUSSION

In these studies, *A. pleuropneumoniae* stimulated the production of oxygen radicals by porcine neutrophils. This stimulation was followed by an inhibition of the oxidative activity, as indicated by the lack of stimulation by opsonized zymosan. The results of neutral red uptake studies demonstrated that this inhibition of the oxidative activity of porcine neutrophils resulted from killing of the neutrophils. The
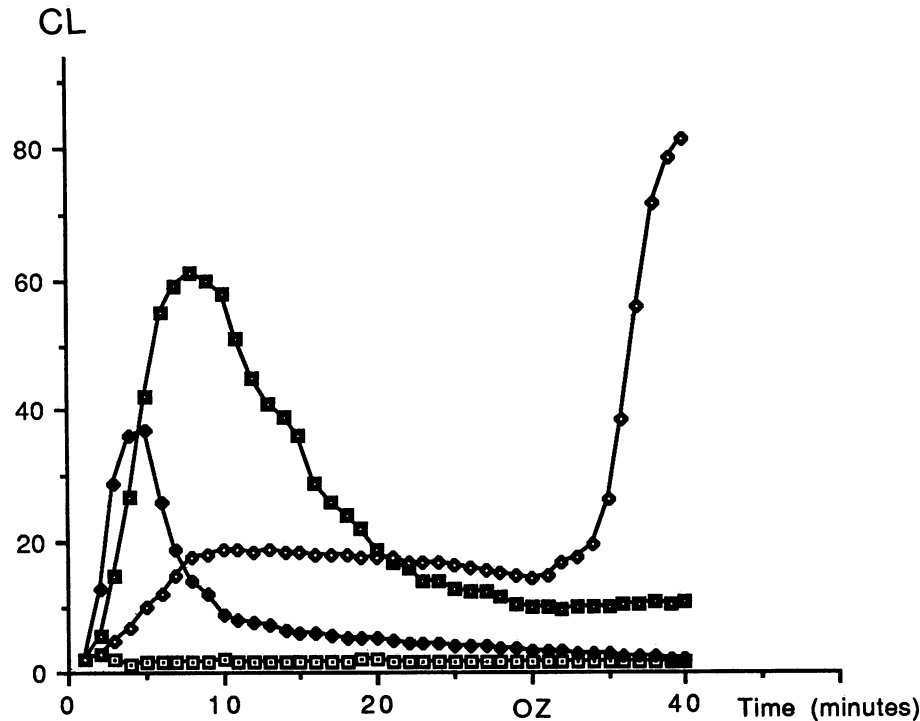
FIG. 2. CL patterns of neutrophils incubated with 1:2 (□), 1:8 (◆), 1:16 (■), and 1:64 (◆) dilutions of *A. pleuropneumoniae* culture supernatants. After 30 min, opsonized zymosan (OZ) was added to all of the samples. CL responses are expressed as counts per minute (in thousands).

cytotoxic activity of *A. pleuropneumoniae* on procine neutrophils is a well-known phenomenon (21). Stimulation of the oxygen burst of porcine neutrophils by *A. pleuropneumoniae*, on the other hand, is a new finding.

Recently, we described stimulation of the oxidative activity of porcine pulmonary alveolar macrophages by *A. pleu-*

TABLE 1. Influence of increasing dilutions of recombinant *A. pleuropneumoniae* ClyII and ClyIII on oxidative activity of neutrophils

Cytolysin and reciprocal dilution	CL response of neutrophils (PV-BV)[a]	Time to PV (min)
ClyII		
10	2 ± 1	1 ± 1
40	14 ± 3	5 ± 1
80	51 ± 6	16 ± 2
160	26 ± 3	20 ± 5
320	5 ± 1	18 ± 7
640	NA	NA
ClyIII		
10	2 ± 1	4 ± 1
40	24 ± 3	11 ± 1
80	11 ± 6	19 ± 2
160	8 ± 1	17 ± 5
320	4 ± 1	16 ± 7
640	2 ± 1	18 ± 8

[a] Values are expressed as means ± standard errors of the means ($n = 4$). PV, peak value in 1,000 cpm of CL induced by dilutions of recombinant cytolysins; BV, background value in 1,000 cpm of CL in the absence of recombinant cytolysins; NA, not applicable (no peak value was induced).

ropneumoniae and its metabolites (7). The generation of oxygen radicals in neutrophils, however, turned out to be much more pronounced than that in macrophages. Under our experimental conditions, the CL responses of 5×10^4 neutrophils to *A. pleuropneumoniae* metabolites were up to three to five times higher than the responses of 10^5 pulmonary alveolar macrophages. Although the oxygen burst is known to be toxic to bacteria as well as to the hosts' cells, it is unlikely that the inactivation of the neutrophils was the consequence of their own production of radicals. The addition of opsonized zymosan to neutrophils results in extensive production of oxygen radicals without cell death (data not shown). Furthermore, high doses of bacteria or undiluted *A. pleuropneumoniae* crude toxin preparation inactivated neutrophils without having stimulated the CL response.

Several findings in these experiments indicate that stimulation and subsequent inhibition of CL by viable bacteria are, at least in part, due to cytolysins secreted by *A. pleuropneumoniae*. Viable *A. pleuropneumoniae* first stimulated and subsequently inhibited oxidative activity, while equivalent doses of inactivated bacteria failed to influence the CL of the neutrophils. Since the point that cell surface components can be denatured by heat inactivation or UV inactivation may be raised, the experiments were repeated with *A. pleuropneumoniae* in the presence of chloramphenicol. *A. pleuropneumoniae* did not induce a CL response in the presence of chloramphenicol. Chloramphenicol inhibits protein synthesis while preserving cell surface components. Furthermore, the stimulating and inhibitory factors produced by *A. pleuropneumoniae* could be filtered from the culture medium and appeared heat labile. Finally, stimulation and inhibition of oxidative activity could be reproduced
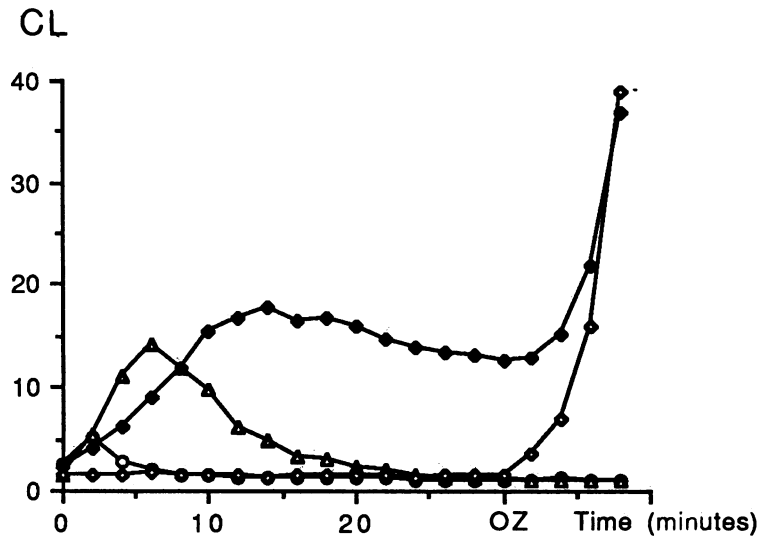
FIG. 3. CL patterns of neutrophils incubated with 1:10 (○), 1:40 (△), and 1:160 (◆) dilutions of recombinant ClyII. The CL pattern of neutrophils incubated with a 1:10 dilution of supernatants of an *E. coli* culture without the *A. pleuropneumoniae* components is also given (◇). After 30 min, opsonized zymosan (OZ) was added to all of the samples. CL responses are expressed as counts per minute (in thousands).

by ClyII and ClyIII produced by recombinant *E. coli*. Both cytolysins have been previously demonstrated to be produced by the *A. pleuropneumoniae* serotype 2 reference strain (16), as well as by the strain used in these studies (data not shown).

Cloning and DNA sequence analysis have provided evidence that the cytolysins of *A. pleuropneumoniae* are members of the RTX family of gram-negative bacterial, pore-forming exotoxins. These include the leukotoxin of *Pasteurella haemolytica* and the alpha-hemolysin of *E. coli* (19, 27). Similar toxins in four other species of *Actinobacillus* (*A. equuli*, *A. lignieresii*, *A. actinomycetemcomitans*, and *A. suis*) and in *Bordetella pertussis* have also been identified (20). It has been demonstrated that low concentrations of partially purified *P. haemolytica* A1 leukotoxin and *E. coli* alpha-hemolysin induce increased production of oxygen radicals by bovine and human neutrophils, respectively (3-5). These findings agree with the results of the present study.

In the present study, we found that the lowest concentrations of the metabolites (1:64 and 1:128 dilutions) induced stimulation without subsequently inhibiting the neutrophils, as indicated by a normal response to opsonized zymosan. Increasing doses of metabolites resulted in higher CL responses, which were followed by inhibition of the oxidative activity (1:16 and 1:32 dilutions). When metabolite concentrations increased further, peak values decreased and were succeeded more quickly by inhibition. Undiluted and the lowest supernatant dilutions caused immediate CL suppression without preceding stimulation. Similar findings were made for the *E. coli* alpha-hemolysin by Bhakdi and Martin (3). They proposed that toxin molecules first bind to the cell membrane without pore formation, thereby possibly interacting with binding sites that relay a signal to the cells. Since the probability of pore formation would increase as a function of time and with the number of molecules bound, only cells loaded with a very small number of toxin molecules would have time to respond to the stimulus.

The stimulation of neutrophils by cytolysins of *A. pleuropneumoniae*, resulting in a respiratory burst, may play a role in the development of lung lesions during pleuropneumonia. Oxygen radicals can degrade hyaluronic acid, denature proteins, and induce cell membrane lipid peroxidation (1, 9). It is conceivable, therefore, that excessive production of oxygen radicals by stimulated neutrophils has deleterious effects on neighboring tissue cells. Furthermore, activated neutrophils can release potent digestive lysosomal enzymes into the surrounding medium (5). All of these processes may promote tissue injury and destruction, contributing to the extensive lung lesions that occur during pleuropneumonia.

The inhibitory and stimulatory effects of *A. pleuropneumoniae* and its metabolites were neutralized by convalescent-phase sera adsorbed with heat-inactivated bacteria but not by sera adsorbed with viable bacteria. The reason for this finding is not clear. It is possible that the heat-labile cytolysins of *A. pleuropneumoniae* are not only secreted in the medium but are also present at the bacterial surface. In this case, however, it remains to be revealed why non-heat-treated bacteria do not influence CL responses in the presence of chloramphenicol.

ACKNOWLEDGMENTS

REFERENCES

1. **Anderson, B. O., J. M. Brown, and A. H. Harken.** 1991. Mechanisms of neutrophil-mediated tissue injury. J. Surg. Res. **51:**170–179.
2. **Bertram, T. A.** 1985. Quantitative morphology of peracute pulmonary lesions in swine induced by *Haemophilus pleuropneumoniae*. Vet. Pathol. **22:**598–609.
3. **Bhakdi, S., and E. Martin.** 1991. Superoxide generation by human neutrophils induced by low doses of *Escherichia coli* hemolysin. Infect. Immun. **59:**2955–2962.

4. Cavalieri, S. J., and I. S. Snyder. 1982. Effect of *Escherichia coli* alpha-hemolysin on human peripheral leukocyte function in vitro. Infect. Immun. **37**:966–974.

5. Czuprynski, C. J., E. J. Noel, O. Ortiz-Carranza, and S. Srikumaran. 1991. Activation of bovine neutrophils by partially purified *Pasteurella haemolytica* leukotoxin. Infect. Immun. **59**:3126–3133.

6. Devenish, J., and S. Rosendal. 1989. Identification of the heat-labile hemolysin of *Actinobacillus pleuropneumoniae* serotype 1. Can. J. Vet. Res. **53**:251–254.

7. Dom, P., F. Haesebrouck, and P. De Baetselier. 1992. Stimulation and suppression of the oxygenation activity of procine pulmonary alveolar macrophages by *Actinobacillus pleuropneumoniae* and its metabolites. Am. J. Vet. Res. **53**:1113–1118.

8. Fenwick, B. W. 1990. Virulence attributes of the lipopolysaccharides of the HAP group organisms. Can. J. Vet. Res. **54**:S28–S32.

9. Freeman, B. A., and J. D. Crapo. 1982. Free radicals and tissue injury. Lab. Invest. **47**:412–417.

10. Frey, J., and J. Nicolet. 1988. Regulation of hemolysin expression in *Actinobacillus pleuropneumoniae* serotype 1 by Ca^{2+}. Infect. Immun. **56**:2570–2575.

11. Frey, J., and J. Nicolet. 1988. Purification and partial characterization of a hemolysin produced by *Actinobacillus pleuropneumoniae* type strain 4074. FEMS Microbiol. Lett. **55**:41–46.

12. Frey, J., and J. Nicolet. 1990. Hemolysin patterns of *Actinobacillus pleuropneumoniae*. J. Clin. Microbiol. **28**:232–236.

13. Gygi, D., J. Nicolet, J. Frey, M. Cross, V. Koronakis, and C. Hughes. 1990. Isolation of the *Actinobacillus pleuropneumoniae* haemolysin gene and the activation and secretion of the prohaemolysin by the HlyC, HlyB and HlyD proteins of *Escherichia coli*. Mol. Microbiol. **4**:123–128.

14. Inzana, T. J. 1990. Capsules and virulence in the HAP group of bacteria. Can. J. Vet. Res. **54**:S22–S27.

15. Jansen, R., J. Briaire, E. M. Kamp, A. L. J. Gielkens, and M. A. Smits. Unpublished data.

16. Kamp, E. M., J. K. Popma, J. Anakotta, and M. A. Smits. 1991. Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. Infect. Immun. **59**:3079–3085.

17. Kamp, E. M., and L. A. M. G. van Leengoed. 1989. Serotype-related differences in production and type of heat-labile hemolysin and heat-labile cytotoxin of *Haemophilus (Actinobacillus) pleuropneumoniae*. J. Clin. Microbiol. **27**:1187–1191.

18. Ligett, A. D., L. R. Harrison, and R. L. Farrell. 1987. Sequential study of lesion development in experimental haemophilus pleuropneumonia. Res. Vet. Sci. **42**:204–212.

19. Lo, R. Y. C. 1990. Molecular characterization of cytotoxins produced by *Haemophilus, Actinobacillus, Pasteurella*. Can. J. Vet. Res. **54**:S33–S35.

20. Merner, L. L., and R. Y. C. Lo. 1991. Characterization of an *Actinobacillus suis* hemolysin, p. 32, B-43. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.

21. Rosendal, S., J. Devenish, I. MacInnes, J. D. Lumsden, S. Watson, and H. Xun. 1988. Evaluation of heat-sensitive, neutrophil-toxic, and hemolytic activity of *Haemophilus (Actinobacillus) pleuropneumoniae*. Am. J. Vet. Res. **49**:1053–1058.

22. Rycroft, A. N., D. Williams, J. M. Cullen, and J. Macdonald. 1991. The cytotoxin of *Actinobacillus pleuropneumoniae* (pleurotoxin) is distinct from the haemolysin and is associated with a 120 kDa polypeptide. J. Gen. Microbiol. **137**:561–568.

23. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

24. Sebunya, T. N. K., and J. R. Saunders. 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182**:1331–1337.

25. Serebrin, S., S. Rosendal, A. Valdivieso-Garcia, and P. B. Little. 1991. Endothelial cytotoxicity of *Actinobacillus pleuropneumoniae*. Res. Vet. Sci. **50**:18–22.

26. Smits, M. A., J. Briaire, R. Jansen, H. E. Smith, E. M. Kamp, and A. L. J. Gielkens. 1991. Cytolysins of *Actinobacillus pleuropneumoniae* serotype 9. Infect. Immun. **59**:4497–4504.

27. Strathdee, C. A., and R. Y. C. Lo. 1987. Extensive homology between the leukotoxin of *Pasteurella hemolytica* A1 and the alpha-haemolysin of *Escherichia coli*. Infect. Immun. **55**:3233–3236.

28. Utrera, V., and C. Pijoan. 1991. Fimbriae in *A. pleuropneumoniae* strains isolated from pig respiratory tracts. Vet. Rec. **128**:357–358.