INFECTION AND IMMUNITY, Sept. 1981, p. 673-676
0019-9567/81/090673-04$02.00/0

Vol. 33, No. 3

Toxicity of *Haemophilus pleuropneumoniae* for Porcine Lung Macrophages, Peripheral Blood Monocytes, and Testicular Cells

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Received 17 February 1981/Accepted 22 May 1981

Strain CM5 of *Haemophilus pleuropneumoniae* was toxic for porcine lung macrophages in minimum doses of 10^6 colony-forming units per ml and for peripheral blood monocytes at 10^7 colony-forming units per ml. The organism was not toxic for porcine testicular cells in a concentration of 10^8 colony-forming units per ml. Filtered sterile culture supernatant diluted 10^0, 10^{-1}, and 10^{-2} was toxic for porcine pulmonary lavage cells. In dilutions of 10^0 and 10^{-1}, culture supernatant was toxic for peripheral blood monocytes, and at 10^0 it was toxic for testicular cells. Toxicity associated with bacterial cells was sensitive to heating (60°, 60 min), whereas that of the culture supernatant was heat stable (100°C, 15 min). Swine convalescent serum neutralized supernatant toxicity in dilutions of ≥1:320. The findings promote the understanding of the pathogenesis of pleuropneumonia in swine caused by *H. pleuropneumoniae*.

Porcine pleuropneumonia caused by *Haemophilus pleuropneumoniae* is either acute extensive and fibrinohemorrhagic or chronic localized and necrotizing. Each type is associated with pleuritis (4). Pigs may die within 8 h of experimental inoculation. Bacteria-free culture supernatants of *H. pleuropneumoniae* or sonicated nonviable bacteria can induce localized pneumonia which is similar microscopically to pneumonia in spontaneously infected pigs (3). In this study, the toxicity of *H. pleuropneumoniae* is explored further by examining the effect in vitro of viable bacteria, heat-killed bacteria, filtered sterile supernatant, and boiled sterile supernatant on porcine pulmonary lavage cells, peripheral blood mononuclear cells, and cultured testicular cells.

MATERIALS AND METHODS

Bacteria and culture supernatants. *H. pleuropneumoniae* strain CM5, isolated from a case of acute porcine pleuropneumonia, was used in all experiments. Roux flasks with 150 ml of tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 0.1% nicotinamide adenine dinucleotide (Eastman Kodak Co., Rochester, N.Y.) were inoculated with approximately 10^7 colony-forming units (CFU) of bacteria removed from a plate culture incubated overnight. The flask cultures were incubated at 37°C for 6 h, after which 100 ml of RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) was added and incubation was continued for an additional 12 h to reach the late logarithmic growth phase as determined in preliminary experiments. The liquid phase was harvested and centrifuged for 10 min at 10,000 × *g*. The supernatant was filtered through a 200-nm membrane filter (Millipore Corp., Bedford, Mass.), cultured to determine its sterility and stored at −70°C in 8-ml samples. Individual samples were heated at 100°C for 15 min. Both unheated and heated culture supernatants were tested for cytotoxicity. The bacterial pellet was suspended in RPMI 1640 medium, dispensed in 8-ml samples, and stored at −70°C. After the samples were thawed, the number of CFU per ml was determined by the standard plate counting technique. Selected samples of bacterial suspension were inactivated by heating at 60°C for 60 min. Unheated as well as heated bacteria were tested for cytotoxicity on substrate cells. The liquid phase from uninoculated diphasic medium was processed, as was that from inoculated medium, for comparison in experiments evaluating cytotoxicity.

Pulmonary lavage cells. Cells were obtained from porcine lungs by lavage, using fiberoptic bronchoscopy essentially as described for cattle (2). Yorkshire pigs weighing 40 to 50 kg each from a herd free of *Haemophilus* infection were premedicated with a fentanyl and droperidol mixture (Innovar-Vet, 1 ml/25 kg; Pitman-Moore, Inc., Washington Crossing, N.J.) and anesthetized by the intravenous injection of a combination of 3% thiopental sodium (Pentothal; Abbott Laboratories, North Chicago, Ill.) and 3% sodium pentobarbital (Nembutal; Abbott Laboratories) in physiological saline. A fiberoptic bronchoscope (Machida model MUGS 155; Therascope, Toronto, Ontario, Canada) was placed in the lumen of the smallest possible airway arising from the main bronchus. A total of 250 ml of 0.1 M phosphate-buffered saline, pH

7.4, containing 50 μg of streptomycin, 100 U of penicillin, and 100 U of mycostatin per ml was delivered into the lung. The lavage fluid was recovered by moderate suction applied by a vacuum pump. This procedure was performed on each lung. The pulmonary lavage fluid was centrifuged at 400 × *g* for 10 min, and the cell pellet was suspended in RPMI 1640 medium containing 100 U of penicillin and 50 μg of streptomycin per ml.

Peripheral blood mononuclear cells. Blood from 6- to 8-week-old Yorkshire pigs was collected in evacuated glass tubes containing ethylenediaminetetraacetic acid (Vacutainer; Becton, Dickinson & Co., Mississauga, Ontario). Mononuclear cells were separated on a Ficoll-Hypaque gradient (5) and suspended to yield 4×10^6 to 6×10^6 cells per ml in RPMI 1640 medium containing 20% newborn calf serum, 100 U of penicillin, and 50 μg of streptomycin per ml. The cells were incubated in plastic tissue culture flasks at 37°C in 5% CO_2. The medium was renewed, and nonadherent cells were discarded after 24 h. Adherent cells were left for 6 days to mature into macrophages. These cells were detached by using warm (38°C) phosphate-buffered saline (P. H. Bendixen, Am. J. Vet. Res., in press) and resuspended in RPMI 1640 medium.

Swine testicular cells. Cells of a stable swine testicular cell line (Diamond Laboratories, Des Moines, Iowa) were cultured in plastic tissue culture flasks for 4 days in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. Before use, cells were detached by trypsinization, washed once, and resuspended in RPMI 1640 medium.

^{51}Cr labeling and determination of cytotoxicity. Cells were counted and adjusted to 7×10^6 to 9×10^6 cells per ml. A 50-μCi amount of [^{51}Cr]sodium chromate with a specific activity of 1 mCi/ml (Amersham Corp., Oakville, Ontario) was added per ml of cell suspension, and the cells were incubated for 1 h at 37°C on a rocking platform. Cells were washed six times to remove unbound ^{51}Cr, adjusted to 10^6 cells per ml in RPMI 1640 medium, and dispensed in 1.5-ml samples in Leighton tubes containing glass cover slips. Cells were allowed to adhere to the cover slips for 1 h at 37°C. Cover slips were then transferred to Leighton tubes containing 1.5 ml of the test suspension of bacteria or dilutions of culture supernatant. All tests were performed in triplicate. After a further 1-h incubation, the radioactivity remaining on the cover slips and that released into the medium were counted in an automatic gamma counter (model 1805; Nuclear-Chicago Corp., Des Plaines, Ill.). Radioactivity measured in the medium was expressed as a percentage of the total radioactivity by adding values obtained for cover slip and medium. The resultant value was taken to indicate release of ^{51}Cr-related activity from injured cells or detachment of formerly adherent cells from cover slips.

Neutralization of toxicity. Serum with precipitating antibodies against *H. pleuropneumoniae* CM5 was obtained from pigs with chronic pleuropneumonia. The serum was filtered through a 200-nm Millipore filter and cultured to confirm sterility. Normal swine serum was obtained from pigs uninfected with *H. pleuropneumoniae*. Bacterial culture supernatants diluted 1:10 in RPMI 1640 medium were incubated for 30 min at 37°C with an equal volume of the appropriate serum dilution and added to cultured labeled pulmonary lavage cells. Controls without serum and controls without supernatant were included.

RESULTS

Lung lavage samples contained 80 to 90% mononuclear cells, yielding an adherent cell population composed of alveolar macrophages.

After exposure to viable *H. pleuropneumoniae* or its culture supernatants, radioactivity decreased in adherent target cells and increased in the medium. This result could be due to cell lysis, to cell detachment, or to both. This cytopathic effect was recognized morphologically as rounding and detachment of cells from cover slips and by increased uptake of trypan blue dye by adherent cells.

H. pleuropneumoniae CM5 was cytotoxic for pulmonary lavage cells in concentrations of $\geq 10^6$ CFU/ml (Fig. 1). Doses of $\leq 10^5$ CFU/ml were not different from control cultures without bacteria. Peripheral blood mononuclear cells were susceptible to bacteria in concentrations of $\geq 10^7$ CFU/ml. No cytotoxic effect was detected in testicular cell cultures incubated with 10^8 CFU/ml, the highest dose of bacteria tested.

Filtered sterile *H. pleuropneumoniae* culture supernatant was toxic for pulmonary lavage cells even in dilutions of 10^{-2} (Fig. 2). Undiluted supernatant and 10^{-1} dilutions were toxic for peripheral blood mononuclear cells. Toxicity for testicular cells was detectable only in undiluted supernatant and was not pronounced.

Heat inactivation of 10^8 CFU of *H. pleuropneumoniae* CM5 per ml eliminated toxicity of the bacteria for pulmonary lavage cells. How-

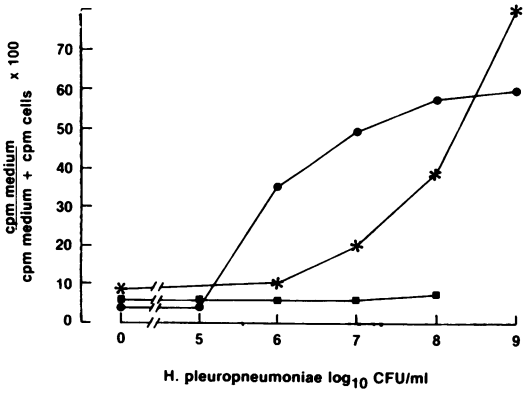

FIG. 1. *Cytotoxicity of H. pleuropneumoniae for porcine pulmonary lavage cells (●), peripheral blood mononuclear cells (*), and testicular cells (■). All data had standard deviations of ≤3.*

ever, culture supernatant diluted 10^{-1} retained most of its toxicity after being heated to 100°C for 15 min (Fig. 3).

The toxicity of culture supernatant for pulmonary lavage cells was partially neutralized by normal swine serum diluted 1:10 but not by 1:80 or 1:320 dilutions. Immune serum from infected pigs neutralized toxicity in dilutions of 1:10, 1:80, and 1:320 (Fig. 4). Swine serum alone was not toxic for pulmonary lavage cells.

DISCUSSION

Necrosis is the predominant feature of acute and chronic porcine pleuropneumonia associated with *H. pleuropneumoniae* infection. It is not known whether necrosis is due to a direct cytotoxic effect of the bacteria, to bacterial products, or to ischemia after thrombosis. However, bacteria rendered nonviable by sonication or filtered sterile culture supernatant can induce localized pleuropneumonia when instilled into porcine lung. In both cases, vascular changes with thrombosis are marked (3). It may be that the cytotoxicity demonstrated here by in vitro assays could be related to the changes seen in the lungs of diseased pigs.

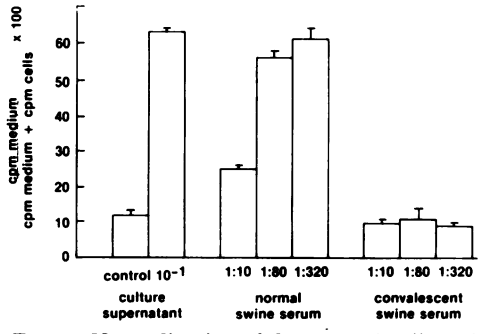

FIG. 4. *Neutralization of the cytotoxic effect of H. pleuropneumoniae culture supernatant after incubation with various dilutions of normal or convalescent swine serum.*

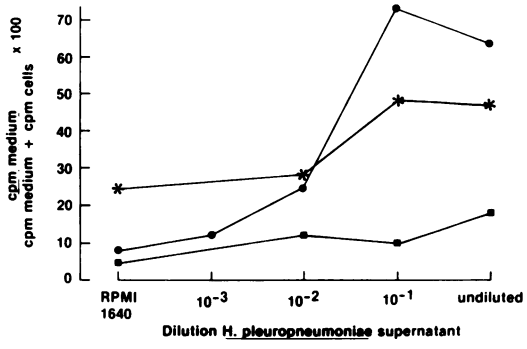

FIG. 2. *Cytotoxicity of sterile culture supernatant from H. pleuropneumoniae for porcine pulmonary lavage cells (●), peripheral blood mononuclear cells (*), and testicular cells (■). All data had standard deviations of ≤6.*

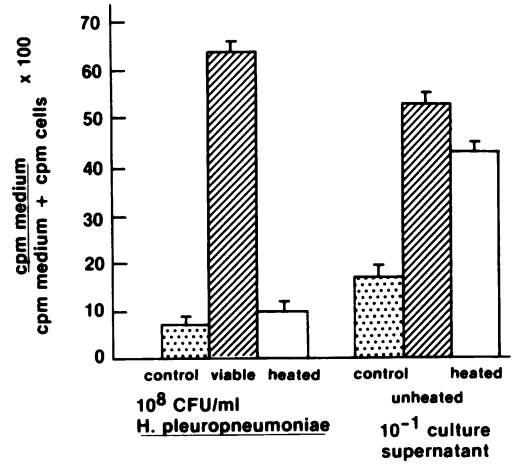

FIG. 3. *Effect of heat treatment on the cytotoxicity of H. pleuropneumoniae for pulmonary lavage cells. Bacteria were treated at 60°C for 1 h, and culture supernatant was treated at 100°C for 15 min.*

Viable *H. pleuropneumoniae* cells were toxic for adherent porcine pulmonary lavage cells and for peripheral blood mononuclear cells, but not for testicular cells up to 10^8 CFU/ml. For pulmonary cells, cytotoxicity was observed even at the ratio of 1 bacterium per target cell (10^6 CFU/ml), making phagocytic engorgement an unlikely explanation for the phenomenon. Further, heat-killed bacteria exerted no detrimental effect, suggesting that cytotoxicity was due to metabolically active organisms or their products.

It is known that *H. pleuropneumoniae* releases toxic substances during growth (3). In this study, filtered sterile bacterial culture supernatants were toxic for pulmonary lavage cells and, to a lesser extent, for cultured peripheral blood monocytes. Swine testicular cells were only slightly affected by undiluted supernatant. The toxin in the supernatant was heat stable, as it retained activity after 15 min at 100°C. Furthermore, the toxin could be neutralized by antibodies present in the sera of pigs with chronic infection. This finding may have important implications for attempts to control *H. pleuropneumoniae* infection by vaccination. The moderate neutralizing capacity of normal swine serum in dilutions of 10^{-1} may be due to low levels of specific or cross-reacting antibodies or to nonspecific toxin-neutralizing factors. Recent studies indicate that gram-negative bacterial endo-

toxin may be detrimental to human alveolar macrophages (1). Perhaps normal swine serum exerts its effect by neutralizing an endotoxic component of *H. pleuropneumoniae*. The resistance of the *H. pleuropneumoniae* toxin to heating at 100°C is compatible with a major characteristic of gram-negative bacillary endotoxin.

It seems reasonable to assume that toxicity to macrophages confers a considerable advantage to any pulmonary pathogen. Previously, *Pasteurella haemolytica*, the major agent of bovine bacterial pneumonia, was shown to produce a potent, heat-labile cytotoxin injurious to bovine alveolar macrophages (2). Preliminary investigation indicates that the toxin produced by *P. haemolytica* does not affect porcine lung macrophages, whereas *H. pleuropneumoniae* culture supernatants are equally toxic for cultured bovine and porcine pulmonary cells.

If the toxicity for the alveolar macrophage demonstrated for *H. pleuropneumoniae* in vitro prevails also in vivo, it may contribute to the frequently rapid and fulminant course of this disease.

ACKNOWLEDGMENTS

We thank W. N. McDonell, Department of Clinical Studies, for advice on anesthesia and J. Dimoor, G. Woodall, and S. Hobson for technical assistance.

This work was supported by grants from the Ontario Ministry of Agriculture and Food and the Ontario Pork Producers Association. P.H.B. and P.E.S. are fellows of the Medical Research Council of Canada.

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