

# Acute Inflammatory Effects of Intratracheally Instilled *Escherichia coli* Endotoxin and Sonicated Suspension of *Haemophilus pleuropneumoniae* in Swine

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## ABSTRACT

A single bolus of either *Escherichia coli* endotoxin, sonicated suspension of *Haemophilus pleuropneumoniae*, or pyrogen-free normal saline was intratracheally instilled in six week old specific-pathogen-free pigs. Pigs exposed to *E. coli* endotoxin developed fever, leukopenia followed by leukocytosis, and endotoxemia. Leukocytosis was the only clinical abnormality noted in pigs receiving the sonicated suspension of *H. pleuropneumoniae*. At one day post-exposure, focal areas of atelectasis and consolidation were observed in the caudal lung lobes of animals receiving either *E. coli* endotoxin or the sonicated suspension of *H. pleuropneumoniae*. Lesions were characterized by a neutrophilic bronchitis and bronchiolitis with alveolitis in the surrounding tissue. Increased numbers of alveolar macrophages and evidence of phagocytosis were observed by light and electron microscopy. No clinical abnormalities or lesions were observed in animals receiving normal saline. Lesions typical of acute porcine *Haemophilus pleuropneumoniae* were not produced by either *E. coli* endotoxin or sonicated suspension of *H. pleuropneumoniae*, indicating that multiple virulence factors are probably involved in lesion development.

**Key words:** Pulmonary, endotoxin, *Escherichia coli*, *Haemophilus pleuropneumoniae*.

## RÉSUMÉ

Cette expérience portait sur des porcelets exempts de germes pathogènes spécifiques et âgés de six semaines; elle consistait à les soumettre à une instillation trachéale d'endotoxine d'*Escherichia coli* ou d'une suspension d'*Haemophilus pleuropneumoniae*, soumise aux ultrasons, ou de saline conventionnelle, dépourvue d'agent pyrogène. Les porcelets qui avaient reçu l'endotoxine d'*E. coli* développèrent de la fièvre, une leucopénie, suivie de leucocytose, et une endotoxémie. Ceux qui avaient reçu la suspension d'*H. pleuropneumoniae* ne développèrent cependant qu'une leucocytose. Au bout de 24 heures, les porcelets des deux groupes précités affichèrent des foyers d'atélectasie et de consolidation, dans leurs lobes pulmonaires caudaux. L'histopathologie révéla la présence de bronchite et de bronchiolite purulentes, ainsi que d'une alvéolite avoisinante. La microscopie, tant photonique qu'électronique, permit de constater une augmentation du nombre de macrophages alvéolaires et une activité phagocytaire. Les témoins ne développèrent pas de signes cliniques ou de lésions. L'endotoxine et la suspension précitées ne provoquèrent pas le développement de lésions typiques de la pleuro-pneumonie porcine aiguë imputable à *H. pleuropneumoniae*, indice de l'implication probable de plusieurs facteurs virulents dans le développement des lésions.

**Mots clés:** poumons, endotoxine, *Escherichia coli*, *Haemophilus pleuropneumoniae*.

## INTRODUCTION

Outbreaks of porcine *Haemophilus pleuropneumoniae* (PHP) often have a peracute onset with commonly observed signs of fever, severe respiratory distress, vomiting, and epistaxis (1,2). Gross and histopathological lesions are usually confined to the thoracic cavity (2). In acute cases, the gross lesions are a fibrinous pleuritis and necrotizing hemorrhagic pneumonia (1). Histologically the lesion is characterized by hemorrhage, necrosis, exudation of fibrin, infiltration of an undefined population of degenerate mononuclear cells, and thrombosis of pulmonary vessels (1,2,3,4). The interlobular septa are distended by edema and a mononuclear cell infiltrate with fibrin clots in lymphatic vessels (1). In most reports of PHP, neutrophils are absent or not a significant component of the infiltrates (1,2,3,4).

Smith has stated that Gram-negative bacteria can mediate damage to host tissue by liberation of endotoxin, production of exotoxin, tissue invasion, and evocation of hypersensitivity (5). These mechanisms appear to vary in importance with the species and strain of bacteria involved. To a limited extent, all of these mechanisms have been proposed

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to explain lesion development in PHP. Although endotoxin production by *H. pleuropneumoniae* (and more specifically, endotoxemia in PHP) has not been demonstrated, the clinical signs, gross findings, and histological lesions of PHP have been compared to those of endotoxic shock (1,2,6). Exotoxins with cytotoxic activity *in vitro* for porcine macrophages and monocytes and red blood cells of other species have recently been demonstrated (7,8). Tissue invasion with resultant septicemia has been reported in both naturally occurring and experimental PHP (9,10). Pijoan has proposed that the vasculitis and mononuclear infiltrates observed in PHP are suggestive of a hypersensitivity reaction (11).

Liberation of endotoxin has been the most frequently proposed explanation for lesion development. The objectives of this experiment were to determine the effects of intratracheal instillation of a single bolus of sonicated suspension of *H. pleuropneumoniae* or *E. coli* endotoxin on rectal temperature, total white blood cell count, and lesion development in the lung at the gross, microscopic, and ultrastructural levels with emphasis on the inflammatory reaction. In addition, plasma samples were tested by the limulus amoebocyte lysate (LAL) assay for endotoxin.

## MATERIALS AND METHODS

### INJECTABLES

A strain of *H. pleuropneumoniae* serotype 5 isolated from a pneumonic lung in an acute outbreak of PHP was cultured in trypticase soy broth (TSB) containing 0.1 mg per mL of nicotinamide adenine dinucleotide (NAD) for six hours at 37°C in 5% carbon dioxide in air. One mL portions of the culture were inoculated into tubes containing 10 mL of TSB and NAD and incubated as above for 12 hours. Broth cultures were centrifuged at 3,000 x g for ten minutes at 8°C. The pelleted bacteria were resuspended in 10 mL of sterile pyrogen-free normal saline and centrifuged. The last step was repeated three times. After the final rinse, the pellet was resuspended in 3 mL of sterile pyrogen-free normal saline, transferred to a plastic tube,

and sonicated for three minutes in a cell disruptor (Heat Systems, Ultrasonic Inc., Plainview, New York) with a cuphorn attachment connected to a circulating waterbath at 8°C. Sonicated suspensions were held at 4°C pending verification of sterility by culture on bovine blood agar (BBA) plates with a centrally placed NAD-saturated disk at 37°C in 5% carbon dioxide in air and on BBA plates at 37°C in air. *Escherichia coli* endotoxin (0128:B12) (Difco Laboratories, Detroit, Michigan) was obtained from a commercial source. All injectables were warmed to 37°C prior to injection.

### MATERIALS

All glassware and screw caps used in the preparation of the sonicated suspension and LAL procedure were rendered pyrogen-free by an overnight soak in E-Toxa-Clean (Sigma Chemical, St. Louis, Missouri) followed by ten rinses with warm tap water and five rinses in distilled water. The glassware and screw caps were then wrapped in paper and autoclaved at 121°C for one hour followed by heating at 180°C for four hours. Single-use pyrogen-free sterile needles and syringes were used as prepared by the manufacturer.

### EXPERIMENTAL DESIGN AND ANIMALS

Pregnant specific-pathogen-free sows were purchased and farrowed in an isolation facility. Litters were maintained in individual rooms in the facility throughout all phases of the experiment. At six weeks of age, the pigs were randomly assigned to one of three groups of six pigs each. Group I pigs received 1 mg *E. coli* endotoxin per kg body weight via endotracheal injection. Group II pigs received 1 mL of a sonicated suspension of *H. pleuropneumoniae* per 5 kg body weight via the same route. Pyrogen-free sterile normal saline was added as required to make the total volume injected 6 mL in both groups. Group III received 6 mL of pyrogen-free sterile normal saline via the same route. Pigs were held upright with head extended for one minute following injection. Injection and venipuncture sites were prepared by cleansing with an iodine solution and 70%

alcohol. Blood samples were collected in EDTA tubes and pyrogen-free heparinized vials (Sigma Chemical, St. Louis, Missouri) immediately prior to and at 1 and 24 hours after injection. Clinical signs and rectal temperatures were monitored at the same time intervals. An elevation of 0.5°C above the mean of the baseline was reported as fever. White blood cell counts were determined with a Coulter counter. Leukopenia and leukocytosis were reported when mean values were below or above the normal range (11 to 22 x 10<sup>3</sup>/mm<sup>3</sup>), respectively (12).

At 24 hours postinjection, all animals were anesthetized with thio-pental and killed by exsanguination. The trachea was exposed and cannulated. Trump's fixative (13) at 4°C was infused into the trachea from a height of 20 cm. After approximately 30 minutes, 5 mm thick sagittal sections and 1 mm cubes of lung were cut and placed in fresh fixative. Portions of sagittal sections were dehydrated, infiltrated, and embedded in either paraffin or glycol methacrylate (JB4 kit, Polyscience Inc., Warrington, Pennsylvania). Paraffin blocks were sectioned at 6 μ and stained with hematoxylin and eosin (H&E). Glycol methacrylate blocks were sectioned at 2 μ and stained either with H&E or with Turner's modification of Lillie's hematoxylin-toluidine blue-phloxinate for light microscopy (14). Cubes of tissue were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer and then dehydrated through a series of ethanol and acetone rinses prior to embedding in plastic. Ultrathin sections were stained with lead citrate and uranyl acetate and examined in a transmission electron microscope.

### LIMULUS AMEBOCYTE LYSATE TEST

Plasma extraction was conducted according to a heat extraction procedure outlined by Berg *et al* (15). Single test vial LAL kits (Whittaker M.A. Bioproducts, Walkersville, Maryland) were used in accordance with the manufacturer's test procedures. Vials were incubated at 37°C for one hour in a modular heat block. Positive, negative, and inhibition controls were performed with each trial. At the end of the incubation period, the vials were inverted and examined. A positive test was reported when a clot formed and a

negative test was reported when no clot formed.

#### STATISTICAL ANALYSIS

Total white blood cell count and rectal temperature data were evaluated using analysis of variance and Newman-Keuls method for comparison of means (16).

### RESULTS

Fever and a marked leukopenia were detected in group I animals at one hour after exposure (Tables I and II). Additional observations in this group were transient vomiting and diarrhea, depression, and partial anorexia. A mild leukocytosis developed in groups I and II at 24 hours postinjection. No abnormalities were observed in group III.

Results of the LAL test are shown (Table III). Negative controls were negative and positive and inhibition controls were positive for each trial. Manufacturer's stated sensitivity of tests was 1.14 ng of *E. coli* (0111:B4) endotoxin.

Gross examination of visceral organs revealed lesions confined to the lungs of animals in groups I and II; no lesions were observed in group III. The pulmonary lesions in groups I and II were similar and characterized by focal areas of atelectasis and consolidation in the caudal lobes involving single or multiple lobules. In sagittal sections, areas of consolidation were centered around bronchi and bronchioles.

Microscopic examination of caudal lung lobe sections at low magnification from group I and II animals revealed areas of increased cellularity and partial collapse of alveolar spaces involving portions of confluent lobules. Bronchi and bronchioles were patent, and interlobular septa were mildly dilated.

Histologically, lesions were confined to the areas observed grossly in group I and II animals. Bronchi and bronchioles at all levels had moderate numbers of neutrophils in the lamina propria and between epithelial cells (Fig. 1). Some bronchial and bronchiolar surfaces were incompletely lined by a layer of neutrophils, and a few small bronchioles contained plugs of

**TABLE I. Evaluation of Mean Rectal Temperatures in Groups of Six Pigs Each after Intratracheal Injection of Endotoxin, Sonicated Bacteria, or Saline**

Group	Hours after Injection		
	0 <sup>a</sup>	I	24
I (endotoxin)	39.6	40.7 (P > 0.05)	40.2
II (sonicated bacteria)	39.5	39.8	40.0
III (saline)	39.5	39.8	39.9

<sup>a</sup>Measurements taken immediately prior to injection. Data expressed in degrees C

**TABLE II. Evaluation of Mean Total White Blood Cell Counts in Groups of Six Pigs Each after Intratracheal Injection of Endotoxin, Sonicated Bacteria, or Saline**

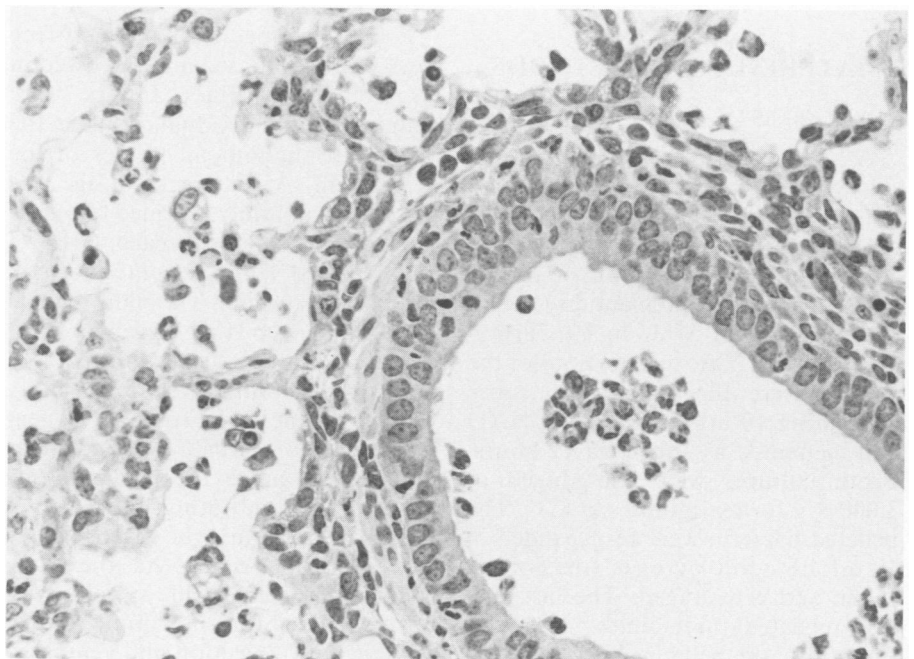
Group No.	Hours after Injection		
	0 <sup>a</sup>	I	24
I (endotoxin)	16.2	5.9 (P > 0.05)	23.3 (P > 0.05)
II (sonicated bacteria)	17.4	17.1	26.8 (P > 0.05)
III (saline)	19.3	19.4	18.0

<sup>a</sup>Samples taken immediately prior to injection. Data expressed as mean WBC count x 10<sup>3</sup>/mm<sup>3</sup>

**TABLE III. Results of Limulus Amebocyte Lysate Assay by Group and Time after Intratracheal Injection of Endotoxin, Sonicated Bacteria, or Saline**

Group No.	Hours after Injection		
	0 <sup>a</sup>	I	24
I (endotoxin)	0/6	6/6	4/6
II (sonicated bacteria)	0/6	0/6	0/6
III (saline)	0/6	0/6	0/6

<sup>a</sup>Samples taken immediately prior to injection. Data expressed as no. of pigs positive/no. examined



**Fig. 1. Lung sections embedded in GMA. Neutrophilic bronchiolitis and alveolitis are observed. *H. pleuropneumoniae* sonicated suspension. H & E. X400.**

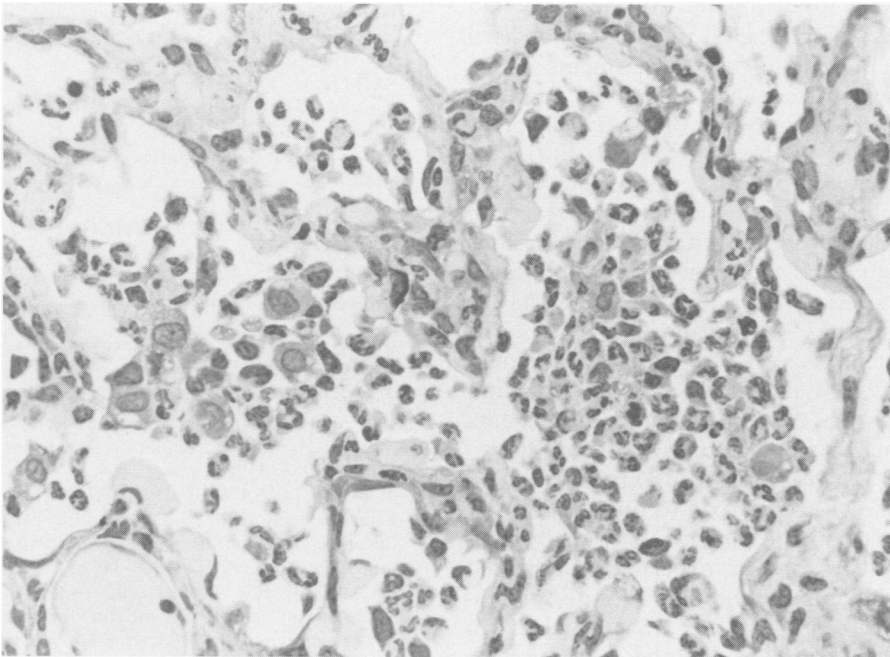


Fig. 2. Alveolar exudate composed predominantly of neutrophils and lesser numbers of macrophages. Septa are thickened and many neutrophils are present in capillaries. *E. coli* endotoxin. H & E. X500.

exudate. Alveolar septa were thickened, and many neutrophils were observed in capillaries. The alveolar infiltrate was composed predominantly of nondegenerate neutrophils

and increased numbers of large macrophages (Fig. 2). Many of the macrophages contained clear vacuoles and phagocytized cells. Type II pneumocytes were conspicuous, due

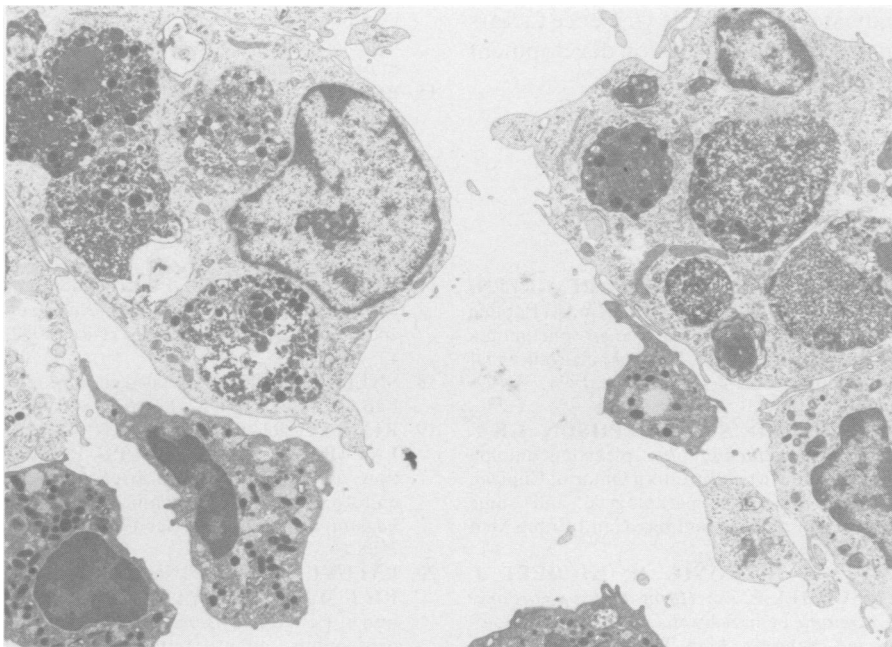


Fig. 3. Macrophages with numerous phagosomes in alveolar exudate. Phagosomes contain neutrophils and lamellar bodies. Uranyl acetate and lead citrate. X4,500.

to an apparent increase in size. The endothelial lining of pulmonary vessels was intact and did not appear swollen. Peribronchovascular and interlobular lymphatic vessels within and along the margins of lesions were mildly dilated and contained from a few to many reactive lymphocytes and lesser numbers of neutrophils and macrophages.

Ultrastructural examination revealed similar findings. The alveolar infiltrate was composed predominantly of neutrophils and macrophages (Fig. 3). Both cell types were nondegenerate and revealed evidence of active phagocytosis. Macrophages often contained from one to several neutrophils, lamellar bodies, and an occasional red blood cell. A few strands of fibrin were admixed with inflammatory cells in some but not all alveolar spaces examined. No abnormalities were observed in type II pneumocytes or endothelial cells.

## DISCUSSION

Fever, leukopenia followed by leukocytosis, and a positive LAL test in group I pigs demonstrate that endotoxin can be rapidly absorbed from the lung in an active form and can persist for at least 18 to 24 hours after exposure. Similar clinical findings have been described in pigs receiving continuous intravenous endotoxin (17) and in rabbits following exposure to aerosolized endotoxin (18). The lack of fever, absence of leukopenia, and a negative LAL test in group II pigs indicate that an endotoxemia was not induced by the methods utilized. However, the endotoxin of *H. pleuropneumonia* has not been fully characterized, and the endotoxin fractions of *Pasteurella haemolytica*, a closely related bacterium, vary in their solubility, pyrogenicity, and ability to cause gelation of LAL (19). It is important to note that the bacterium used in our experiment was in the logarithmic phase of growth. Endotoxin production during this phase of growth may be minimal or absent. Large amounts of endotoxin are produced by older cultures of *Pasteurella haemolytica* (20).

The hypothesis that endotoxin plays a significant role in PHP is based

on indirect evidence from an experiment in which bacteria isolated from a case of Glasser's disease and identified as *H. parainfluenzae* were disintegrated and injected intravenously into pigs, resulting in the generalized Shwartzman reaction (GSR) (21). A similar experiment using both intravenous and aerosol exposure of pigs to a sonicated suspension of *H. pleuropneumoniae* produced no lesions. The GSR was produced only by a sonicated suspension of *H. pleuropneumoniae* infected lung (6). Since both experiments involved intravenous injections of particulate matter (bacterial fragments or tissue factors and components), disseminated intravascular coagulopathy may have occurred.

A neutrophilic bronchitis and bronchiolitis was observed in group I and II animals in our experiment. The neutrophilic response in group I animals is consistent with findings obtained in other experiments in which guinea pigs (22) and rabbits (18) were exposed to aerosolized endotoxin. Hudson (22) suggested that endotoxin was responsible for neutrophil recruitment either by direct chemotactic activity or by interaction with epithelial cells to generate chemotactic factors, but also alluded to the possible role of complement which endotoxin can activate by both the classic and alternate pathways. Since endotoxin was not detected in group II animals, some component of the bacteria other than endotoxin was able to produce the neutrophilic response. Possible explanations for this response are the facts that bacterial cell walls can activate the alternative complement pathway and that particulate matter and microorganisms can stimulate alveolar macrophage secretion of a low molecular weight chemotactic factor for neutrophils (23).

Snell (18) reported a marked proliferation of alveolar macrophages in rabbits exposed to aerosolized endotoxin. When sonicated suspensions of *H. pleuropneumoniae* were used, increased numbers of alveolar macrophages were observed in our study and a similar experiment (24). Endotoxin has also been shown to mediate endothelial injury and to stimulate proliferation of endothelial

cells following intravenous injection (25). Endothelial injury has not been reported in the experiments using aerosolized endotoxin (18,22); however, the presence of extravasated red blood cells and fibrin indicates increased vascular permeability associated with the inflammatory reaction. The absence of ultrastructural lesions in type II pneumocytes in the present experiment is consistent with the findings of Hudson *et al* (22). The atelectasis observed in our experiment suggests an alteration of the surfactant system. Alveolar inflammatory edema inactivates existing surfactant (26).

Although endotoxin has been shown to be toxic to human alveolar macrophages *in vitro* (27), the macrophages in our experiment appeared viable and contained recently phagocytized material in addition to increasing in number. Failure to produce cytotoxicity *in vivo* with a sonicated suspension of *H. pleuropneumoniae* or *E. coli* endotoxin suggests that cytotoxicity in PHP may be due to the recently identified exotoxin of *H. pleuropneumoniae* which is released into the supernatant during the logarithmic phase of growth (7).

Characterization of *H. pleuropneumoniae* endotoxin and establishment of its role in PHP await further studies using purified endotoxin preparations. The findings of our study indicate that multiple virulence factors may be involved in lesion development in PHP.

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