

Low-Molecular-Weight Substance in Peritoneal Exudate Is  
Antibacterial at Febrile Temperature (41794)

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**Abstract.** Peritoneal exudate was collected from rabbits 18 hr after ip injection of shellfish glycogen. The fluid was centrifuged and the peritoneal exudate supernatant (PES) retained for use in growth studies. The growth of *Pasteurella multocida* in PES was inhibited at 41°C (febrile temperature for rabbits) as compared with 39°C (afebrile temperature), suggesting the presence of an antibacterial agent active at febrile temperature. Further studies indicated that this antibacterial substance has a molecular weight less than 5000 Da. Heat treatment (70°C, 1 hr) had no influence upon the activity of the inhibitory factor.

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Activated leukocytes release numerous substances, many of which directly or indirectly inhibit bacterial growth and proliferation. For example, lysozyme, a product of both macrophages and neutrophils, is capable of hydrolyzing the peptidoglycan of some bacterial cell walls (1, 2). Lactoferrin is thought to inhibit the growth of certain bacteria by chelating iron (3, 4), an essential component for the growth of several microorganisms. Cationic proteins and acid hydrolases are among other factors of leukocyte origin which demonstrate antimicrobial activities (5-7). Hence, an inflammatory fluid harboring activated leukocytes and leukocytic products creates a potentially hostile environment for an invading pathogen.

Elevated temperatures during infection have been shown to augment several host defense responses in both *in vivo* and *in vitro* studies (see reviews in 8-10). Evidence exists indicating that at temperatures normally encountered during infection, leukocyte mobility increases (11), the bactericidal activity of leukocytes is augmented (12), and lymphocyte transformation is accelerated or enhanced (13, 14). However, it is unknown whether febrile temperature also stimulates the antibacterial activity of factors released by stimulated leukocytes. Because of the dramatic infiltration of leukocytes into the peritoneal cavity following injection of shellfish glycogen or other chemotactic agents, we chose this peritoneal fluid to test for such a relationship. Peritoneal fluid provides large quantities of stimulated leukocytes and leukocytic products. We have tested the antibacterial activity of this inflam-

matory fluid at temperatures simulating normal and febrile temperatures for rabbits.

**Materials and Methods.** *Peritoneal exudate.* Male New Zealand White rabbits (2.5-3.5 kg) were anesthetized (10 mg/kg xylazine and 35 mg/kg ketamine HCl), and injected intraperitoneally with 420 ml of 0.2% shellfish glycogen in sterile pyrogen-free 0.9% sodium chloride. After 18 hr, peritoneal exudate was collected aseptically, and centrifuged at 900g for 20 min to yield a cell-free supernatant. The supernatant was filtered through Millipore 0.22- $\mu$ m filters to remove bacteria, and samples cultured on blood agar plates at 37°C to assess for contamination. Once it was demonstrated that the supernatant was free of bacterial contamination, exudate supernatant obtained from four to six rabbits was pooled to eliminate individual variations in exudate content. This pooled peritoneal exudate supernatant (PES) was used as a crude preparation or ultrafiltered as described below. (Please note that all future references to PES refer to pooled PES from four to six rabbits rather than from an individual animal.)

*Ultrafiltration of PES.* Crude PES was filtered through Diaflo (Amicon) ultrafiltration membranes (YM5, 5000-mol-wt cutoff, 76-mm diameter). The membranes were utilized in a stirred cell (450-ml volume) with compressed nitrogen (70 psi) as the pressure source. Both filtrate and retentate were retained after ultrafiltration of peritoneal supernatant.

*Heat treatment.* In order to test PES for heat lability, volumes of crude and ultrafiltered PES were placed in pyrogen-free glassware, and subject to heat treatment in an oven set

at 70°C for 1 hr. The resulting heat-treated PES was employed in growth studies outlined below.

**Concentration of PES.** Three-hundred-milliliter vol of PES filtrate (less than 5000 Da) were lyophilized and reconstituted in 30 ml of pH 7.4 phosphate-buffered saline (Sigma), to yield a 10-fold concentration.

**Bacteria.** The *Pasteurella multocida* strain selected for this study was kindly provided and serotyped by Dr. Rimler and Dr. K. Rhoades, National Animal Disease Center, Ames, Iowa and Dr. L. Spanoghe, Laboratory for Microbiology and Infectious Diseases, Rijksuniversiteit, Gent, Belgium. This strain, designated P.3770, serotype 3,12,15:D, was obtained from a rabbit source; and will produce subcutaneous abscesses and pneumonia in infected rabbits.

**Growth studies.** *Pasteurella multocida* ( $1.5 \times 10^7$  cells/ml,  $\log_{10} = 7.2$ ) was suspended in 10-ml vol of crude, heat-treated or ultrafiltered PES. Growth studies were also performed using rabbit plasma as the growth medium. To obtain this plasma, rabbits were anesthetized with xylazine and ketamine HCl, and exsanguinated by cardiac puncture. Blood was collected in heparinized syringes, followed by centrifugation at 430g to remove cellular

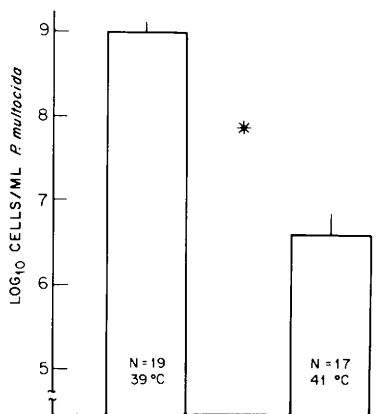


FIG. 1. Effect of crude peritoneal exudate supernatant (PES) on the growth of *P. multocida* at 39 and 41°C. Log<sub>10</sub> viable cells/ml *P. multocida* after an 8-hr incubation. *N* = the number of individual growth studies performed for each set of experimental conditions. Value = mean  $\pm$  1 SE. \**P* < 0.001. Growth medium: crude peritoneal exudate supernatant (PES). At time = 0, log<sub>10</sub> cells/ml = 7.2.

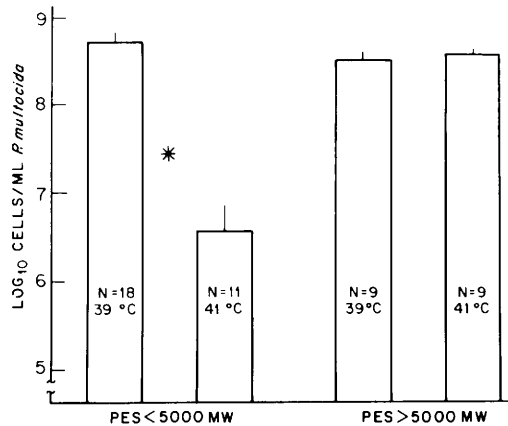


FIG. 2. Effect of ultrafiltration on the antibacterial action of PES. Log<sub>10</sub> viable cells/ml *P. multocida* after an 8-hr incubation. *N* = the number of individual growth studies performed for each set of experimental conditions. Value = mean  $\pm$  1 SE. \**P* < 0.001. Growth medium: PES < 5000 mol wt and PES > 5000 mol wt. At time = 0, log<sub>10</sub> cells/ml = 7.2.

components. Cultures were incubated at 39 and 41  $\pm$  1°C (afebrile and febrile temperatures for rabbits) in shaking water baths (Forma Scientific, modified with RFL Industries proportional temperature controllers) for 8 hr. Temperature was monitored by continual recording on Honeywell Elektronik 112 recorders. At the conclusion of the incubation period, samples were plated on blood agar and viable bacterial cell counts determined.

**Statistics.** Values reported are means  $\pm$  1 SE. Data were analyzed for statistical significance using Student's *t* test, or one-way analysis of variance with Scheffe allowances.

**Results.** Initial growth studies using crude peritoneal exudate supernatant (PES) resulted in a statistically significant (*P* < 0.001) reduction in growth (greater than 2 log units) by 8 hr at febrile temperature (41°C), as compared to afebrile temperature (39°C) (Fig. 1).

To determine the apparent molecular-weight range for the inhibitory factor(s) present in PES active at 41°C, PES was ultrafiltered into greater than and less than 5000-Da fractions. *Pasteurella multocida* grown in PES retentate (>5000 Da) at 39 and 41°C reached final log concentrations of  $8.51 \pm 0.11$  and  $8.57 \pm 0.03$ , respectively (Fig. 2). In contrast, in studies in which *P. multocida* was grown

in PES filtrate (<5000 Da) at 39 and 41°C, 8-hr log concentrations were  $8.70 \pm 0.08$  and  $6.54 \pm 0.28$ , respectively (Fig. 2). These data suggest that the inhibitory factor(s) active at 41°C reside in the less than 5000-Da fraction of PES.

To assess the heat lability of the antibacterial substance(s) in PES, PES fractions (both greater than and less than 5000 Da) were subject to heat treatment (70°C, 1 hr) prior to use as a growth medium. Heat treatment at this temperature did *not* abolish the inhibitory activity observed at 41°C in growth studies with PES filtrate (<5000 Da); nor did such treatment reduce the capacity of PES to support growth of *P. multocida* at afebrile temperature (Fig. 3). The heat treatment actually augmented the antibacterial action of PES filtrate (<5000 Da) at 41°C ( $P < 0.005$ ).

Growth studies were conducted using rabbit plasma as media to which concentrated PES or control saline had been added, to determine whether the inhibitory factor(s) present in PES would exhibit antibacterial activity when added to a nutritive growth medium. The addition of concentrated PES less than 5000 Da to rabbit plasma resulted in a significant difference ( $P < 0.01$ ) in the growth of *P. multocida* at both 39 and 41°C respective to their controls (Fig. 4). A greater reduction in growth

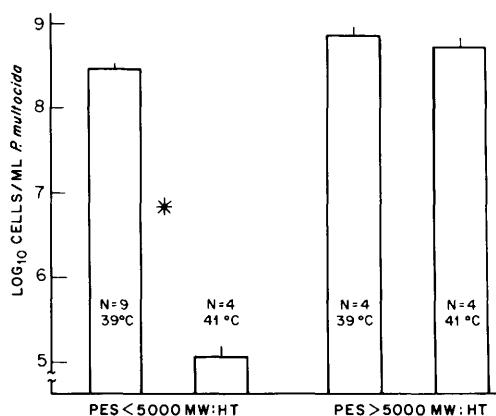


FIG. 3. Effect of heat treatment on the antibacterial activity of PES. Log<sub>10</sub> viable cells/ml *P. multocida* after an 8-hr incubation.  $N$  = the number of individual growth studies performed for each set of experimental conditions. Value = mean  $\pm$  1 SE. \* $P < 0.001$ . Growth medium: heat treated PES < 5000 mol wt and heat treated PES > 5000 mol wt. At time = 0, log<sub>10</sub> cells/ml = 7.2.

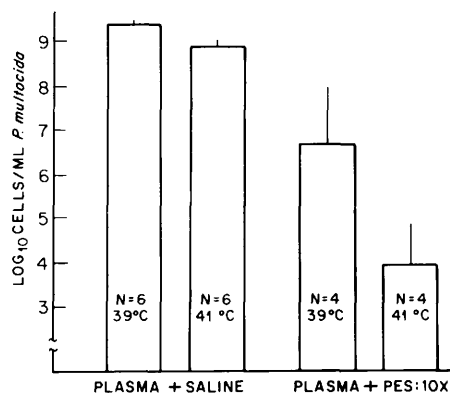


FIG. 4. Effect of concentrated PES on the growth of *P. multocida* in rabbit plasma. Log<sub>10</sub> viable cells/ml *P. multocida* after an 8-hr incubation.  $N$  = the number of individual growth studies performed for each set of experimental conditions. Value = mean  $\pm$  1 SE. Growth medium: rabbit plasma + saline, and rabbit plasma + PES-lyophilized concentrate (PES:10X). At time = 0, log<sub>10</sub> cells/ml = 7.2.

of *P. multocida* in rabbit plasma with concentrated PES was observed at 41°C as compared to 39°C, however, this difference was not statistically significant.

**Discussion.** We conclude that a low-molecular-weight substance is released into the peritoneal fluid of rabbits (following stimulation with shellfish glycogen) which inhibits the growth of *P. multocida* at temperature simulating fever (41°C). Previous studies have shown that the *in vitro* growth of *P. multocida* (ATCC No. 7228) is not directly affected by temperatures in the range of 39 to 42°C (15, 16). Further, in the present study, a comparison of *P. multocida* (P.3770) cultured at 39 and 41°C in plasma + saline (Fig. 4) again shows that there is no direct effect of temperature upon the growth of this organism. Because the presence of shellfish glycogen in the peritoneal cavity has such a marked effect upon leukocyte stimulation and infiltration, we postulate that this inhibitory factor is a product of activated leukocytes. Based upon ultrafiltration experiments, the molecular weight of the antibacterial factor in PES is less than 5000 Da. This substance is markedly smaller in size than other antibacterial agents of leukocytic origin such as lactoferrin and lysozyme. We have demonstrated that the substance in PES active against *P. multocida*

remains potent and might even increase its antibacterial activity at 41°C following heat treatment (70°C, 1 hr). Alternatively, heat treatment may abolish a factor that facilitates growth at 41°C. It remains to be seen whether this factor(s) will retain its inhibitory activity when heated to temperatures approaching 100°C.

These data generate many questions, several of which are subjects of current investigation which include determining: (i) the exact cell source of this factor (can it be isolated from peripheral monocytes or neutrophils?), (ii) the biochemical characteristics (protein vs non-protein, apparent molecular weight based upon gel filtration etc.) of the factor, and (iii) the activity of this inhibitory factor upon other microorganisms, both gram positive and negative.

Temperatures simulating fever seem to have a beneficial influence upon several host resistance mechanisms. The results of this study suggest a permissive or synergistic relationship between temperature and the antibacterial activity of a low-molecular-weight substance found in peritoneal exudate.

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