

# Stimulation by Fibronectin of Macrophage-Mediated Phagocytosis of *Pseudomonas aeruginosa*

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In a previous investigation it was determined that *Pseudomonas aeruginosa* cells taken directly from a mouse *in vivo* growth system were significantly more susceptible to nonopsonic phagocytosis by macrophages than were similar cells after being washed in buffer (N. M. Kelly, J. L. Battershill, S. Kuo, J. P. Arbuthnott, and R. E. W. Hancock, *Infect. Immun.* 55:2841-2843, 1987). It was demonstrated that a phagocytosis-promoting factor was found in the supernatant obtained from chambers incubated in the peritoneal cavities of laboratory mice or rats. The phagocytosis-promoting factor was effective with both strains of *P. aeruginosa* tested, using both unelicited mouse peritoneal macrophages and the P388<sub>D1</sub> mouse macrophage cell line as the phagocytic cells. Phagocytosis enhancement was observed with *in vivo*-grown bacteria and with bacteria grown *in vitro* on agar plates, but not with bacteria grown *in vitro* with rapid agitation. Supernatants from mice and rats were fractionated using a fast pressure liquid chromatography gel exclusion column. The phagocytosis-promoting factor copurified with fibronectin. Furthermore, antifibronectin sera negated the phagocytosis-promoting activities of *in vivo* chamber supernatant, while commercial bovine fibronectin was itself capable of promoting phagocytosis. The concentrations of fibronectin increased in both rat and mouse peritoneal chambers with time, coincident with the ability of chamber supernatants to promote phagocytosis. It was concluded that fibronectin was the phagocytosis-promoting factor of chamber supernatants. Bacterial presence in the peritoneal chambers was not required to elicit fibronectin uptake into the chambers.

*Pseudomonas aeruginosa* is an opportunistic pathogen capable of causing life-threatening disease in immunocompromised individuals. Those especially at risk include patients with severe burns, cancer, diabetes, or cystic fibrosis. In the case of some patients with cystic fibrosis, initial *P. aeruginosa* infections can be suppressed after antibiotic treatment (5). This may involve not only antibiotic action but also non-immune clearance mechanisms. These latter mechanisms encompass both the mucociliary system and nonopsonic phagocytosis by pulmonary alveolar macrophages (18). Nonopsonic phagocytosis involves association of macrophages and bacteria in the absence of external opsonins. This mode of phagocytosis has been considered to be a relatively inefficient process (1) and thus relatively unimportant to the clearance of this organism.

On repeated infection with *P. aeruginosa*, lung function of an individual with cystic fibrosis rapidly deteriorates, and bacteria are less easily suppressed. Thus, it seemed important to understand the mechanisms by which macrophages initially interact with *P. aeruginosa*. These phagocytes are often the first host immune cells to infiltrate sites of infection, have been proposed to be a primary line of defense against lung infection (16), and play a dual role in bacterial clearance by interacting directly with bacterial cells and releasing potent immunomodulators (17). In addition, they are involved in antigen presentation to T cells as part of antigen recognition in the cellular and humoral immune responses.

In a previous investigation, it was determined that *P. aeruginosa* cells taken directly from an *in vivo* growth system were significantly more susceptible to phagocytosis by mouse macrophage cell line P388<sub>D1</sub> than were identical *P. aeruginosa* cells after being washed in buffer (8). The data presented in this paper demonstrate that the factor involved

in promoting the phagocytosis of *in vivo*-grown bacteria was present in the supernatant removed from these cells by centrifugation. We present studies which identified the factor as fibronectin, and we examine the bacterial and phagocyte requirements for phagocytosis promotion by fibronectin.

## MATERIALS AND METHODS

**Bacterial strains and *in vitro* growth conditions.** *P. aeruginosa* PAO1 strain H103, a laboratory serotype 5 isolate (11), and M2, a strain traditionally used for mouse pathogenicity studies (20), were maintained on Trypticase soy agar (Becton, Dickinson & Co., Cockeysville, Md.). Before assay, *in vitro* cells were inoculated from these plates and grown for 20 h, shaken vigorously (200 rpm), in Trypticase soy broth or for 20 h on Trypticase soy agar at 37°C. These stationary-phase cells, with an optical density at 600 nm greater than 2.0, were washed in phosphate-buffered saline (PBS) (pH 7.2) and suspended to a concentration of 10<sup>9</sup>/ml before experimentation.

**Bacterial growth *in vivo*.** Chambers for implantation into mice were constructed from 1-ml polypropylene syringe barrels as previously described (4). Chambers for implantation into rats were similarly constructed from 3-ml polypropylene syringe barrels (9). The *P. aeruginosa* culture used to inoculate the chambers was grown overnight in proteose peptone no. 2 broth (Difco Laboratories, Detroit, Mich.) and diluted in physiological saline to give a chamber inoculum of approximately 10<sup>4</sup> bacteria per ml. Chambers for implantation into mice received a volume of 100 µl of the diluted culture, whereas chambers for rats received 500 µl. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (Somnotol; M.T.C. Pharmaceuticals, Mississauga, Ontario, Canada) at 0.06 mg/g of body weight for mice and 0.14 mg/g for rats. Chambers were implanted, four per animal, through a small longitudinal incision in the

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abdomen of the animal. The chambers were removed after 3 days, at which stage the bacterial cultures had reached their maximal density of  $10^8$  to  $10^9$  cells per ml (9).

Unwashed in vivo-grown *P. aeruginosa* cells were counted in a Petroff-Hausser bacterial counting chamber (Hausser Scientific, Blue Bell, Pa.) and maintained on ice until used. Washed organisms were centrifuged at  $12,000 \times g$  and gently suspended in PBS twice before assay. The first decanted supernatant from these cells was saved for assessment of phagocytosis enhancement (in vivo supernatant).

**Maintenance of macrophage cell types.** Mouse macrophage cell line P388<sub>D1</sub> was maintained at 37°C in 10% CO<sub>2</sub> in Nunc flat-bottomed flasks (GIBCO, Burlington, Ontario, Canada) using RPMI 1640 medium (GIBCO) supplemented with 44 mM sodium bicarbonate (Fisher Scientific, Vancouver, B.C., Canada), 10% fetal calf serum (GIBCO), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Terochem Laboratories, Vancouver, B.C., Canada), 0.4% (vol/vol) 2-mercaptoethanol (Bio-Rad, Mississauga, Ontario, Canada), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo.), 40 U of penicillin per ml, and 40 µg of streptomycin (GIBCO) (pH 7.2) per ml. Before assay, macrophages were grown for 16 h in Nunclon petri dishes (35 by 10 mm; GIBCO) at a concentration of  $10^6$  cells per dish. Nonadherent cells were removed by gently washing the monolayer with unsupplemented RPMI 1640 (phagocytosis buffer).

Unelicited mouse peritoneal macrophages were obtained from female BALB/c mice by peritoneal lavage as described previously (3). Cells were 67.1% macrophages, 32.5% erythrocytes, and 0.4% granulocytes as determined by Diff-quick (Canlab, Vancouver, B.C., Canada) staining and visual inspection. Macrophages were separated from erythrocytes by centrifugation at 1,000 rpm (Silencer Centrifuge; Western Scientific Co., Vancouver, British Columbia, Canada) for 10 min and were suspended in supplemented RPMI 1640 medium. Macrophages were maintained in samples of  $10^6$  cells for 16 h at 37°C in 10% CO<sub>2</sub> in the Nunclon petri dishes. Before assay, nonadherent cells were removed as described above.

**Phagocytosis assay.** The visual assay for phagocytosis of *P. aeruginosa* was performed as described previously (3). Briefly, 1 ml of phagocytosis buffer was added to a washed macrophage monolayer to give a final concentration of  $10^6$  cells per ml. To assess enhancement of phagocytosis, PBS, in vivo supernatant (100 µl), or 100 µg of bovine plasma fibronectin (Sigma) was added to the assay volume. No additional opsonins or macrophage activators were included in the system. Bacterial cells were used at a *P. aeruginosa*-to-macrophage ratio of 20:1, and phagocytosis was allowed to occur for 90 min in 10% CO<sub>2</sub> at 37°C. Uptake was assessed visually after Diff-quick staining. Speert et al. (19) previously showed that results obtained with this visual phagocytosis assay gave data comparable to the findings of chemiluminescence and electron microscopy studies.

When utilized, goat anti-human fibronectin serum (Sigma) was incubated with supernatant or fibronectin for 15 min at room temperature before addition to the macrophage monolayer. Antibody was used at the recommended ratio of 1 µg/µg of fibronectin.

**Characterization of the phagocytosis-promoting factor.** To characterize the phagocytosis-promoting factor, in vivo supernatant was passed over a fast pressure liquid chromatography (FPLC) Superose 12 gel sieving column (Pharmacia, Dorval, Quebec, Canada). The flow rate was 30 ml/h, and elution buffer consisted of 20 mM Tris (ICN Biomedicals,

TABLE 1. Enhancement of the association of in vivo-grown *P. aeruginosa* M2 with P388<sub>D1</sub> cells, using supernatant from mouse chambers

Treatment of in vivo-grown M2	Addition to bacteria	No. of bacteria associated per macrophage
Unwashed	PBS	9.4 ± 3.8 <sup>a</sup>
Washed	PBS	2.9 ± 1.4
Washed	100% in vivo supernatant	7.7 ± 3.4 <sup>b</sup>
Washed	75% in vivo supernatant	6.1 <sup>c</sup>
Washed	50% in vivo supernatant	4.3 <sup>d</sup>
Washed	25% in vivo supernatant	3.8 <sup>d</sup>

<sup>a</sup>  $P < 0.005$  (by Student's *t* test) in eight of eight assays as compared to the washed + PBS control.

<sup>b</sup>  $P < 0.005$  in seven of eight assays and  $P < 0.1$  in one of eight assays as compared to the washed + PBS control.

<sup>c</sup>  $P < 0.005$  in the one assay performed, as compared to the washed + PBS control.

<sup>d</sup> Not significantly greater than the washed + PBS control.

Cleveland, Ohio)—0.1 M NaCl (BDH Chemicals, Toronto, Ontario, Canada) (pH 7.5). Fractions were collected, lyophilized, suspended in distilled water, and dialyzed extensively against distilled water. The final suspension volume was the same as the volume of supernatant initially added to the column.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7) and Western immunoblotting techniques (10) were performed as described previously. For Western immunoblots, 100 µg of antifibronectin antibody was utilized per blot.

## RESULTS

**Enhancement of phagocytosis by in vivo supernatant.** In a previous investigation it was determined that *P. aeruginosa* M2 cells taken directly from an in vivo system were significantly more susceptible to phagocytosis by mouse macrophage cell line P388<sub>D1</sub> than were identical *P. aeruginosa* cells that had been centrifuged and suspended in buffer (8). *P. aeruginosa* cells were grown for 3 days in the peritoneal cavity of laboratory mice. Bacteria were contained in 1-cm-long plastic chambers, sealed at both ends with 0.22-µm-pore-size membrane filters (4), allowing free exchange of peritoneal fluids and bacterial products while prohibiting access by immune cells or escape of bacteria. Upon removal of chambers, bacteria were separated from the fluid in the chamber by centrifugation. This decanted fluid was called the in vivo supernatant.

Initially, we wished to determine whether the decreased phagocytosis of washed in vivo-grown bacteria was related to effects of the washing procedure on the organisms or was due to removal of some phagocytosis-promoting factor in the in vivo supernatant. To determine whether in vivo supernatant could be added back to the washed bacteria and still facilitate uptake of bacteria by macrophages, a visual assay of phagocytosis was performed (3). Mouse in vivo-grown M2 cells and mouse macrophage cell line P388<sub>D1</sub> were used in these initial studies to provide continuity with the previous study.

It was observed that there was indeed a phagocytosis-promoting factor obtained from in vivo chambers, which could be separated from the bacteria easily by centrifugation at  $12,000 \times g$  for 10 min and added back to again facilitate

TABLE 2. Enhancement of the association of *P. aeruginosa* strains M2 and H103 with unelicited mouse peritoneal macrophages and the P388<sub>D1</sub> macrophage cell line, using in vivo supernatant from rat chambers

Macrophage cells	Bacterial cells <sup>a</sup>	No. of bacteria associated per phagocyte		
		Unwashed bacteria	Washed bacteria + PBS	Washed bacteria + rat in vivo supernatant
P388 <sub>D1</sub>	In vivo-grown M2	7.3 ± 3.3 <sup>b</sup>	3.0 ± 2.3	10.6 ± 3.7 <sup>b</sup>
P388 <sub>D1</sub>	In vivo-grown H103	9.0 ± 3.5 <sup>b</sup>	2.2 ± 1.8	6.6 ± 1.8 <sup>b</sup>
P388 <sub>D1</sub>	In vitro-grown H103		5.8 ± 2.3	13.9 ± 5.1 <sup>b</sup>
Mouse peritoneal macrophages	In vitro-grown H103		2.1 ± 1.0	7.0 ± 3.1 <sup>b</sup>

<sup>a</sup> In vitro bacteria were grown on Trypticase soy agar plates; in vivo bacteria were grown in peritoneal chambers in rats.

<sup>b</sup>  $P < 0.005$  (by Student's *t* test) in three of three assays performed, as compared to the washed + PBS control.

bacterial association with macrophages (Table 1). At a bacteria-to-macrophage ratio of 20 to 1, an average of 2.9 washed bacteria became associated per macrophage as compared to 7.7 bacteria taken up in the presence of in vivo supernatant. This difference was found to be statistically significant in seven of eight assays performed ( $P < 0.005$ , Student's *t* test) and marginally significant ( $P < 0.1$ ) in the other assay. This phagocytosis-enhancing factor was titerable in this system, as decreasing amounts of added supernatant resulted in progressively decreasing levels of bacterial uptake (Table 1).

To determine whether these phenomena were restricted to the mouse in vivo system or to this particular strain of *P. aeruginosa*, the same studies were carried out using rat-grown bacteria. Almost identical results were obtained in the rat model, utilizing both strain M2 and laboratory wild-type strain H103 (Table 2). Additionally, rat supernatant from in vivo chambers could effectively promote uptake of in vitro-grown strain H103 on Trypticase soy agar (Table 2). Similar results were obtained using in vitro-grown strain H103 and unelicited mouse peritoneal macrophages. In this case, addition of rat in vivo supernatant increased bacterial association from 2.1 to 7.0 bacteria per phagocyte. These differences were found to be statistically significant in all assays performed ( $P < 0.005$ , Student's *t* test). It was thus evident that the mouse macrophage cell line P388<sub>D1</sub> could be used as a model for unelicited mouse peritoneal macrophages in this system.

Thus, the remainder of our studies centered on measuring promotion of association of in vitro-grown strain H103 with P388<sub>D1</sub> cells. This particular system was chosen because of the relative ease of working with in vitro-grown organisms. However, it was necessary to determine whether growth conditions affected in vivo supernatant-promoted phagocytosis. Strain H103 was grown either in rapidly agitated (200

rpm) Trypticase soy broth or on a plate of Trypticase soy agar. Although uptake of agitated bacteria could not be promoted by using in vivo supernatant from rat peritoneal chambers, phagocytosis of plate-grown organisms was significantly enhanced (Table 3). Thus, strain H103 cells grown

TABLE 3. In vitro growth conditions affecting the ability of in vivo supernatant to enhance association of *P. aeruginosa* H103 with P388<sub>D1</sub> cells

H103 growth condition <sup>a</sup>	Addition to bacteria	No. of bacteria associated per macrophage
Agitated broth (TSB)	PBS	8.2 ± 0.3
Agitated broth (TSB)	Rat chamber supernatant	7.2 ± 0.4 <sup>b</sup>
Plate (TSA)	PBS	5.9 ± 2.2
Plate (TSA)	Rat chamber supernatant	13.9 ± 4.3 <sup>c</sup>

<sup>a</sup> TSB, Trypticase soy broth; TSA, Trypticase soy agar.

<sup>b</sup> Not significantly different from the PBS control in three of three assays performed (Student's *t* test).

<sup>c</sup>  $P < 0.005$  in three of three assays as compared to the PBS control.

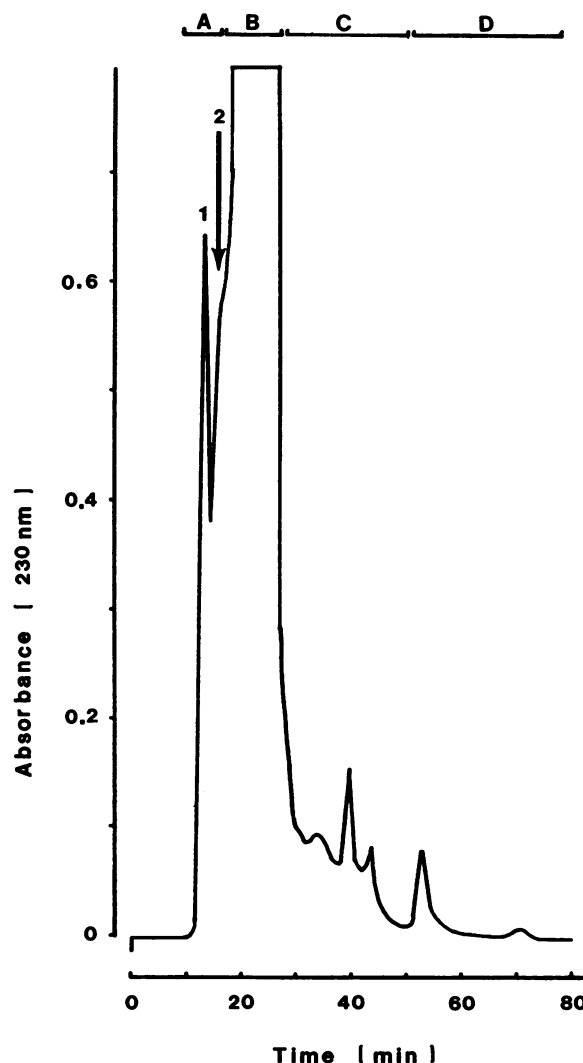


FIG. 1. FPLC gel sieving fractionation of in vivo supernatant from rat chambers. The elution profile at an absorbance at 230 nm is shown. Peaks were collected in four pools (A through D). Peaks 1 and 2 of pool A are labeled.

TABLE 4. Enhancement of the association of *P. aeruginosa* H103 with P388<sub>D1</sub> cells, using pooled fractions collected from an FPLC gel sieving fractionation of in vivo supernatant from rat chambers

Addition	No. of bacteria associated per macrophage
PBS.....	5.0 ± 0.4
In vivo supernatant.....	13.5 ± 1.4 <sup>a</sup>
Pool A supernatant.....	10.5 ± 1.9 <sup>b</sup>
Pool B supernatant.....	8.5 ± 5.7 <sup>c</sup>
Pool C supernatant.....	4.2 ± 0.9 <sup>d</sup>
Pool D supernatant.....	5.6 ± 0.1 <sup>d</sup>
100°C-treated supernatant.....	16.4 ± 9.3 <sup>b</sup>

<sup>a</sup>  $P < 0.005$  (by Student's *t* test) in 11 of 11 assays performed, as compared to the PBS control.

<sup>b</sup>  $P < 0.005$  in two of two assays as compared to the PBS control.

<sup>c</sup>  $P < 0.005$  in one of two assays as compared to the PBS control;  $P > 0.5$  in the other assay.

<sup>d</sup> Not significantly different from the PBS control in two of two assays.

on Trypticase soy agar plates were used for subsequent studies.

**Characterization of the phagocytosis-promoting factor.** To further characterize the phagocytosis-promoting factor, in vivo supernatant was fractionated on an FPLC Superose 12 gel sieving column, and peaks were collected in four pools (Fig. 1). After extensive dialysis and concentration, the phagocytosis-promoting activity of these pools was assessed. It was observed that pool A strongly enhanced phagocytosis, pool B was less effective, and pools C and D were ineffective (Table 4).

These data established that the factor was of high molecular weight. Thus, we considered three possible candidates for the phagocytosis-promoting factor, namely, antibody, complement, and fibronectin, each of which had been previously shown to promote phagocytosis (14). The ease of dissociation of the factor from cells (by centrifugation and washing) and the use of naive mice in our experiments seemed to rule out antibody. In addition, the activity was stable to heating to 100°C for 10 min (Table 4), thus ruling out complement, which is inactivated at this temperature. Therefore we tested for the presence of fibronectin by using a commercially available anti-human fibronectin antibody preparation. Use of this antiserum was possible owing to the strong evolutionary and antigenic conservation of fibronectin (12). By Western immunoblotting with goat anti-human fibronectin antiserum, in vivo supernatant and pool A reacted strongly, pool B reacted less strongly, and pools C and D failed to react. Thus the order of reactivity matched the ability to enhance phagocytosis (Table 4). Pool A was partially resolved by FPLC into two peaks, 1 and 2 (Fig. 1). Fibronectin was present in peak 2 (Fig. 2, lane 2) but not peak 1 (Fig. 2, lane 1). Small amounts of fibronectin were detectable in pool B (Fig. 2, lane 3) but none in pools C or D. This suggested that the fibronectin content, and thus the phagocytosis-promoting activity of pool B, was probably due to incomplete separation of peak 2 and pool B.

To confirm fibronectin as the phagocytosis-promoting factor, antifibronectin serum was incubated with in vivo supernatant for 15 min before phagocytosis assays. The antiserum significantly reduced, to the level of the PBS control, the phagocytosis-promoting ability of the in vivo supernatant (Table 5).

A commercially available preparation of bovine plasma fibronectin was tested for phagocytosis-promoting activity at 100 µg/ml. This concentration of fibronectin increased bac-

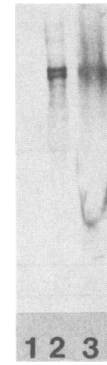


FIG. 2. Western immunoblots of fractions collected from an FPLC gel sieving fractionation of in vivo supernatant from rat chambers probed with goat anti-human fibronectin serum. Lane 1, FPLC peak 1; lane 2, FPLC peak 2; lane 3, FPLC pool B.

terial association with P388<sub>D1</sub> cells from 5.2 to 14.1 bacteria per macrophage (Table 5). If fibronectin was incubated with antifibronectin prior to assay, phagocytosis was not significantly enhanced (Table 5). In further experiments, it was determined that concentrations as small as 12 µg of fibronectin per ml resulted in enhancement of phagocytosis.

**Requirement for bacteria.** To determine whether bacteria must be present to allow production or possibly activation of this factor, saline-containing chambers were incubated in vivo, and their fluid contents were assessed for phagocytosis promotion. It was observed that saline chamber supernatants from both mice and rats were indeed capable of enhancing phagocytosis of in vitro-grown *P. aeruginosa* H103 from 4.5 to 24.1 and 11.5 bacteria per macrophage, respectively (Table 6). The phagocytosis-promoting factor in saline-containing chamber supernatants was apparently fibronectin, since antifibronectin antibodies significantly reduced the ability of these supernatants to promote phagocytosis (data not shown).

To establish the time at which the phagocytosis-promoting factor appeared in vivo, bacteria- and saline-containing chambers were incubated in the peritoneums of mice and rats for 4, 8, 16, 24, 44, and 68 h. Supernatants from these chambers were tested for phagocytosis enhancement. In both saline and bacterial chambers, phagocytosis-promoting activity appeared within 4 h in mice and within 16 h in rats (Table 6). The degree of phagocytosis enhancement did not differ significantly between bacteria-containing and saline-containing chambers in either animal system, although

TABLE 5. Antifibronectin serum inhibition of phagocytosis-promoting activities of bovine fibronectin and of in vivo supernatant from rat peritoneal chambers

Addition to assay	No. of bacteria associated per P388 <sub>D1</sub> cell	
	No antifibronectin serum	With antifibronectin serum
PBS	5.2 ± 0.7	Not done
In vivo supernatant	12.2 ± 1.5 <sup>a</sup>	6.5 ± 1.8 <sup>b</sup>
100 µg of bovine fibronectin	14.1 ± 4.6 <sup>a</sup>	5.5 ± 2.4 <sup>b</sup>

<sup>a</sup>  $P < 0.005$  (Student's *t* test) in all assays performed as compared to the PBS control.

<sup>b</sup> Not significantly different from the PBS control in both assays (fibronectin) and in all four assays (in vivo supernatant), respectively;  $P < 0.005$  as compared to the assay in the absence of antifibronectin serum.

TABLE 6. Time course of emergence of phagocytosis-promoting activity in rat and mouse peritoneal chambers containing strain H103 or saline

Time (h) of chamber implantation <sup>a</sup>	No. of bacteria associated per macrophage, using supernatant of chambers from:			
	Mice		Rats	
	Bacteria-containing chambers	Saline-containing chambers	Bacteria-containing chambers	Saline-containing chambers
0 (PBS control)	4.5			
4	18.4 <sup>b</sup>	20.2 <sup>b</sup>	3.3	2.9
8	21.6 <sup>b</sup>	24.1 <sup>b</sup>	4.6	2.6
16	24.7 <sup>b</sup>	26.3 <sup>b</sup>	7.4 <sup>b</sup>	6.8 <sup>b</sup>
24	22.3 <sup>b</sup>	23.4 <sup>b</sup>	11.8 <sup>b</sup>	7.5 <sup>b</sup>
44	25.3 <sup>b</sup>	21.8 <sup>b</sup>	12.0 <sup>b</sup>	10.8 <sup>b</sup>
68	25.5 <sup>b</sup>	24.1 <sup>b</sup>	13.4 <sup>b</sup>	11.5 <sup>b</sup>

<sup>a</sup> Time between implantation of chambers in animals and removal for harvesting of supernatant.

<sup>b</sup>  $P < 0.005$  (by Student's *t* test) compared to the PBS control; all other data not significantly different.

mouse supernatants were substantially more active than their rat counterparts.

The emergence of fibronectin in these chambers correlated well with the ability to enhance bacterial association with P388<sub>D1</sub> cells. Fibronectin was not detectable in Western blots of rat 4-h supernatants but was easily seen at 24 h (Fig. 3). In mice, however, this protein was found at a substantial concentration at all time points (Fig. 3). By comparison with the intensity of staining on Western immunoblots of a standard fibronectin preparation (Fig. 3, lane 13), we could estimate that the concentration of fibronectin in the chambers reached levels as high as or higher than 1 mg/ml (i.e., 100 µg per mouse chamber, 500 µg per rat chamber).

### DISCUSSION

The data in this paper demonstrated that a phagocytosis-promoting factor was found in the supernatant obtained from

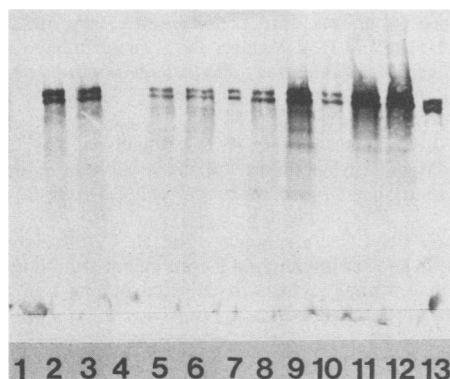


FIG. 3. Western immunoblots after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the supernatants of bacteria- and saline-containing chambers that had been incubated in the peritoneums of mice and rats for 4, 24, and 68 h, probed with goat anti-human fibronectin serum. Lanes 1, 2, and 3, Supernatants from bacterial chambers incubated in rat peritoneal cavities for 4, 24, and 68 h, respectively; lanes 4, 5, and 6, supernatants from saline chambers incubated in rat peritoneal cavities for 4, 24, and 68 h, respectively; lanes 7, 8, and 9, supernatants from bacterial chambers incubated in mouse peritoneal cavities for 4, 24, and 68 h, respectively; lanes 10, 11, and 12, supernatants from saline chambers incubated in mouse peritoneal cavities for 4, 24, and 68 h, respectively; lane 13, 12 µg of purified bovine fibronectin.

*P. aeruginosa* chambers incubated in the peritoneal cavities of laboratory mice and rats. This factor could be separated easily from bacteria by centrifugation and then added back to again facilitate association of *P. aeruginosa* with mouse unelicited peritoneal macrophages or macrophage cell line P388<sub>D1</sub> (Tables 1 and 2). The ease of removal of this factor suggested that it was not an opsonin such as antibody or complement, which typically have very high binding affinities and thus remain attached to cells during centrifugation. Furthermore, treatment of *in vivo* supernatant at 100°C for 10 min failed to affect its ability to enhance phagocytosis (Table 4). This treatment would have inactivated any complement or antibody proteins present.

This phenomenon was reproduced with two *P. aeruginosa* strains of different serotypes, using either mice or rats as the *in vivo* chamber host. Additionally, rat *in vivo* supernatant could effectively promote uptake of plate-grown bacteria by P388<sub>D1</sub> cells or unelicited mouse peritoneal macrophages. The similar results obtained with these two macrophage cell types confirmed that mouse macrophage cell line P388<sub>D1</sub> is an appropriate model for unelicited mouse peritoneal macrophages, as previously demonstrated in studies of opsonized phagocytosis of *P. aeruginosa* (3).

The fact that fibronectin copurified with phagocytosis-promoting activity on a gel sieving column suggested to us that fibronectin might be the factor in question. Fibronectin has been shown previously to activate macrophages for increased adherence (2), C3 and Fc receptor-mediated phagocytosis of coated erythrocytes (13, 22), and maintenance of antistaphylococcal activity (15). However, no reports of its effects on nonopsonic phagocytosis or on phagocytosis of a gram-negative bacterium have appeared to date. In our studies, the phagocytosis-promoting activity of a commercially available fibronectin preparation and the negating effects of antifibronectin antibodies (Table 5) strongly supported the conclusion that fibronectin was the active component of the *in vivo* supernatant. Whether this protein was acting as an opsonin or macrophage activator is currently being determined. This latter possibility seems more likely, however, as fibronectin displays a low binding affinity for *P. aeruginosa* (21).

The similar phagocytosis enhancement produced by *in vivo* supernatants from chambers containing bacteria or saline (Table 6) suggested that bacterial presence was not required to elicit the fibronectin response. Confirmation of this was obtained by using Western blotting techniques (Fig. 3). These data suggested that fibronectin increased in concentration over time in the peritoneal chambers, possibly due to surgical injury or implantation of a foreign body (the chamber), but not necessarily in response to the bacteria. There is a considerable precedent for this, as fibronectin is commonly found at wound sites (6). It should be noted that *P. aeruginosa* commonly initiates infections at sites of injury, including wounds and burns.

The presence of fibronectin early in the mouse time-course experiments may have indicated a higher level of fibronectin in the normal peritoneum of mice as compared to rats. Alternatively, the mouse system may have had more efficient delivery of fibronectin to the site of injury. The lower maximal levels of bacterial association with macrophages, found using rat *in vivo* supernatant, may however reflect a macrophage preference for homologous fibronectin, since the macrophages used were derived from mice.

The observation that the enhancement of phagocytosis of *in vitro*-grown organisms was dependent on whether they were grown on plates or in shaken broth suggested that there

was some alterable property of *P. aeruginosa* required for in vivo supernatant-mediated phagocytosis. *P. aeruginosa* grown with little or no agitation are thought to have increased quantities of pili, alginate exopolysaccharide, and possibly slime. One or more of these factors may be required for in vivo fibronectin-promoted phagocytosis. In previous papers it has been suggested that fibronectin is incapable of promoting association of mammalian cells and *P. aeruginosa* (21). In these earlier studies, however, bacteria were grown with rapid agitation, and thus their uptake by phagocytes would not be expected to be influenced by fibronectin (Table 3).

Our current working hypothesis is that fibronectin activates macrophages to increase their level of nonopsonic uptake. Experiments are being performed to test this hypothesis and to determine the nature of the alterable bacterial property crucial to this system.

Nonopsonic uptake could be seen as a very important clearance mechanism at the site of *P. aeruginosa* infection. These areas often display tissue injury and may therefore contain relatively high levels of fibronectin. If this is true, we would predict, based on the data presented here, that macrophages infiltrating the area could be acted upon by fibronectin and begin efficient nonopsonic clearance of *P. aeruginosa*. Indeed, very high levels of uptake were observed in the experiments reported here, with up to 100% of added bacteria taken up per macrophage (Table 6).

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