INFECTION AND IMMUNITY, Sept. 1989, p. 2782–2785
0019-9567/89/092782-04$02.00/0

Vol. 57, No. 9

Fibronectin as an Enhancer of Nonopsonic Phagocytosis of *Pseudomonas aeruginosa* by Macrophages

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Received 18 April 1989/Accepted 12 June 1989

Fibronectin is capable of enhancing uptake by macrophages of *Pseudomonas aeruginosa* grown in vivo in rats or mice or in vitro on nutrient agar plates. It was demonstrated that concentrations as low as 27 nM fibronectin produced significant enhancement of macrophage phagocytosis. Washing of fibronectin-treated macrophages did not prevent phagocytosis enhancement, but washing of fibronectin-treated bacteria did. The tetrapeptide arginine-glycine-aspartic acid-serine, which comprises the eucaryotic cell-binding domain of fibronectin, was also capable of promoting bacterial uptake, whereas the control tetrapeptide tetraglycine was not. Fibronectin caused depolarization of the mouse macrophage cell line $P388_{D1}$ plasma membrane, as demonstrated by using a polarization-sensitive fluorescent probe. These data indicate that promotion by fibronectin of nonopsonic phagocytosis is mediated by the action of fibronectin on the macrophages.

Macrophages are important host cells in the prevention of and defense against bacterial infections (19). In addition to their role in bacterial phagocytosis, they play an important part in amplifying the host immune response. Through release of potent immunomodulators, inflammation and wound healing occur at an accelerated rate (19). Furthermore, antigen presentation by macrophages is an important event in the functioning of cell populations in both the humoral and cellular immune responses.

In previous investigations (7, 8), it was determined that *Pseudomonas aeruginosa* cells taken directly from mouse or rat peritoneal chamber growth systems were significantly more susceptible to macrophage phagocytosis than were the same cells after being washed in buffer. The phagocytosis-promoting factor was partially purified from the supernatant of centrifuged in vivo-grown bacteria (in vivo supernatant) and determined to be fibronectin. Its phagocytosis-promoting activity could be mimicked by purified bovine plasma fibronectin (8). Furthermore, its activity could be completely neutralized by specific antifibronectin serum. Western immunoblotting demonstrated that this serum recognized only fibronectin in these preparations, indicating that fibronectin was responsible for phagocytosis promotion. The presence of bacteria was not required for entry of the phagocytosis-promoting activity into the chambers, since we observed similar effects with the fluid contents of saline-containing chambers that had been implanted in the peritoneal cavities of mice and rats. These data seemed to eliminate a role for other potential macrophage activators, including endotoxin.

Fibronectin is a large dimeric molecule (M_r 440,000) which has specific binding sites for mammalian cells, viral glycoproteins, and bacterial surfaces (15). It has been shown to enhance the activity of macrophages in adherence (2), C3- and Fc-receptor-mediated phagocytosis of coated erythrocytes (14, 22), and killing of staphylococci (17). It has been hypothesized to act as an opsonin in the promotion of staphylococcal adhesion (16). The possibility of fibronectin opsonizing *P. aeruginosa*, however, seemed unlikely, since *P. aeruginosa* binds poorly to epithelial cells with high contents of surface fibronectin and binds well to cells with low contents of this molecule (1, 21). In this paper, we provide evidence that fibronectin acts directly on macrophages to stimulate increased phagocytosis of bacteria.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* H103 (11) and M2, a strain often used for mouse pathogenicity studies (19), were maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Prior to assay, cells were inoculated and grown for 20 h on Trypticase soy agar at 37°C. These stationary-phase cells were suspended in phosphate-buffered saline (PBS), pH 7.2, to a concentration of 10^9/ml before experimentation.

Maintenance of macrophage cell line. Mouse macrophage cell line $P388_{D1}$ was maintained at 37°C, 10% CO_2, in supplemented RPMI 1640 medium as described previously (9). Prior to assay, macrophages were grown for 16 h in petri dishes (35 by 10 mm) (Nuclon; GIBCO, Burlington, Ontario, Canada) at a concentration of 10^6 cells per dish. Nonadherent cells were removed by gently washing the monolayer with unsupplemented RPMI 1640 medium (phagocytosis buffer).

Preparation of in vivo supernatant. *P. aeruginosa* H103 was grown in vivo as previously described (5, 7). Briefly, organisms were isolated in plastic chambers sealed on either end with 0.22-μm filters. On insertion of these chambers into the peritoneal cavities of laboratory rats, these filters allowed free exchange of peritoneal fluids and small bacterial products while prohibiting bacterial escape or direct immune-cell access to the organisms. Bacteria were grown for 72 h in vivo prior to harvesting. Organisms were centrifuged at 12,000 × *g*, and the decanted supernatant was saved for assessment of phagocytosis enhancement (in vivo supernatant).

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/ml. To assess enhancement of phagocytosis, PBS, 100 μl of in vivo supernatant, various concentrations of bovine plasma fibronectin (Sigma Chemical Co., St. Louis, Mo.), arginine-glycine-aspartic acid-serine (RGDS), or tetraglycine peptides (Sigma) were added. The affinity-purified bovine

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plasma fibronectin was found by *Limulus* amoebocyte lysate assay to contain less than 0.2 ng of endotoxin per 100 μg of protein (the maximal amount used in phagocytosis assays). This is several orders of magnitude less than the levels of endotoxin that optimally activate $P388_{D1}$ cells or mouse peritoneal macrophages (12). Control experiments demonstrated that macrophage assays with similar levels of endotoxin did not result in enhanced *P. aeruginosa* uptake (data not shown). 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 at 37°C, 10% CO_2. Uptake was assessed visually after Diff-quik (Canlab, Vancouver, British Columbia, Canada) staining.

Fluorescence assay. $P388_{D1}$ cells were suspended with a pipette from the bottom of flat-bottomed flasks (GIBCO), centrifuged at 1,000 rpm for 10 min, suspended at a concentration of 2.5×10^6/ml in fresh supplemented RPMI 1640 medium, and grown for 16 h prior to assay in screw-cap Teflon jars (Savillex, Minnetonka, Minn.). After gentle resuspension, cells were washed and suspended in an experimental solution which approximated the ion composition of RPMI 1640 medium (18). KCl was added to a final concentration of 0.3 mM to create a K^+ concentration gradient across the plasma membrane. Cells were dispersed in 1-ml assay volumes, and the carbocyanine dye 3,3'-dipropylthiocarbocyanine iodide ($diSC_3$ [5]; Molecular Probes, Eugene, Oreg.) was added at a concentration of 2×10^{-6} M. Carbocyanine dyes are lipophilic probes which are highly fluorescent in aqueous environments and minimally fluorescent in the hydrophobic environment of the membrane (18). When ion fluxes are generated across the membrane, either the probe is shunted out of the membrane to become more fluorescent (upon depolarization, decreasing electrical potential gradients across the plasma membrane) or more probe is inserted into the membrane to become less fluorescent (upon hyperpolarization). To test the effects of fibronectin on the plasma membrane of macrophages, various concentrations were added to cells equilibrated with $diSC_3$ (5) and the change in fluorescence (excitation, 620 nm; emission, 670 nm) was measured in a spectrofluorimeter (model 650-10S; Perkin-Elmer Corp., Norwalk, Conn.). All samples were added in 100-μl aliquots (volumes equalized with PBS, pH 7.2). The K^+ ionophore valinomycin (Sigma), at a concentration of 2×10^{-6} M, was used as a positive control for depolarization. Rates of depolarization were measured from the maximal slopes obtained from the spectrofluorimeter trace. Data were obtained from independent experiments on two separate days.

RESULTS

Enhancement of phagocytosis of *P. aeruginosa* by macrophages by using fibronectin. In a previous investigation it was determined that 230 nM fibronectin was capable of enhancing uptake of statically grown *P. aeruginosa* by mouse macrophage cell line $P388_{D1}$ and unelicited mouse peritoneal macrophages (8). To determine the threshold level of fibronectin required for stimulation of macrophage-mediated nonopsonic uptake, various concentrations were incubated with the $P388_{D1}$ cells prior to addition of *P. aeruginosa* (Fig. 1). While concentrations of 13 nM and lower caused no response, a significant level of phagocytosis enhancement was observed at concentrations at and above 27 nM. Maximal stimulation of macrophages was obtained at concentrations around 50 nM fibronectin.

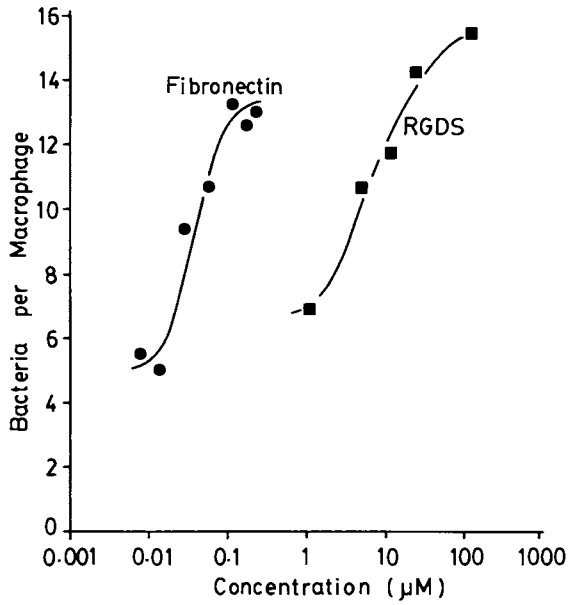

FIG. 1. Effect of increasing concentrations of fibronectin (circles) and RGDS (squares) on the level of uptake of *P. aeruginosa* H103 by mouse macrophage cell line $P388_{D1}$. Each data point represents the average uptake observed in three independent experiments, with standard deviations around 10% of the mean.

Previous studies (7, 8) indicated that fibronectin did not act as an opsonin, since it could be removed from bacteria by simple centrifugation and suspension in buffer (Table 1). Western immunoblotting, with antifibronectin sera, of washed bacteria after solubilization and sodium dodecyl sulfate-polyacrylamide gel electrophoresis confirmed the lack of fibronectin associated with washed bacterial cells (data not shown). However, a very different result was obtained in experiments in which fibronectin was added to macrophages for 15 min at 37°C and in which the macrophages were then washed twice prior to addition of bacteria and assessment of phagocytosis. In this case, washing failed to prevent stimulation of macrophages for increased uptake of *P. aeruginosa* (Table 1). Control experiments using antifibronectin serum demonstrated that fibronectin was responsible for the observed enhancement of phagocytosis.

Determination of the active domain of fibronectin. Fibronectin is a large dimeric glycoprotein with numerous

TABLE 1. Effect of washing on fibronectin-mediated enhancement of nonopsonic macrophage phagocytosis of *P. aeruginosa* H103

Addition to:		Treatment prior to mixing of bacteria with macrophages	Avg no. of bacteria associated per macrophage
Bacteria	Macrophages		
PBS	PBS	None	3.7 ± 0.8
Fibronectin[a]	PBS	None	10.1 ± 0.1[b]
Fibronectin	PBS	Bacteria washed	4.8 ± 1.8[c]
PBS	Fibronectin	None	8.9 ± 3.3[b]
PBS	Fibronectin	Macrophages washed	10.0 ± 0.4[b]

[a] Source of fibronectin was in vivo peritoneal chamber supernatant from rats in which the only phagocytosis-promoting factor was fibronectin (8). Part of the assay was reproduced by using purified bovine fibronectin, with similar results.

[b] $P < 0.005$ (Student's t test) in all assays performed (two to four individual experiments) when compared with the PBS control.

[c] Not significantly different from the PBS control in two of two assays.

specific binding sites, including those for mammalian cells and bacterial surfaces (15). One of these regions of the molecule, the eucaryotic cell-binding domain, has been shown to interact with various mammalian cell types, including macrophages (4, 13, 15). A four-amino-acid sequence, RGDS, has been proven to be the smallest portion of the eucaryotic cell-binding domain capable of interaction with mammalian cells (4, 13). As such, we considered this sequence a likely candidate for the macrophage-stimulating region of the fibronectin molecule.

To test this hypothesis, a commercially available preparation of RGDS was incubated with the macrophages for 15 min prior to addition of bacteria. This four-amino-acid sequence significantly increased bacterial association with $P388_{D1}$ cells in a concentration-dependent manner (Fig. 1). The maximal level of enhancement was similar to that obtained with a purified bovine fibronectin preparation (Fig. 1), and when it was used at a concentration of 100 μM the value was found to be statistically greater than that of the PBS control in all four assays performed ($P < 0.005$ by Student's t test) and not significantly different from the value obtained with 230 nM fibronectin. Concentrations as low as 2 μM RGDS were capable of significantly enhancing phagocytosis. As a control, we demonstrated that 800 μM tetraglycine failed to stimulate phagocytosis above the PBS control level.

Depolarization of the macrophage plasma membrane by fibronectin. The initial step in macrophage phagocytosis involves receptor-mediated interactions of the macrophage plasma membrane with the bacterial surface (20). For fibronectin to stimulate macrophages for enhanced phagocytosis, subsequent to fibronectin binding, a signal must be passed to the interior of the cell to activate the macrophage and presumably upregulate nonopsonic receptors. Past studies have suggested that phagocytic activation signals involve the generation of ion fluxes across the macrophage membrane (23). Using the polarization-sensitive fluorescent probe $diSC_3$ (5), it was determined that fibronectin did indeed produce depolarization of the $P388_{D1}$ plasma membrane (Fig. 2). The rate of ion flux generation increased as a function of the concentration of fibronectin added. The rates of depolarization attained at higher fibronectin concentrations were similar to that caused by the positive control ionophore, valinomycin, in dissipation of an imposed high K^+ concentration gradient. Fibronectin-mediated ion fluxes were generated virtually immediately across the macrophage membrane, and maximal rates were attained within 1 min of stimulus addition. It should be noted that lipopolysaccharide, which is also active on macrophages, failed to generate such ion fluxes (9).

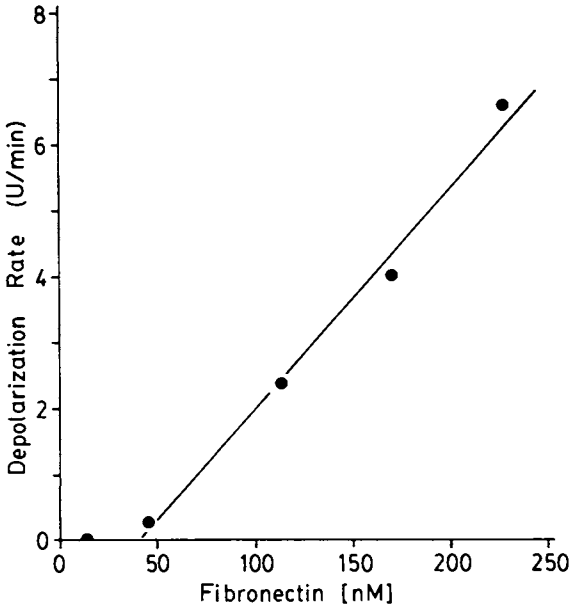

FIG. 2. Effect of increasing concentrations of fibronectin on the rate of increase (i.e., depolarization) of $diSC_3$ (5) fluorescence. The rates were calculated from spectrofluorimeter traces and represent the means of two independent experiments.

DISCUSSION

The data presented in this paper demonstrate that fibronectin acts directly on macrophages to stimulate increased phagocytosis of *P. aeruginosa*. The fact that macrophages retained enhanced ability to phagocytose *P. aeruginosa* after incubation with and subsequent removal of fibronectin-containing in vivo supernatant strongly favored this interpretation. In contrast, treatment of bacteria with this supernatant and subsequent washing prior to mixing with macrophages resulted in only background levels of phagocytosis. As previously demonstrated (8), such washed bacteria are still susceptible to fibronectin-mediated enhancement of phagocytosis. Thus, these data reflect the lack of high-affinity binding of fibronectin to bacteria rather than the removal of a surface structure required for phagocytosis. Furthermore, the tetrapeptide eucaryotic cell-binding domain was sufficient to activate macrophages, and fibronectin caused macrophage depolarization in a manner reminiscent of other macrophage activators (23). It is thus apparent that fibronectin acted not as an opsonin, lectin, or ligand in this system but acted directly on the macrophages to stimulate uptake. The concentration giving half maximal stimulation of macrophages by fibronectin (6×10^{-8} M; Fig. 1) correlated well with the known affinity of fibronectin for its cell surface receptors on platelets, hepatocytes, and fibroblasts (kilodaltons = 10^{-7} to 10^{-8} [10]).

Fibronectin is a large dimeric glycoprotein whose structure can be divided into several functional domains. These areas have been named according to the substances which bind in those regions of the molecule (15). The eucaryotic cell adhesion region had been previously shown to interact with various mammalian cell types, including macrophages (4, 12). A four-amino-acid sequence, RGDS, in this cell-binding domain has been shown to interact with the mammalian cell surface glycoprotein IIb/IIIa (3). In our studies, a commercially available RGDS preparation was able to significantly increase nonopsonic uptake of *P. aeruginosa* by mouse macrophage cell line $P388_{D1}$ (Fig. 1). Although 80-fold-higher molar concentrations of RGDS than fibronectin were required to observe this effect, this is consistent with previous studies (10, 13) and the notion that the structure of the tetrapeptide would be conformationally constrained in the intact fibronectin molecule.

Using the polarization-sensitive fluorescent probe $diSC_3$ (5), it was determined that fibronectin could generate a strong ion flux across the macrophage membrane at concentrations as low as 27 nM (Fig. 2). This result correlated well with studies of the concentration requirements of fibronectin in promoting phagocytosis (Fig. 1). While maximal phagocytosis was observed at concentrations around 50 nM, the rate of ion flux generation increased as a function of the level

of fibronectin to at least 225 nM. This suggested that while higher concentrations of fibronectin can produce greater ion fluxes across the macrophage plasma membrane, lower concentrations and their correspondingly lower rates of ion flux were sufficient to maximally enhance phagocytosis at a bacterium-to-macrophage ratio of 20:1. It is possible that this perturbation of ion gradients across the membrane may be the phagocytic activation signal which triggers enhanced nonopsonic uptake of *P. aeruginosa*.

Nonopsonic phagocytosis could be seen as an important clearance mechanism at sites of potential *P. aeruginosa* colonization and infection. These areas usually display tissue injury and would therefore possess a high concentration of fibronectin (6). As such, macrophages in the area could be activated by the fibronectin and begin efficient nonopsonic clearance of *P. aeruginosa*. Alternatively, since plasma concentrations of fibronectin are around 0.7 μM in healthy individuals (10), a concentration resulting in maximal enhancement of macrophage function (Fig. 1), it may be that fibronectin-enhanced phagocytosis represents the basal level of nonopsonic phagocytosis required for fulfilment of important macrophage functions in nonspecific and immune defenses against *P. aeruginosa*.

ACKNOWLEDGMENTS

This research was financially supported by a grant from the Medical Research Council of Canada to R.E.W.H. and a fellowship from the Canadian Cystic Fibrosis Foundation to N.M.K.

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