INFECTION AND IMMUNITY, Mar. 1985, p. 629–637
0019-9567/85/030629-09$02.00/0

Vol. 47, No. 3

Role of Fibronectin in Human Monocyte and Macrophage Bactericidal Activity

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Received 8 August 1984/Accepted 26 November 1984

Fibronectin is a high-molecular-weight glycoprotein found as a soluble dimer in plasma and as an insoluble multimer in tissues. It has been proposed that plasma fibronectin facilitates phagocytic removal of lysed cells and damaged tissues. Fibronectin binds avidly to several species of gram-positive bacteria and enhances staphylococcal and streptococcal attachment to cultured cells. Determination of whether fibronectin will enhance the bactericidal activity of monocytes and macrophages has not been reported. The bactericidal activity of freshly isolated monocytes, cultured monocytes, or lymphokine-activated macrophages was tested in the presence of either dimeric or multimeric fibronectin. Freshly isolated monocytes and lymphokine-activated macrophages killed *Staphylococcus aureus* effectively in the absence of fibronectin or whole serum. In contrast, monocytes cultured for 7 to 10 days had diminished staphylocidal capacity. When the monocytes were cultured with either dimeric or multimeric fibronectin, however, bactericidal capacity was maintained. Thus, although fibronectin did not enhance the bactericidal activity of mononuclear phagocytes, both multimeric and dimeric fibronectin were effective at maintaining the bactericidal capacity.

Fibronectin is a 400,000- to 500,000-dalton glycoprotein found as a soluble dimer in plasma and as an insoluble multimer in the basement membrane and intercellular matrix (23, 38, 56). Although both forms of fibronectin show many similarities, including affinities for fibrin, collagen, hyaluronic acids, heparin, and actin, differences in structure (16, 20), solubility (65), immunogenicity (3), and biological properties (65) have been noted. Interactions between gram-positive bacteria and plasma (4, 30, 41, 51, 57, 60–62) or tissue-bound (1, 50, 53, 63) fibronectin have been reported. The *Staphylococcus aureus* binding site (41) and the mammalian cell binding site (38) are well separated on the fibronectin molecule. Thus, fibronectin could theoretically act as a bacterial opsonin by bridging bacteria and phagocytic cells. This hypothesis is supported by studies that demonstrate opsonic activity for the removal of lysed cells and damaged tissues (18, 32, 58, 61). Moreover, monocytes have a fibronectin receptor(s) that enhances phagocytosis of erythrocytes (10–13, 47).

The possibility that fibronectin might promote killing of bacteria by mononuclear phagocytes has not been investigated in detail. Fibronectin was thought to enhance the uptake of glutaraldehyde-fixed bacteria by Kuppfer cells (36). These studies, however, relied on a liver slice assay that may measure adherence and not phagocytosis (8, 35). A more recent communication, with macrophage-like cell lines, mouse peritoneal exudate cells, and rabbit alveolar macrophages, failed to show any loss of phagocytic activity when fibronectin-depleted serum was used as an opsonin or any positive response when fibronectin alone was added (60). *S. aureus* and *Salmonella typhimurium* were used in these studies. However, the incubation times were short (only 20 min) for assessing enhancement of macrophage phagocytic activity by fibronectin, and it is known that human alveolar macrophages will phagocytose *S. aureus* in the absence of opsonins (21, 22).

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In the present paper, we characterize the effects of dimeric and multimeric fibronectin on the bactericidal activity of human monocytes, cultured monocytes, and activated macrophages. We also have studied the effects of time in culture and activation state of the phagocytes as these relate to the opsonic activity of fibronectin. Coating staphylococci with fibronectin did not enhance the bactericidal activity of mononuclear phagocytes, but both monomeric and dimeric fibronectin did provide a melieu in which monocytes maintained their staphylocidal activity while being held in tissue culture for 7 to 10 days.

MATERIALS AND METHODS

Neutrophils. Neutrophils were harvested from human blood by dextrose sedimentation and Ficoll-Hypaque fraction as described by Böyum (7).

Monocytes. Samples (120 to 180 ml) of fresh human blood were sedimented with dextran (Sigma Chemical Co., St. Louis, Mo.), supernatants were centrifuged at 180 × *g* for 5 min, and pellets were combined and suspended in 20 ml of Hanks buffered salt solution, pH 7.4 (HBSS). This was layered over 13 ml of Ficoll-Hypaque (Sigma type F-P) and centrifuged at room temperature at 900 × *g* for 20 min. The resulting band between the supernatant and Ficoll contained the monocytes and lymphocytes. These were washed three times in HBSS, suspended by gentle agitation in 1 ml of HBSS, containing 0.1% bovine serum albumin, counted, and adjusted to 10^6 cells per ml by the addition of RPMI-1640 medium with L-glutamine, phenol red (M.A. Bioproducts, Walkersville, Md.), 50 μg of gentamicin (Sigma) per ml, and 0.3% bovine serum albumin. Depending on the experiment, the medium also contained fibronectin at 0.3 mg/ml, 7% isologous serum, 7% fibronectin-depleted serum (mixed donors), or no serum in a total volume of 1 ml. A 1-ml sample of this suspension was added to each well of a Falcon tissue culture plate (LB Labware, Oxnard, Calif.) and incubated at 37°C in 6% CO_2 for 1 h to allow attachment of monocytes. After 1 h of adhesion, the cells in each well were gently agitated and washed to release most of the lymphocytes. The

plates were then incubated an additional 2 h and then washed three times with HBSS to remove remaining lymphocytes and any antibiotic. The bactericidal assays were then performed on these cells. Cells were identified as monocytes by morphology (Wright stain), nonspecific esterase staining, and phagocytic activity.

Cultured monocytes and activated macrophages. Monocytes were isolated as indicated above. In the case of the cultured monocytes, 1.5 ml of RPMI-1640 with the additives described above was added to each well and allowed to incubate for the indicated number of days with replacement of half the medium every 48 to 72 h. To activate the macrophages, 1.2 ml of the above-mentioned medium and 0.3 ml of lymphokines were added to each well. The cells were viewed under a microscope periodically to look for characteristic morphological changes and tested for O_2^- production and viability (trypan blue exclusion) on day 1 and on the last day. Testing was performed only when the viability was greater than 95% and when fewer than 10% of the cells were lost during medium changes.

The lymphokines were isologous and produced by concanavalin A stimulation as previously described (44) and briefly summarized here. Lymphocytes were obtained by dextran sedimentation and Ficoll-Hypaque gradients from human donors whose monocytes would later be used. To each of five tubes was added 1 ml of lymphocytes (in HBSS) at a concentration of 2×10^6/ml, 1 ml of isologous plasma, 3 ml of RPMI-1640 with 50 μg of gentamicin per ml, and 50 μl of concanavalin A (grade IV; Sigma) at a concentration of 0.4 mg/ml. These tubes were incubated at 37°C in 6% CO_2 for 4 days and then centrifuged at 200 × *g* for 5 min, and the supernatant was passed through a 0.45-μm filter. This filtrate contained the lymphokines.

Of note, all additives (serum, balanced salt solutions, albumin, lyphokines, plasma, Ficoll-Hypaque, and dextran) were negative by the *Limulus* amebocyte lysate test, whose sensitivity was less than 0.1 ng of endotoxin per ml. Endotoxin testing was performed on additives before they were diluted to increase sensitivity.

O_2^- production of monocytes and macrophages and protein determination. The cells were washed twice with HBSS and then incubated for 10 min at 37°C with 10 μg of cytochalasin B (Sigma) in 0.5 ml of HBSS. This did not result in the release of cells from monolayers. The cells in four wells received 1 μg of phorbol myristate acetate (Consolidated Midland Corp., Brewster, N.Y.) and 0.6 mg of horse ferricytochrome *c* (Sigma) in a final volume of 1.0 ml of HBSS. Two of the wells also contained 35 U of superoxide dismutase (Sigma). After 30 min of incubation at 37°C, the supernatant was removed, and absorbance was measured at 550 nm. Nanomoles of O_2^- were calculated from the difference in absorbance in the absence and presence of superoxide dismutase as previously described (49). The protein content of monocytes and macrophages in each well was determined by first washing off all medium containing any contaminating protein and then adding 250 μl of 1 N NaOH to each well to solubilize the cells. The wells were also scraped with a rubber policeman and rinsed twice with 250 μl of HBSS. The scrapings and rinsings were combined, and a standard Lowry protein assay was performed.

Phagocyte bactericidal assays. Assays of monocytes were performed on the day of isolation. Cultured monocytes and macrophages were assayed after 7 to 10 days in culture. There were approximately 10^6 cells adherent in each reaction well. These were washed twice with HBSS containing 0.1% bovine serum albumin. As controls, some sets of wells contained no phagocytes. Reaction mixtures contained HBSS with no additives, 0.3 mg of fibronectin per ml, 7% human serum, or 7% fibronectin-depleted human serum as noted in the tables and figure legends. In wells without serum, the protein concentration was maintained by adding bovine serum albumin to make the final protein concentration 3 mg/ml. To each well was added *S. aureus* ATCC 25923 that were harvested in the logarithmic phase of growth from unbuffered trypic soy broth. Bacterial inocula were counted in a Petroff-Hausser counting chamber and adjusted to 2×10^7 bacteria per ml. The volume of bacterial suspension added was adjusted so that the ratio of bacteria to monocytes and macrophages would approximate 2 bacteria per phagocyte. The actual counts ranged from 1 to 4 bacteria per phagocyte. The cells were incubated without agitation at 37°C for 0, 2, 4, or 6 h; at those times, the contents of the wells were scraped with a rubber policeman, put into a plastic tube, sonicated for 10 s, serially diluted in sterile saline, and cultured in pour plates. All counts were performed in triplicate on plates with between 30 and 300 colonies. In 24 to 48 h, the CFU were counted to determine whether the various times or conditions had an effect on the growth or killing of the *S. aureus*.

The neutrophil bactericidal assay was performed as previously described (53) in the presence of multimeric fibronectin. Collagen-fibronectin matrices were made as previously described (50).

Electron microscopy. Monocytes and macrophages were harvested as described above and then cultured on glass cover slips. These cells received various treatments such as lymphokines, culturing with multimeric fibronectin, etc., as noted in the figure legends. The monocytes were washed three times in phosphate buffered saline, pH 7.2 (PBS), treated with 1.0% glutaraldehyde in PBS for 5 min at room temperature to fix the cells and prevent phagocytsis of markers, and washed three times in PBS. Two mililiters of a suspension containing anti-fibronectin antibodies labeled with gold beads (described below) was added to petri dishes containing the cover slips. The reaction was allowed to proceed for 20 min at room temperature with gentle shaking. The cover slips were washed three times with PBS and fixed overnight in 2% glutaraldehyde in PBS at 4°C. The cells were dehydrated by passing them through a series of graded enthanol solutions, critical point dried, and mounted on scanning electron microscopy stubs. The samples were coated with 3 nm of gold-palladium and viewed in a JEOL 35C scanning electron microscope or a JEOL 200 CX STEM operated scanning electron microscope.

Gold bead labeling of anti-fibronectin antibodies. Anti-fibronectin antibodies were prepared in rabbits immunized by subcutaneous injections in the back according to the following schedule. The first injection consisted of 150 μg of human fibronectin in complete Freund adjuvant. Three weekly injections of the same dose of fibronectin in saline followed. Anti-fibronectin and normal rabbit immunoglobulin G were purified by affinity chromatography on protein A-Sepharose (Sigma). Anti-*S. aureus* antiserum was prepared as previously described (46).

Preparation of colloidal gold markers was carried out as previously described (19). Briefly, to make the gold beads, 0.5 ml of 4% $HAuCl_4$ was added to 200 ml of distilled water. A reducing agent was then added. The reducing agent used and the amount used varied with the size of bead desired. For example, to make 30-nm beads, 3.0 ml of 1.0% sodium citrate was added. The solution was refluxed for 30 min and then cooled on ice. Proteins to be adsorbed onto the beads

were dialyzed overnight against 0.005 M sodium phosphate (pH 7.5), and the protein and gold beads were mixed. Five minutes later 0.5 ml of 1.0% polyethylene glycol (20,000 daltons) was added to fully stabilize the gold beads against flocculation. The solution was centrifuged to remove unreacted protein and polyethylene glycol. The pelleted gold beads were suspended in distilled water and stored at 4°C. Beads 5 nm in diameter were used in transmission electron microscopy studies, and 30-nm beads were used in scanning electron microscopy studies. Gold beads were kindly prepared by Joseph Loftus and Ralph Albrecht of the University of Wisconsin School of Pharmacy.

Fibronectin. Plasma fibronectin was purified from a fibronectin- and fibrinogen-rich human plasma protein fraction (kindly provided by Mike Hrinda, Revlon Industries, Tuckahoe, N.Y.) by gelatin affinity chromatography as previously described (37, 51). Fibronectin was concentrated by precipitation with 20% (wt/vol) ammonium sulfate. The final product was dissolved in and dialyzed against 0.01 M Tris–0.14 sodium chloride (pH 7.4). Portions were snap-frozen at concentrations of 10 to 15 mg/ml and stored at −70°C. Human serum >90% depleted of fibronectin was made by treating 1 U of fresh frozen plasma (Badger Red Cross, Madison, Wis.) with thrombin (Parke Davis & Co., Detroit, Mich.) and calcium chloride at 4°C (37). Fibronectin multimers were produced by exposing purified dimeric plasma fibronectin to 3 M guanidine as previously described (40). [^{125}I]fibronectin was prepared by the chloramine T method as previously described (51).

Bacteria. *S. aureus* ATCC 25923 was obtained from the American Type Culture Collection, Rockville, Md. Bacteria were harvested in the logarithmic phase of growth for tryptic soy broth (Difco Laboratories, Detroit, Mich.), counted in a Petroff-Hausser chamber, and also plated on blood agar base (Difco) to count the number of viable bacteria as previously described (52).

RESULTS

To evaluate fully the possibility that fibronectin might enhance phagocytosis, a number of experimental conditions were studied. One of the variables was the state of fibronectin. Multimeric (40) and dimeric fibronectin were used because it was thought that multivalent interaction might enhance phagocytosis. Dimeric fibronectin bound to *S. aureus* ATCC 25923 as previously reported (51), whereas ^{125}I-labeled multimeric fibronectin showed a nonlinear pattern (Fig. 1). The data are plotted after the method of Scatchard, and the amount bound per 10^9 *S. aureus* organisms is specific (total bound minus the amount bound in the presence of excess, unlabeled, multimeric fibronectin). The dimeric fibronectin demonstrated 2,100 molecules of fibronectin per organism (using a molecular weight for fibronectin of 440,000) and an affinity constant of 1.77×10^{-9} M. Because the number of molecules in a multimer may vary, the numbers of multimeric fibronectin molecules were expressed as dimeric equivalents. The multimeric fibronectin had 1,500 dimeric fibronectin molecular equivalents per *S. aureus* organism for the high-affinity receptor (K_a, 2.1×10^{-9} M) and 29,000 dimeric fibronectin molecular equivalents per bacterium for the low-affinity receptor (K_a, 172×10^{-9} M). The nonlinearity may be due to positive cooperativity or due to a second binding site.

Freshly isolated monocytes alone were as effective at killing *S. aureus* as those monocytes receiving serum, dimeric fibronectin, or multimeric fibronectin in the bacterial test reaction mixture (Tables 1 and 2). The number of *S. aureus*

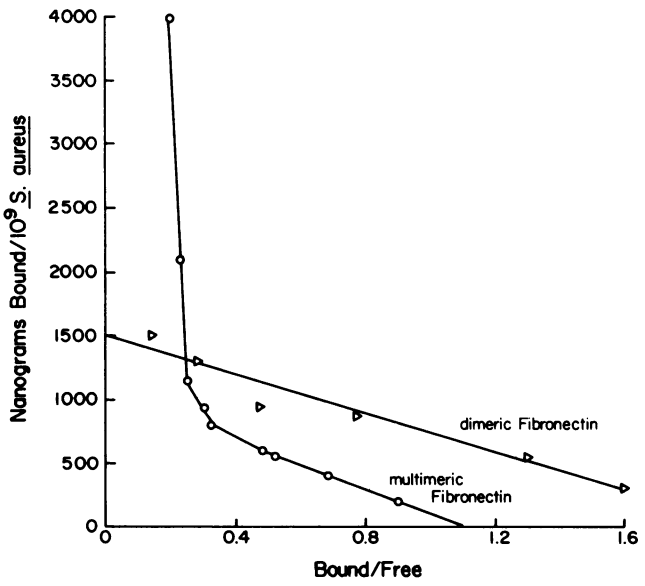


FIG. 1. Specific binding of ^{125}I-labeled dimeric and multimeric fibronectin to *S. aureus* ATCC 25923 harvested in the logarithmic phase of growth from unbuffered tryptic soy broth as previously described (51).

organisms increased slightly in the presence of buffer or fibronectin. In contrast, when serum was added to the buffer, a 30-fold increase in CFU occurred during the 6-h test for bactericidal activity. The growth-promoting effects of serum probably leads to underestimating the bactericidal activity of phagocytes in 7% serum. Fibronectin-depleted serum was as effective as whole serum in supporting the bactericidal activity of monocytes. The addition of serum or multimeric fibronectin to the monocytes during the 2-h in incubation period before bacterial challenge did not alter the monocyte bactericidal activity (Tables 1 and 2). Increasing the *S. aureus* cell monocyte ratio up to 20:1 did not allow fibronectin to have an effect. In ratios above 20:1, the bacteria overwhelmed the ability of the monocytes to kill the *S. aureus*.

Macrophage bactericidal activity was similar to that of monocytes. The presence of serum or dimeric fibronectin in the bactericidal assay mixture or in the culture fluid for 7 to 10 days did not enhance the macrophage bactericidal activity, whereas the addition of multimeric fibronectin did result in some enhancement (Tables 1 and 2). Multimeric fibronectin enhanced the bactericidal activity of macrophages, especially when present during the initial culturing and in the bactericidal assay mixture (Table 2). Although the macrophages in Table 1 showed greater staphylocidal capacity than those in Table 2, this comparison is not valid because the macrophages showed different levels of activity depending upon the donor. Thus, comparisons can only be made with the controls run simultaneously.

Cultured monocytes (7 to 10 days in culture without activators) killed *S. aureus* less effectively than did freshly harvested monocytes (Table 1). Serum did not enhance cultured monocyte bactericidal activity because the growth-stimulating effect of serum was dominant over any opsonic effect, although some bacteria were seen to be cell associated. When dimeric fibronectin was added to the bactericidal reaction mixture, monocytes cultured in buffer did not show enhanced killing (Table 1). However, the addition of dimeric fibronectin (purified or as contained in serum) during the

TABLE 1. Role of lymphokines and dimeric fibronectin on the bactericidal activity of mononuclear phagocytes

Cell type	Protein in initial culturing suspension	Bactericidal assay reaction condition	n	Surviving bacteria (% of initial inoculum, $\bar{x}$ ± SEM)		
				2 h	4 h	6 h
Freshly isolated monocytes	Albumin	Albumin	3	86 ± 14	31 ± 4	29 ± 4
	Albumin	Serum	3	95 ± 9	23 ± 5	16 ± 2
	Albumin	Dimeric FN[a]	3	79 ± 11	41 ± 10	17 ± 2
	Serum	Albumin	6	107 ± 21	50 ± 6	27 ± 8
	Serum	Serum	6	91 ± 17	24 ± 7	24 ± 9
	Serum	Dimeric FN	6	89 ± 6	24 ± 5	19 ± 4
Cultured monocytes	Albumin	Albumin	3	180 ± 43	162 ± 38	140 ± 18
	Albumin	Serum	2	140 ± 26	>400	>400
	Albumin	Dimeric FN	3	146 ± 19	137 ± 24	121 ± 12
	Serum	Albumin	3	86 ± 4	79 ± 8	61 ± 7
	Serum	Serum	3	143 ± 11	192 ± 17	314 ± 51
	Serum	Dimeric FN	3	97 ± 10	96 ± 10	70 ± 2
Macrophages	Lymphokines	Albumin	3	49 ± 8	18 ± 2	12 ± 2
	Lymphokines	Serum	2	86 ± 1	68 ± 5	19 ± 3
	Lymphokines	Dimeric FN	3	30 ± 3	17 ± 3	14 ± 1

[a] FN, Fibronectin.

culturing period did increase the bactericidal capacity of these cultured monocytes (Table 1 and 2). Phase-contrast microscopy demonstrated greater phagocytosis in those monocytes that had been cultured in the presence of fibronectin. Of note, after the monocytes had been cultured for 5 days in the absence of fibronectin, culturing these cells with monomeric or dimeric fibronectin for 2 to 5 days did not restore their bactericidal capacity. Also, monocytes not cultured with fibronectin showed a progressive loss of bactericidal capacity over a 5-day period. Fibronectin added during this period prevented further loss of bactericidal activity, but did not return it to levels demonstrated by freshly harvested monocytes.

The reason(s) for the decreased killing activity of cultured monocytes in the absence of fibronectin may be multiple, but one major reason seems to be the decreased ability of these cells to produce superoxide as compared with freshly harvested monocytes or activated macrophages (nanomoles per milligram of protein per 15 min ± standard error of the mean for five to nine determinations: monocytes, 124 ± 22; cultured monocytes, 45 ± 11; macrophages, 124 ± 15; $P < 0.02$, Student t test). The cultured monocytes had the morphological appearance of macrophages, were positive for nonspecific esterase, were phagocytic by light microscopy, and were viable as assessed by trypan blue exclusion. The presence of fibronectin did not alter viability. However, culturing monocytes in the presence of multimeric fibronectin gave the following results for superoxide production (nanomoles per milligram of protein per 15 min: monocytes, 172 ± 22; monocytes cultured in fibronectin, 131 ± 14;

TABLE 2. Effects of dimeric and multimeric fibronectin on phagocyte bactericidal activity

Cell type	Protein in initial culturing suspension	Bactericidal assay reaction condition	n	Surviving bacteria (% of initial inoculum, $\bar{x}$ ± SEM)		
				2 h	4 h	6 h
Monocytes	Albumin	Albumin	3	59 ± 12	31 ± 8	14 ± 9
	Albumin	Dimeric FN[a]	3	52 ± 7	13 ± 2	10 ± 2
	Albumin	Multimeric FN	3	58 ± 8	16 ± 1	3 ± 0
	Multimeric FN	Albumin	3	55 ± 12	59 ± 16	23 ± 6
	Multimeric FN	Dimeric FN	3	53 ± 7	36 ± 4	2 ± 0
	Multimeric FN	Multimeric FN	3	36 ± 5	20 ± 5	12 ± 4
Cultured monocytes	Albumin	Albumin	3	193 ± 36	168 ± 61	128 ± 8
	Albumin	Dimeric FN	3	158 ± 41	139 ± 39	126 ± 15
	Albumin	Multimeric FN	3	132 ± 43	120 ± 40	98 ± 17
	Dimeric FN	Albumin	3	90 ± 14	80 ± 6	66 ± 8
	Dimeric FN	Dimeric FN	3	98 ± 11	71 ± 3	72 ± 7
	Dimeric FN	Multimeric FN	3	86 ± 7	64 ± 8	59 ± 8
	Multimeric FN	Albumin	3	113 ± 28	84 ± 13	56 ± 5
	Multimeric FN	Dimeric FN	3	111 ± 24	62 ± 6	41 ± 5
	Multimeric FN	Multimeric FN	3	66 ± 16	38 ± 11	25 ± 4
Macrophages	Lymphokines	Albumin	3	112 ± 14	81 ± 10	50 ± 3
	Lymphokines	Dimeric FN	3	99 ± 12	73 ± 9	48 ± 4
	Lymphokines	Multimeric FN	3	66 ± 4	29 ± 2	16 ± 1
	Lymphokines plus dimeric FN	Albumin	3	74 ± 7	68 ± 8	56 ± 6
	Lymphokines plus dimeric FN	Dimeric FN	3	44 ± 14	43 ± 3	37 ± 4
	Lymphokines plus dimeric FN	Multimeric FN	3	52 ± 9	66 ± 2	30 ± 1
	Lymphokines plus multimeric FN	Albumin	3	105 ± 8	60 ± 12	68 ± 7
	Lymphokines plus multimeric FN	Dimeric FN	3	100 ± 16	65 ± 7	47 ± 6
	Lymphokines plus multimeric FN	Multimeric FN	3	67 ± 5	52 ± 4	37 ± 4

[a] FN, Fibronectin.

TABLE 3. Effects of multimeric fibronectin and culturing on neutrophil bactericidal activity

Cell type	Protein in initial culturing suspension	Bactericidal assay reaction condition	n	Surviving bacteria (% of initial inoculum, $\bar{x} \pm$ SEM)		
				2 h	4 h	6 h
Neutrophils in suspension	Albumin	Albumin	3	103 ± 8	96 ± 8	98 ± 9
	Albumin	Serum	3	17 ± 2	8 ± 1	2 ± 0
	Albumin	Multimeric FN[a]	3	100 ± 12	92 ± 10	94 ± 8
Neutrophil monolayer	Collagen	Albumin	3	85 ± 9	49 ± 4	37 ± 3
	Collagen plus dimeric FN	Albumin	3	67 ± 11	53 ± 5	35 ± 3
	Collagen plus multimeric FN	Albumin	3	72 ± 6	54 ± 7	27 ± 3

[a] FN, Fibronectin.

macrophages, 194 ± 39. Culturing cells with dimeric fibronectin did not alter the superoxide production by monocytes or macrophages, but it did increase the release of superoxide of cultured monocytes to 112 ± 19.

Neurophils did not show increased bactericidal activity in the presence of multimeric fibronectin (Table 3). Because neutrophils demonstrate enhanced phagocytosis on surfaces (54), we tested their bactericidal capacity on collagen-fibronectin matrices. Placing neutrophils on a protein matrix did enhance the killing of *S. aureus*, but fibronectin stimulated no further killing.

In Fig. 2, scanning electron microscopy studies of monocytes, cultured monocytes, and macrophages are shown. Albumin (3 mg/ml) was the only protein to which the monocytes and cultured monocytes were exposed until 30 min before fixation (Fig. 2A through K). On day 4, concanavalin A-treated lymphokines were added to the cells to produce macrophages (Fig. 2L and M). The serum used during the preparation of the lymphokines was depleted of fibronectin before it was added to the lymphocyte cultures. Addition of the lymphokines to the monocytes gave a final concentration of 4% fibronectin-depleted serum. Fibronectin on the phagocyte surface was identified with gold bead-labeled, anti-fibronectin antibodies (Fig. 2A through K). The normal rabbit immunoglobulin did not adhere to the phagocytes under any of the test conditions.

Only rare gold beads were seen associated with monocytes under any of the conditions noted in Fig. 2A through E. This is in agreement with a previous report that found no fibronectin on human monocytes (2). When lymphokines were added to fresh monocytes (Fig. 2F and G), many gold beads were found to be adherent to these monocytes. In contrast, the addition of multimeric fibronectin plus lymphokines led to few anti-fibronectin-gold beads adhering to fresh monocytes (Fig. 2H). The presence of anti-fibronectin-gold beads on cultured monocytes without any added fibronectin suggests that fibronectin was synthesized by these cells in tissue culture and then remained adherent (Fig. 2I). The addition of dimeric FN (Fig. 2J) or multimeric FN (Fig. 2K) to cultured monocytes resulted in large numbers of anti-fibronectin-gold beads attaching to the cell surface. Few gold beads were seen on lymphokine-activated macrophages (Fig. 2L). When fresh serum was added, the fibronectin rapidly associated with macrophages in a patchy distribution (Fig. 2M).

DISCUSSION

The addition of fibronectin to the bactericidal assay mixture did not enhance the bactericidal capacity of phagocytes, except for the combination of multimeric fibronectin and activated macrophages, which resulted in a modest increase in bactericidal activity. In contrast, the addition of either dimeric or multimeric fibronectin to cultured monocytes while they were maintained in tissue culture resulted in a marked increased bactericidal activity. Otherwise, the high bactericidal capacity of freshly harvested monocytes was lost when the monocytes were cultured for several days.

The nonlinear Scatchard plot for multimeric fibronectin may be due to the presence of two types of receptors: low-number, high-affinity receptors and high-number, low-affinity receptors. Alternatively, there might be positive cooperativity between multimeric fibronectin molecules. These results are very similar to those reported by Doran and Raynor (14). Of interest, the procedure used by Doran and Raynor (14) to prepare fibronectin may have favored the development of some multimers due to the presence of the reducing agent mercaptoethanol (40).

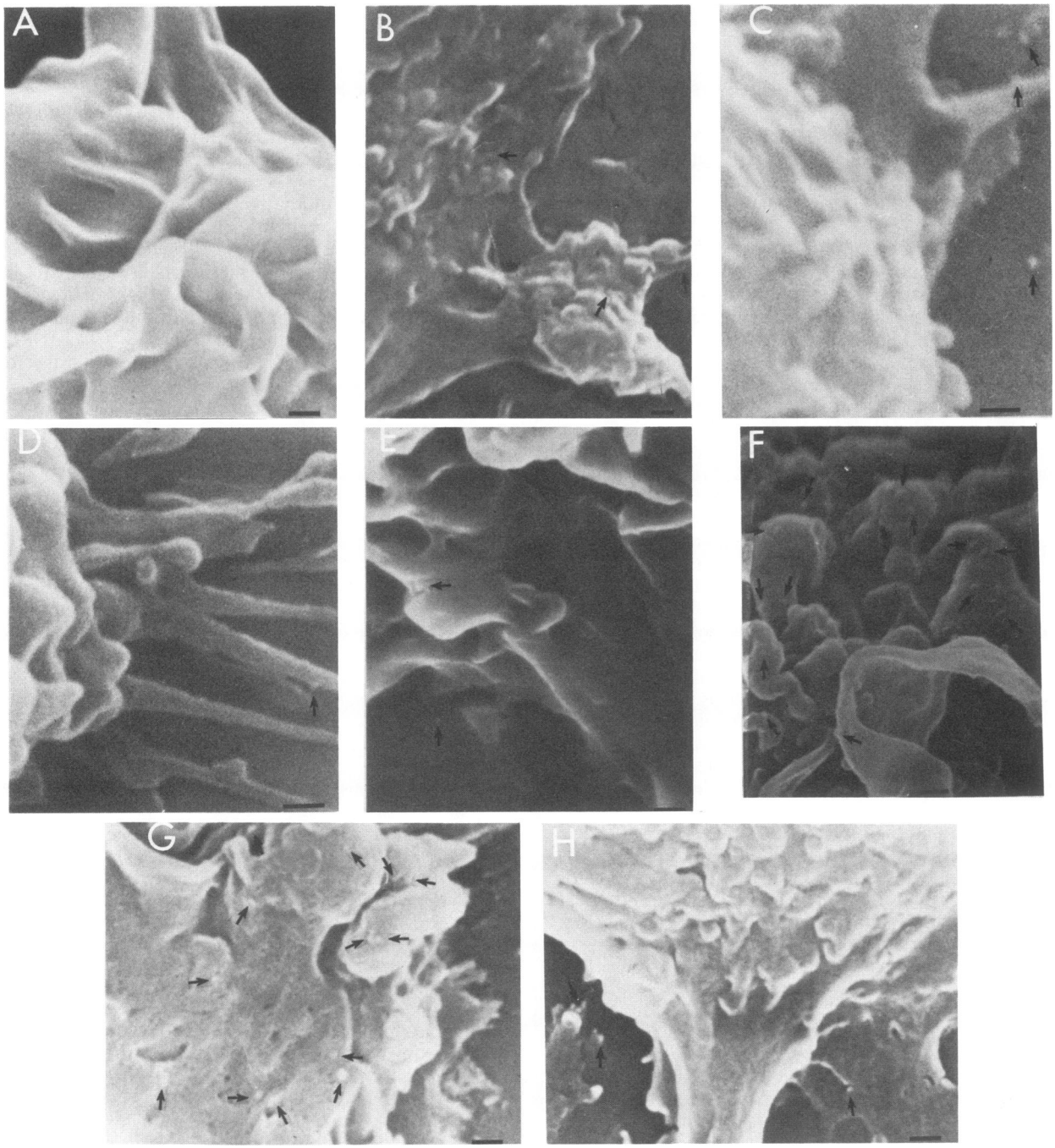
Fibronectin is known to preserve and promote the functions of several types of cells. Fibronectin encourages cellular adhesion to substrata, cystoskeletal organization, differentiation, migration during wound healing and embryogenesis, aggregation, and particle uptake by fibroblasts (5, 24, 25, 27, 29, 32, 34, 39, 56). Fibronectin is also known to enhance some monocyte functions. Soluble fibronectin has been shown to enhance monocyte and macrophage tumoricidal activity, whereas coating plates with dimeric fibronectin did not enhance monocyte or macrophage tumoricidal activity (45). Similarly, human monocytes that are exposed to plasma fibronectin and then washed acquire the ability to phagocytize sheep erythrocytes bearing C3b, whereas those monocytes not exposed to fibronectin only demonstrate adherence of erythrocytes to their plasma membranes (47, 48). In our studies, even when the cultured monocytes were washed after being incubated with either dimeric or multimeric fibronectin, an enhanced bactericidal capacity was maintained.

Several mechanisms by which fibronectin preserves cultured monocyte bactericidal activity are possible. Fibronectin may help to maintain the phagocytic capacity of cultured monocytes. Fibronectin receptors on phagocytes are known to exist (6, 10, 11, 42). Fibronectin might promote cultured monocyte bactericidal activity (i) by increased chemotactic activity (26, 43), (ii) by greater cytoskeletal organization (24, 27, 29), or (iii) by expression of other receptors (6, 48). Changes such as these are not mutually exclusive and might all favor phagocytosis and movement of lysosomal granules. Fibronectin may promote cultured monocyte bactericidal activity by enhancing the bactericidal systems as well as promoting phagocytosis. Indeed, one group has suggested that plasma fibronectin is closely related to the T-cell lymphokine, macrophage aggregating factor (17). Also cellular, but not plasma, fibronectin was able to restore macrophage inhibitory factor responsiveness to trypsinized guinea pig

macrophages (55). In addition, the increased bactericidal activity of monocytes cultured with fibronectin may relate to increased protein synthesis. Fibronectin increases human monocyte protein synthesis (33), and protein synthesis is a necessary step in monocyte activation (9). Thus fibronectin may play a role in monocyte activation either directly as a lymphokine or indirectly by promoting cellular interactions with lymphokines (or both). Perhaps these lymphokine-like activities also account for the increased superoxide-producing and bactericidal activity of cultured monocytes. Details of the mechanism(s) by which fibronectin promotes cultured monocyte bactericidal capacity await further study.

The presence of fibronectin on cultured monocytes is in contrast to a previous study (2) that reported synthesis, but little surface localization by indirect immunofluorescence. This difference may be more apparent than real because they did find some cell surface fibronectin. The scanning electron microscopic techniques we used will identify very small amounts of surface fibronectin, whereas Alitalo et al. (2) were studying pericellular fibronectin matrices.

The finding that monocytes kill *S. aureus* in the absence of opsonins was of interest. Previous work has shown that phagocytosis of *S. aureus* was opsonin independent in alveolar macrophages, but not human monocytes (21, 22).



However, phagocytosis was only followed for 60 min. In the first 60 min of our incubations, phagocytosis and killing were minimal. Over a 6-h period, the monocytes did show significant bactericidal activity.

Fibronectin has also been suggested as an opsonin for gram-positive bacteria in neutrophil systems (31, 54). However, others have found that fibronectin promotes adherence to neutrophils, but not phagocytosis (53, 59, 60, 62). When direct comparisons are made between antibody and complement-mediated (fresh serum) versus fibronectin-mediated opsonic activity, antibody and complement enhance the association of streptococci with neutrophils 2 orders of magnitude greater than fibronectin (59). Also the kinetics of *S. aureus* uptake by neutrophils were approximately 20-fold slower in the presence of fibronectin as compared with antibody and complement (54). In the absence of any serum proteins, *S. aureus* will attach to neutrophil monolayers (28). Phagocytosis of *S. aureus* by neutrophils in monolayers is reminiscent of the "surface phagocytosis" as proposed by Wood (64). However, it would not be hard to envisage some of these adherent staphylococci becoming entrapped between the neutrophil and the culture plate. These entrapped, but not phagocytosed, bacteria might then be resistant to lysostaphin. Also metabolic inhibitors and 4°C temperatures would inhibit any chemotaxis and thus appear to be inhibiting phagocytosis. Because fibronectin has been shown to increase neutrophil (26) and monocyte (43) chemotaxis, the chances for nonphagocytic entrapement would be much enhanced by adding fibronectin. Indeed, we found that neutrophils in monolayers would kill *S. aureus* in the absence of opsonins, but that neither multimeric or dimeric fibronection would further enhance their killing capacity. In our studies, the contents of the wells were scraped with a rubber policeman to free entrapped *S. aureus* and then

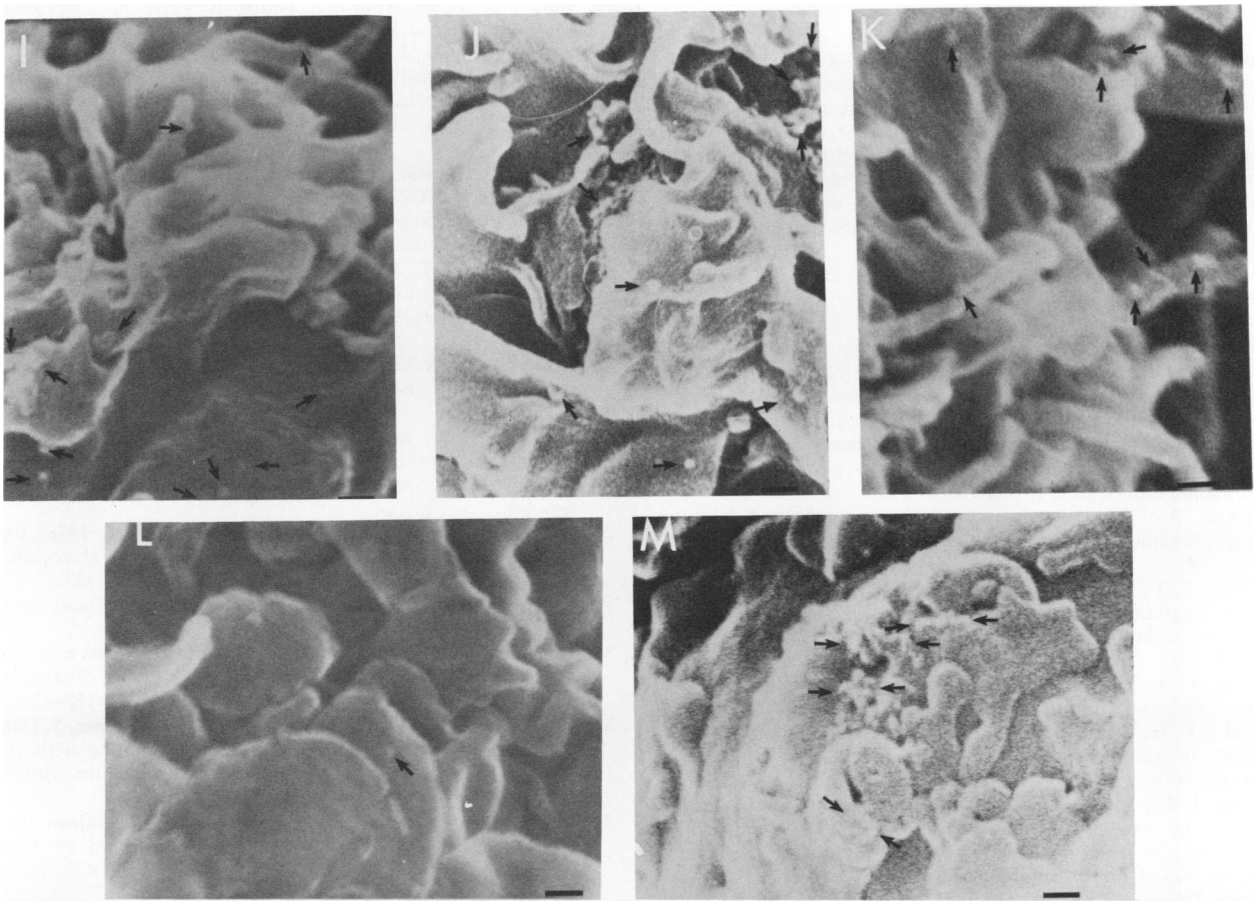


FIG. 2. Scanning electron micrographs of monocytes (A through H), cultured monocytes (I through K), and macrophages (L and M). These cells were tested for the presence of surface fibronectin by gold bead-labeled anti-fibronectin antibodies. Gold bead markers are identified by arrows. Bars, 200 nm. (A) Monocytes with anti-fibronectin-labeled gold beads (anti-fibronectin-gold). (B) Monocytes incubated with 0.3 mg of dimeric fibronectin per ml for 30 min, and with anti-fibronectin-gold beads. (C) Monocytes with 0.3 mg of multimeric fibronectin per ml added 30 min before fixation, when anti-fibronectin-gold beads were added. (D) Monocytes with 7% serum added at room temperature 30 min before fixation, when anti-fibronectin-gold beads were added. (E) Monocytes with 7% fibronectin-depleted serum added 30 min before fixation, when anti-fibronectin-gold beads were added. (F) Monocytes with lymphokines added 30 min before fixation, when anti-fibronectin-gold beads were added. (G) Monocytes with lymphokines and 0.3 mg of dimeric fibronectin per ml added 30 min before fixation, when anti-fibronectin-gold beads were added. (H) Monocytes with lymphokines and 0.3 mg of multimeric fibronectin per ml added 30 min before fixation, when anti-fibronectin-gold beads were added. (I) Cultured monocytes (7 days) with anti-fibronectin-gold beads. (J) Cultured monocytes (7 days) with 0.3 mg dimeric fibronectin per ml added 30 min before fixation, when anti-fibronectin-gold beads were added. (K) Cultured monocytes (7 days) with 0.3 mg multimeric fibronectin per ml added 30 min before fixation, when anti-fibronectin-gold beads were added. (L) Macrophages washed free of lymphokines and with anti-fibronectin-gold beads. (M) Macrophages washed free of lymphokines, incubated for 30 min with 7% serum, and washed again; anti-fibronectin-gold beads added.

sonicated to remove fibronectin-induced *S. aureus* aggregates.

In summary, direct opsonization of *S. aureus* by dimeric or multimeric fibronectin for mononuclear or polymorphonuclear phagocytes has not been found. However, fibronectin did enhance the bactericidal capacity of monocytes held in tissue culture. Dimeric fibronectin might be important for the maintenance of circulating monocytes, whereas multimeric fibronectin may help to sustain their bactericidal activity after they leave the vascular compartment.

ACKNOWLEDGMENTS

This work was supported by funds from the Graduate School and Medical School, from the Department of Medicine Research Fund, and from Public Health Service grants AI16881, HL24885, and HL21644 from the National Institutes of Health. Special thanks go to Carol Fritsch, who prepared the manuscript.

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