

FIBRINOGEN/FIBRIN ON THE SURFACE OF MACROPHAGES:
DETECTION, DISTRIBUTION,
BINDING REQUIREMENTS, AND POSSIBLE ROLE IN
MACROPHAGE ADHERENCE PHENOMENA*

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After intraperitoneal (i.p.) injection of antigen into guinea pigs primed for delayed hypersensitivity, peritoneal macrophages adhere to each other and to the peritoneal serosal cells (1–3). This phenomenon, termed the macrophage disappearance reaction (MDR)¹ by Nelson, is inhibited by warfarin or heparin (2) and is mimicked by i.p. administration of thrombin to unsensitized animals (4). Although these data suggest that macrophage adherence may be mediated by activation of the clotting system, initial attempts to identify fibrin on macrophages by electron microscopy were unsuccessful (3). Because fibrin deposition has recently been shown to be a characteristic feature of classic cell-mediated hypersensitivity reactions in guinea pigs and man (5, 6), we have re-examined the possibility that mononuclear cells might interact directly with fibrinogen or fibrin (Fib). By means of immunofluorescent techniques, we have found that a substantial population of peritoneal mononuclear cells have a particular avidity for binding Fib to their cell surface.

Materials and Methods

Cell Preparation. Normal, 300–500 g male Hartley guinea pigs² were lightly anesthetized with ether, exsanguinated by cardiac puncture, and the peritoneal cavities lavaged with 100 ml of ice-

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¹ *Abbreviations used in this paper:* CTA, Committee on Thrombolytic Agents; CU, caseinolytic unit; Fib, fibrinogen, fibrin, and their antigenically related fragments which react with rabbit antiguinea pig fibrinogen; HBSS, Hanks' balanced salt solution; MDR, macrophage disappearance reaction; SBTI, soy bean trypsin inhibitor; TBS, 0.14 M NaCl-0.01 M Tris (hydroxymethyl) aminomethane, pH 7.4; TBS-Ca-Mg, TBS containing 1.3 mM Ca⁺⁺ and 0.8 mM Mg⁺⁺; TAME, tosyl arginine methyl ester.

² In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

cold buffer. Peritoneal cells were harvested by means of polyethylene tubing and washed three times at 4°C (144 g × 8 min) in 12 x 75 mm polypropylene tubes (Falcon Plastics, Div. of BioQuest, Los Angeles, Calif.). The buffers used were Hanks' balanced salt solution (HBSS) and 0.14 M NaCl-0.01 M Tris (hydroxymethyl) aminomethane, pH 7.4 (TBS). Heparin (10 U/ml, preservative free, Flow Laboratories, Inc., Rockville, Md.), calcium (as CaCl₂), and/or magnesium (as MgSO₄) were added as indicated in individual experiments. Cells were also obtained from animals with peritoneal exudates induced by oil (7) and glycogen (2) 4 days previously. The majority (>70%) of normal and exudate peritoneal cells consisted of macrophages (see below).

Pulmonary alveolar macrophages were obtained by a modification of the method of Myrvik et al. (8). Guinea pigs were sacrificed as above. The trachea was then incised and cannulated with a polyethylene catheter and the lungs were lavaged with 10–20 ml of cold HBSS containing 10 U/ml heparin. The cell population obtained was rich in macrophages, which accounted for 76.6 ± 4.9% of the mononuclear cells by functional criteria (neutral red uptake, see below).

Mononuclear cells were obtained by a standard Ficoll-Hypaque method (9) from heparinized (10 U/ml) guinea pig and human blood, using a Ficoll-Hypaque mixture with a specific gravity of 1.077. The resulting preparations consisted largely of lymphocytes but also included larger mononuclear cells (mean 4.5 and 7.4% for guinea pig and human blood, respectively) which pinocytosed neutral red and were presumed to be monocytes. Cells from lymph nodes and thymus were obtained by mincing these organs in cold heparinized HBSS and passing the suspensions through a 37 μm nylon sieve (Tobler, Ernst, and Traber, Inc., Elmsford, N. Y.).

Cells were tested for neutral red uptake by incubation in 200 μg/ml of this dye in HBSS for 10 min at room temperature (reference 10 and footnote 3). The percent of cells that ingested neutral red were then promptly scored by light microscopy.

Antisera. A fibrinogen-rich (91% clottable) fraction of normal guinea pig plasma, prepared by the ethanol-precipitation technique (11) in the laboratory of Dr. Robert W. Colman (University of Pennsylvania, Philadelphia, Pa.), was used to immunize rabbits. The pooled rabbit antisera were absorbed twice by passage through a column of guinea pig serum conjugated (12) to CNBr-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). The absorbed antiserum (anti-Fib) formed a single precipitin band against guinea pig plasma in agarose gel diffusion plates and had no activity against guinea pig serum, IgG, or albumin. Antibody activity, as measured by gel diffusion or immunofluorescence, could be abolished by absorption with a washed fibrin clot prepared from guinea pig fibrinogen and bovine thrombin. Antisera to guinea pig plasminogen were obtained by immunization of rabbits with guinea pig plasminogen prepared on a lysine-Sepharose affinity column by the method of Deutsch and Mertz (13). The antiserum, absorbed twice with plasminogen-depleted guinea pig plasma conjugated to Sepharose, formed a single line by gel diffusion and immunoelectrophoresis against guinea pig serum, plasma, and purified plasminogen.

The γ-globulin fraction (50% NH₄SO₄) of these antisera and of pooled normal rabbit serum were conjugated with fluorescein isothiocyanate (14). The IgG fractions obtained by DEAE-cellulose chromatography had a fluorescein/protein ratio of 1.8–3.2 and were used at concentrations of 0.15–0.26 mg/ml. Fluorescein-conjugated rabbit antisera (IgG fraction) to guinea pig IgG (reactive with IgG₁ and IgG₂) and albumin, and to human Fib and IgG were obtained from Cappel Laboratories, Inc., Downingtown, Pa. The specificity was confirmed by agarose gel diffusion and immunoelectrophoresis. Fab fragments were obtained by papain digestion of the IgG fraction from the rabbit antisera to guinea pig fibrinogen and purified by chromatography on carboxymethyl cellulose by the method of Porter (15). The Fab fraction was conjugated with fluorescein as above.

Immunofluorescence Technique. Washed cells (10⁶–10⁷/tube) were resuspended in 50 μl of appropriate dilutions of fluorescein-conjugated antisera in HBSS or TBS containing 1.3 mM Ca⁺⁺ and 0.8 mM Mg⁺⁺ (TBS-Ca-Mg) and incubated for 30 min at 4°C. Cells were then washed three times in cold buffer, resuspended in 10% glycerol in phosphate-buffered saline and viewed by fluorescence darkfield microscopy, as described (5). Mononuclear cells with and without surface fluorescence were enumerated; 200–400 cells were counted per sample. Cells with bright surface fluorescence were scored as positive. Alternating phase and fluorescence microscopy were used to aid in identifying positive cells.

³ Hammond, E. H., S. S. Salvaggio, and H. F. Dvorak. Manuscript submitted for publication.

Digestion and Reconstitution of Surface Fib. Enzymatic removal of surface Fib was attempted with plasmin. Guinea pig plasminogen was purified (13) to a specific activity of 27.2 caseinolytic units (CU)/OD₂₈₀ unit, representing a 360-fold purification from plasma. Washed cells ($4-8 \times 10^6$ /ml) in HBSS were incubated for 10 min at 37°C in reaction mixtures (0.25 ml) containing 0.60 C.U./ml plasminogen, and/or 160 Committee on Thrombolytic Agents (CTA) U/ml urokinase (Abbott Laboratories, North Chicago, Ill.), or in 20 tosyl arginine methyl ester (TAME) U/ml trypsin (TRL 100S, Worthington Biochemical Corp., Freehold, N. J.). Soy bean trypsin inhibitor (SBTI, Worthington Biochemical Corp.) was then added to a final concentration of 80 µg/ml and the cells were washed three times in HBSS and stained for Fib and IgG as described above.

Reconstitution of surface Fib was attempted on peritoneal cells collected and washed in the absence of divalent cations (TBS). These cells, which lack surface Fib (see Results), were incubated for 15 min at 37°C in various concentrations of fresh, heparinized guinea pig plasma diluted in TBS-Ca-Mg. The cells were then washed three times in buffer before fluorescent staining.

Anticoagulation. Animals were anticoagulated with warfarin as described previously (6). At the time of cell harvest, 2 days after receiving 25 mg warfarin i.p., these animals had whole blood clotting times of 10–20 min (normal <5 min). Cells were collected, washed, and stained in the presence of 10 U/ml heparin.

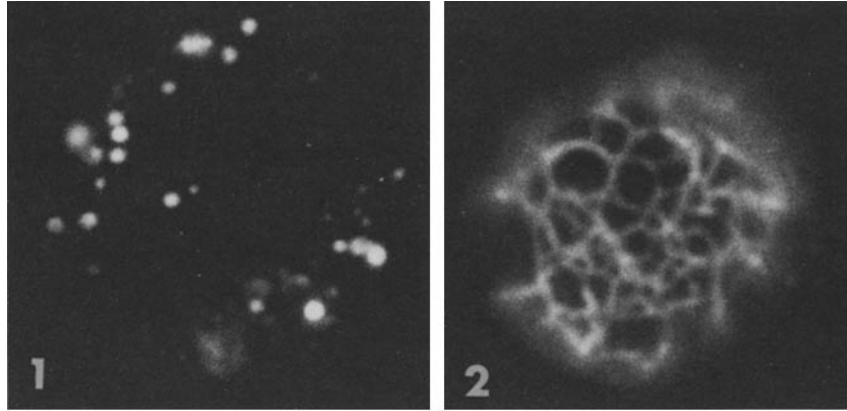
Quantitation of Fibrinogen. 5 µl of normal peritoneal fluid, obtained from five exsanguinated guinea pigs via a small midline abdominal incision, were immediately diluted in buffer containing EDTA and ε-aminocaproic acid (16). These samples and samples of citrated plasma from the same guinea pigs were assayed in duplicate in radial immunodiffusion plates prepared by the method of Edgington (16), using the rabbit anti-Fib antiserum described above, and standardized with purified guinea pig fibrinogen.

Results

Fib-Bearing Peritoneal Cells. Viable macrophages comprised the principal cell type lavaged from the normal guinea pig peritoneal cavity; $90 \pm 2\%$ of the cells avidly took up neutral red (but nuclei did not stain), and over 90% excluded trypan blue. The remainder of the cells consisted of lymphocytes, granulocytes, and rare erythrocytes.

When peritoneal cell suspensions were stained with fluorescein-conjugated anti-Fib, a substantial proportion of the mononuclear cells showed bright fluorescence on the cell surface (Figs. 1 and 2). Fib-bearing cells represented 34.2–51.2% of the mononuclear cells obtained by lavage of the normal peritoneal cavity with buffers containing Ca⁺⁺ (HBSS or TBS-Ca-Mg; Tables I, II, and VI). Two patterns of fluorescent staining were observed with anti-Fib. In one, the entire cell surface was covered with fine speckles or patches of fluorescence (Fig. 1), similar to the pattern observed with anti-IgG (see below). A second, quite distinctive, net or lace pattern (Fig. 2) was present on 27.5% of the mononuclear cells. The delicate, but brightly fluorescent fibrils demonstrated by immunofluorescence formed an interconnected network, closely associated with the cell surface, which usually completely surrounded the cell. Occasionally cells were present in small clumps, and these were almost always strongly positive for Fib in the net pattern (Fig. 3). Although different mononuclear cells in the same cell suspension exhibited the net or speckled pattern of surface Fib, the two patterns were not detected simultaneously on an individual cell. No cap formation was found. Similar results were obtained with exudates induced with oil and glycogen (mean, 37 and 68% Fib-positive cells, respectively). Absorption of antisera with a washed fibrin clot abolished the cell surface binding of the anti-Fib but not that of the anti-IgG conjugate.

To test whether antibody bridging or Fc receptors contributed to the observed



FIGS. 1 and 2. Fluorescence photomicrographs of mononuclear cells obtained by peritoneal lavage of normal guinea pigs with TBS-Ca-Mg buffer and stained with fluorescein-conjugated antiguinea pig Fib. Typical examples of the two patterns of Fib staining that were found in this study are illustrated. *Fig. 1.* The Fib is distributed over the cell surface in discrete patches, or speckles. *Fig. 2.* The entire cell surface is covered with a loose meshwork of brightly fluorescent fibrils which form a net pattern. Both $\times 3,900$.

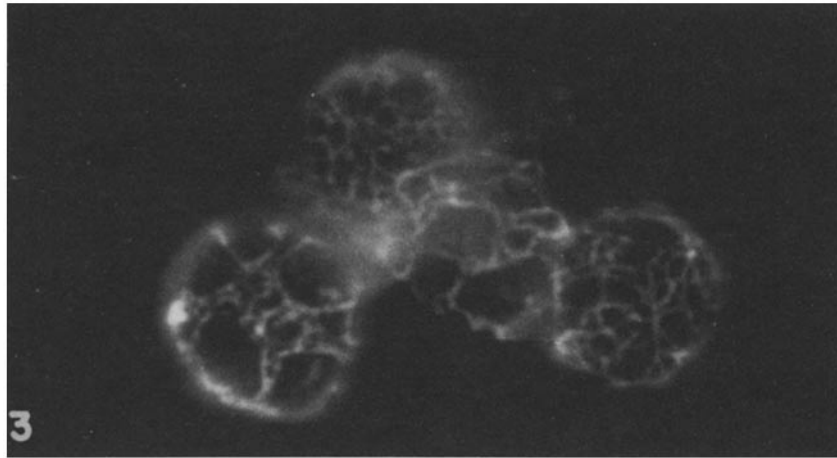


FIG. 3. Fluorescence photomicrograph of a small cluster of peritoneal macrophages stained for surface Fib as in Figs. 1 and 2. The adherent cells all exhibit a net pattern of Fib staining. This pattern was usually present on those cells that were adherent to each other in suspension. $\times 2,300$.

pattern of Fib staining, three pools of normal peritoneal macrophages were stained with fluorescein-conjugated Fab fragments prepared from rabbit anti-Fib. No differences were found in appearance or frequency of the net and speckled Fib patterns when macrophages were stained with this reagent (mean of $22.4 \pm 1.2\%$ of nets and $19.8 \pm 1.6\%$ speckles).

Anti-IgG stained 69% of the cells, always in a finely speckled pattern, consistent with that previously described for cytoplasmic IgG on guinea pig macrophages (17). Net patterns or caps were not found. In contrast, no cell surface staining

TABLE I
Cell Surface Fib and IgG on Normal Peritoneal Mononuclear Cells

Anticoagulants‡	N§	% Positive cells*			
		Fib			IgG
		Total	Speckles	Nets	
Exp. 1					
None	5	45.1 ± 7.6	17.7 ± 5.3	27.4 ± 6.2	69.4 ± 7.9
Warfarin heparin	4	31.7 ± 12.8	31.7 ± 12.8	0.0 ± 0.0	47.6 ± 15.9
Exp. 2					
None	6	34.2 ± 6.5	8.8 ± 1.6	25.4 ± 6.4	28.8 ± 10.7
Heparin	5	51.2 ± 4.8	18.0 ± 3.0	25.7 ± 8.3	26.4 ± 10.7

* The mean percent of mononuclear cells exhibiting surface fluorescence ± standard error. A total of 200-400 cells were counted in each sample. The patterns are described in the text.

‡ Cells were harvested from the peritoneal cavity of normal guinea pigs in HBSS (exp. 2) or TBS-Ca-Mg (exp. 1) containing no anticoagulants or 10 U/ml heparin as indicated. Cells were washed and stained in the same buffer with fluoresceinated anti-Fib or anti-IgG.

§ Number of animals.

|| These animals were pretreated with warfarin as described in the Materials and Methods.

whatsoever was detected with fluoresceinated antialbumin, antiplasminogen, or normal rabbit IgG.

Conditions Necessary for Cell Surface Fib Binding. The net pattern of staining of cell surface Fib suggested that fibrin was formed directly on the cell surface and that the alternative speckled pattern might be due to fibrinogen. Support for this hypothesis was obtained by study of guinea pigs anticoagulated with warfarin. Peritoneal cells from warfarin-treated animals were harvested, washed, and stained in the presence of 10 U/ml heparin. Under these conditions, the net pattern of surface Fib was absent, but the frequency of cells with the speckled pattern was almost doubled (exp. 1, Table I). In contrast, in animals not pretreated with warfarin, heparin in the lavage, wash, and staining steps did not prevent formation of the net pattern (exp. 2, Table I).

Preliminary studies revealed that harvest of cells in the presence of 5 mM EDTA in HBSS yielded cells with no detectable cell surface Fib. We then further explored the role of divalent cations on Fib binding (Table II). Peritoneal cells harvested in TBS lacking Ca⁺⁺ and Mg⁺⁺ consistently had almost no cell surface Fib (1.9%). However, with 1.3 mM Ca⁺⁺ in the TBS harvest and wash buffers, Fib-positive cells were present in their usual frequencies (32.5%). In contrast, substitution of Mg⁺⁺ for Ca⁺⁺ in these buffers, even in concentrations equal to the total divalent cation content of HBSS (2.1 mM), resulted in only rare Fib-positive cells. Thus Ca⁺⁺, but not Mg⁺⁺, was essential to cell surface Fib binding. As expected, neither cation influenced cytophilic IgG binding (18).

The affinity of the Fib for the cell surface in the presence of Ca⁺⁺ was indicated by its resistance to removal by repeated washing in HBSS (Table III). Only after seven washes was there evidence that any Fib had been removed from the cell surface.

TABLE II
Calcium Dependence of Fib Binding to Peritoneal Mononuclear Cells

Divalent cations present*			% Positive cells‡			
Ca	Mg	N‡	Fib			IgG
			Total	Speckles	Nets	
<i>mM</i>	<i>mM</i>					
1.3	0.8	5	45.1 ± 7.6	17.7	27.4	69.4 ± 7.9
0	0	7	1.9 ± 0.8	0.6	1.3	42.3 ± 14.3
1.3	0	6	32.5 ± 13.0	12.6	19.9	57.5 ± 12.6
2.1	0	6	50.7 ± 12.4	3.5	47.3	71.9 ± 9.7
0	0.8	6	7.5 ± 4.3	1.1	6.4	38.6 ± 12.7
0	2.1	6	4.3 ± 0.8	1.2	3.1	75.9 ± 8.3

* Cells were harvested from the peritoneal cavities of normal guinea pigs in TBS supplemented with Ca⁺⁺ or Mg⁺⁺ as indicated. Cells were washed three times in the same buffer and stained with fluoresceinated anti-Fib or anti-IgG in TBS-Ca-Mg.

‡ Same as Table I.

TABLE III
Resistance of Surface Fib to Repeated Washing*

No. of washes	% Positive cells‡		
	Total	Speckles	Nets
3	22.8	9.4	13.3
5	21.6	11.5	10.2
7	21.5	11.8	9.6
10	13.2	7.2	6.0

* Normal peritoneal cells were harvested and washed in HBSS (144 g × 8 min) at 4°C for the number of washes indicated. The cells were then stained with anti-Fib, washed three times subsequently, and positive cells were counted. Data are means of duplicate determinations from cells pooled from two donors.

‡ Same as Table I.

Reconstitution of Cell Surface Fib. The ability to obtain peritoneal cells that lacked cell surface Fib by harvest and washing in Ca⁺⁺-free medium permitted study of the capacity of these cells to bind extracellular fibrinogen. For these experiments, normal peritoneal cells were harvested and washed in TBS without Ca⁺⁺ or Mg⁺⁺. Cells were then incubated 15 min at 37°C in TBS-Ca-Mg containing 10 U/ml heparin and 0–100% fresh heparinized guinea pig plasma. Cells were washed three times in plasma-free buffer and stained with anti-Fib or anti-IgG. Reconstitution of cell surface Fib was demonstrable in the presence of as little as 5% plasma and reached maximal levels in 20% plasma (Table IV). By comparison, IgG staining also increased to saturation levels in the range of 10–50% plasma. Nearly all Fib-positive cells exhibited the speckled staining pattern, although occasionally cells revealed a net pattern. Control incubations

TABLE IV
*Reconstitution of Cell Surface Fib**

% Plasma during incubation	% Positive cells‡			
	Fib			IgG
	Total	Speckles	Nets	
0	1.5 ± 0.8	0.6	0.9	42.7
5	11.1 ± 2.8	9.5	1.6	75.6
10	8.9 ± 3.0	7.2	1.7	85.8
20	26.7 ± 4.7	25.5	1.2	79.2
50	20.6 ± 3.3	19.5	1.0	92.4
100	20.4 ± 4.5	17.7	2.7	91.6
Serum (10%)	0.4	0.2	0.2	94.0

* Normal peritoneal cells were harvested and washed three times in TBS without Ca^{++} or Mg^{++} as in Table II. Cells were then pooled from two or more guinea pigs and divided into aliquots for incubation in TBS-Ca-Mg containing 10 U/ml heparin and the indicated amount of fresh normal heparinized guinea pig plasma or serum. After 15 min. at 37°C cells were washed and stained as in Table I. Data represent means from five separate experiments.

‡ Same as Table I.

in serum (10%) were as effective as plasma in increasing the frequency of IgG-positive cells, but were totally ineffective in restoring surface Fib.

Because peritoneal cells had the capacity to bind Fib to their surfaces in the presence of extracellular fibrinogen, it was of interest to determine whether fibrinogen might be present in normal peritoneal fluid. By radial immunodiffusion, fibrinogen concentration averaged 0.58 ± 0.05 mg/ml in peritoneal fluid from five normal guinea pigs, whose mean plasma fibrinogen level was 3.25 ± 0.12 mg/ml. Thus, peritoneal fibrinogen content was equivalent to 17.9% that of plasma, close to the 20% plasma concentration found to result in maximal cell surface Fib binding in vitro.

Removal of Cell Surface Fib by Plasmin. As a further test that the fluorescent staining surface material we observed was actually fibrinogen/fibrin and to determine whether this material was accessible to proteolytic digestion, normal peritoneal cells were exposed to plasmin, the principal plasma fibrinolytic enzyme. Peritoneal cells collected in HBSS ($2-8 \times 10^6$ /ml) were incubated for 10 min at 37°C in 0.6 CU/ml of purified guinea pig plasminogen which was activated with 160 CTA U/ml urokinase. SBTI (80 $\mu\text{g}/\text{ml}$) was added and the cells were washed three times in HBSS and stained with anti-Fib and anti-IgG (Table V). Virtually all of the cell surface Fib was removed by this treatment (1.4% Fib-positive cells remained), while control incubations of cells without added enzymes, or with plasminogen or urokinase alone, resulted in 49–52% Fib-positive cells. In contrast, cytophilic IgG was not appreciably removed from the cell surface under these conditions. Cell surface Fib was also more sensitive than IgG to another proteolytic enzyme, trypsin, when used at 20 U/ml in simultaneous assays (Table V).

TABLE V
Removal of Cell Surface Fib by Plasmin and Trypsin

Incubation*		% Positive cells‡	
Plasminogen	Urokinase	Fib	IgG
0	0	51.9 ± 4.5	56.7 ± 18.5
+	+	1.4 ± 0.4	58.3 ± 15.8
+	0	49.4 ± 4.2	81.8 ± 5.8
0	+	50.3 ± 3.9	86.4 ± 1.1
Trypsin		0.3 ± 0.3	68.4 ± 13.8

* Normal peritoneal cells harvested and washed in HBSS from two or more guinea pigs were pooled and divided for incubation at 37°C for 10 min. in 0.6 CU/ml plasminogen, 160 U/ml urokinase, and/or 20 U/ml trypsin as indicated (1–2 × 10⁶ cells in 0.25 ml). SBTI (80 μg/ml) was added and the cells were washed and stained at 4°C as in Table I. Values represent means from four experiments.

‡ Same as Table I.

Identification and Distribution of Fib-Bearing Cells. Because over 90% of the mononuclear cells obtained by peritoneal lavage from normal guinea pigs were macrophages by morphologic (phase microscopy) and functional (neutral red uptake) criteria and 30–50% of this cell population had demonstrable cell surface Fib, it seemed likely that the majority of Fib-bearing cells were macrophages. Moreover, when individual cells were viewed with alternating fluorescence and phase microscopy, most Fib-positive cells could be identified as typical macrophages, with abundant cytoplasm containing scattered vacuoles or granules and an eccentric round or indented nucleus (Fig. 4). Occasional Fib-positive cells were intermediate in size between typical macrophages and small lymphocytes and could not be classified with certainty. Small lymphocytes, granulocytes, and erythrocytes never exhibited Fib staining.

Fib-bearing cells were sought in a number of other organs outside the peritoneal cavity. Only rare Fib-bearing cells were found among the cells from peripheral blood, lymph nodes, thymus, and tracheal lavage (chiefly alveolar macrophages) (Table VI). Because more manipulations were necessary to obtain cells from certain of these organs, it is conceivable that some Fib-bearing cells were lost in the course of preparation. However, macrophages from the lung were obtained by simple tracheal lavage and yet very few (0.8%) had cell surface Fib, in marked contrast to the macrophages from the peritoneal cavity of the same animals (51.2%). The alveolar and peritoneal cell populations had similar percentages of mononuclear cells positive for cytophilic IgG (22.7 and 26.4%, respectively) and cells which avidly ingested neutral red (76.6 and 90.2%, respectively).

Fib-bearing Cells in Human Peripheral Blood. Human peripheral blood leukocytes were obtained by sedimentation over Ficoll-Hypaque of eight samples of heparinized blood from five normal adults. Such cell preparations chiefly contain lymphocytes, but also contain a considerable percentage of monocytes (19). In our samples, 7.4 ± 1.9% of the mononuclear cells took up neutral red

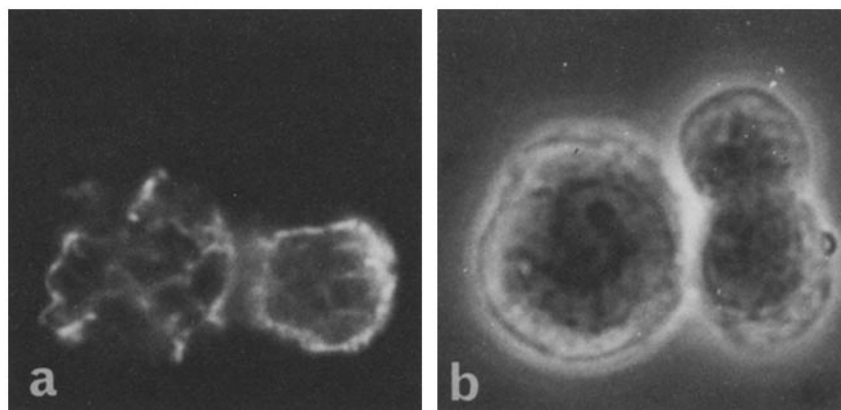


FIG. 4. A cluster of three peritoneal mononuclear cells stained for surface Fib as in Figs. 1 and 2. (a) Fluorescence photomicrograph. The two lower cells have bright staining surface Fib. The Fib-negative cell above them is barely visible. (b) The same cells, viewed by phase microscopy. The irregular nucleus and the cytoplasmic granules and vacuoles indicate that the two lower cells are macrophages. Although the identity is less certain, the small, Fib-negative cell is probably a lymphocyte. $\times 2,400$.

TABLE VI
*Distribution of Fib-Bearing Mononuclear Cells**

Cell source	N‡	% Positive cells‡	
		Fib	IgG
Blood	6	1.4 \pm 0.7	10.3 \pm 1.9
Lymph node	4	0.4 \pm 0.2	25.1 \pm 4.4
Thymus	4	0.0 \pm 0.0	0.7 \pm 0.1
Lung§	4	0.8 \pm 0.5	22.7 \pm 12.2
Peritoneal fluid	6	51.2 \pm 4.8	26.4 \pm 10.7

* Cells were obtained as described in the Materials and Methods from normal guinea pigs.

‡ See Table I. Values represent the total percent of positive cells. All Fib-positive cells exhibited the speckled pattern; the net pattern was demonstrable only on the peritoneal cells.

§ The majority of these mononuclear cells (>75%) were alveolar macrophages (see Materials and Methods).

avidly and were believed to be monocytes. When stained with fluoresceinated antihuman Fib, a small fraction ($4.6 \pm 2.1\%$) of the mononuclear cells were positive. Only the speckled pattern was found. The frequency of Fib-bearing cells was directly correlated with the frequency of neutral red-positive cells ($r = 0.925$, $P < 0.01$). Anti-IgG stained $1.5 \pm 0.8\%$ of the cells. Absorption of the conjugates with a washed human fibrin clot removed all activity from the anti-Fib antiserum, but not from the anti-IgG control.

Discussion

These data demonstrate that a significant percentage of normal and exudate macrophages isolated from the guinea pig peritoneal cavity has Fib bound to the

cell surface where it is readily detected by immunofluorescence. Binding of Fib is reversible, requires Ca^{++} , and can occur under conditions in which fibrin formation is prevented by heparin. This unexpected finding raises the possibility that the clotting system may have a significant role in certain functions of the macrophages, a cell thought to be of central importance in cell-mediated immunity (20).

As detected by immunofluorescence, Fib exists on the cell surface in two distinct patterns. Only a particulate or speckled fluorescent pattern of staining is seen when the coagulation system is inhibited *in vivo* by warfarin or *in vitro* during reconstitution of cell surface Fib from heparinized plasma. This pattern resembles that of many other cell surface components, such as alloantigens (21) and cell-bound immunoglobulins (17, 22) when detected on a variety of cells by similar techniques. However cell-surface Fib was also found in a distinctive net pattern, characterized by a meshwork of fibrils covering most or all of the cell surface. The two different patterns are found, on separate cells, when peritoneal cells are collected with standard balanced salt solutions containing calcium. We hypothesize that the net pattern represents fibrin formed by the clotting mechanism from fibrinogen present on the cell surface. Fibrin has rarely been identified on the surface of peritoneal macrophages by its characteristic ultrastructural periodicity (A. M. Dvorak, unpublished observations). Our repeated attempts to induce the net pattern of Fib *in vitro* with thrombin have been complicated by the macrophage clumping caused by this enzyme (R. B. Colvin, E. H. Hammond, and H. F. Dvorak, unpublished observations). Although Fib could be identified at points of cell contact by immunofluorescence, nets covering the entire cell surface were rare.

The binding of Fib to the macrophage surface requires Ca^{++} , but not Mg^{++} , indicating that the fibrinogen receptor is distinct from that for cytophilic IgG, which has no divalent cation requirement (18), and from that for C3, which requires Mg^{++} , but not Ca^{++} (23). One other macrophage receptor, that for homologous IgM in the mouse, has also been found to require Ca^{++} (24).

The identity of the receptor(s) that binds Fib on the surface of the macrophage is unknown at present, nor is it known which portions of the fibrinogen molecule are bound. One Fib-binding protein has recently been identified as a cryoprecipitable globulin present in normal plasma (25) and on the cell surface of fibroblasts derived from various species, including man (26, 27). Published immunofluorescence photomicrographs (26, 28) of the net-like pattern of this binding protein on cell surfaces appear quite similar to net pattern of Fib reported here. However, the failure to demonstrate that cold insoluble globulin could bind Fib at physiologic temperatures (25) is a serious, but perhaps not insurmountable, difficulty in accepting this protein as a normal Fib receptor.

The source of the cell surface Fib is presumed to be the extracellular fluid, because cells lacking in Fib can be converted to Fib-bearing cells by a 15 min incubation in heparinized plasma. Such Fib uptake occurs *in vitro* under conditions in which the clotting system is not activated and at fibrinogen concentrations comparable to those found in normal peritoneal fluid. Studies are in progress to determine whether the macrophages may also synthesize fibrinogen.

Although resistant to washing, cell surface Fib was readily removed by plasmin and, in fact, was more sensitive to plasmin and trypsin than was cell-

bound IgG. Whether this is due to variations in the intrinsic susceptibilities of Fib and IgG to these proteolytic enzymes or to differences in exposure of these molecules on the cell surface remains to be determined. It was important to establish that cell surface Fib was susceptible to plasmin digestion, because others have shown that stimulated macrophages produce a plasminogen activator (29), and plasmin thus generated might act on the cell's surface Fib with consequences affecting such properties as macrophage migration (E. H. Hammond, R. Roblin, H. F. Dvorak, and R. B. Colvin, unpublished data).

Interactions between fibrinogen or fibrin and the surfaces of other cells have been described. Niewiarowski et al. demonstrated that human fibroblasts and, to a lesser extent, peripheral blood leukocytes bind polymerizing fibrinogen and can cause clot retraction (30). Furthermore, he showed that platelet membranes bind fibrinogen, and that Ca^{++} is required for such binding, just as we have shown for macrophages in the present study. Recent evidence indicates that the fibrinogen on the platelet surface mediates thrombin-induced platelet aggregation (31).

By analogy with platelets, Fib bound to the macrophage surface might have a role in intercellular contacts or cell adherence, possibly by means of fibrin bridging. Indeed, previous data suggest a role for fibrin in the MDR, a reaction characterized by the adherence of macrophages to each other and to peritoneal serosal cells after antigenic challenge of sensitized guinea pigs (3). The MDR is inhibited by anticoagulants (2) and can be mimicked by the intraperitoneal injection of thrombin (4). Other evidence that fibrin does play a role in macrophage-macrophage adhesion comes from observations that clumps of macrophages, when present in the cell suspensions studied by immunofluorescence, typically had Fib on their surface and that thrombin can induce macrophage clumping in vitro (R. B. Colvin, E. H. Hammond, and H. F. Dvorak, unpublished observations). Because there is good evidence that the MDR (32) and the closely related macrophage aggregation reaction (33) are mediated by soluble products of stimulated lymphocytes, it will be of particular interest to determine whether lymphokines may interact with the clotting system.

Alveolar macrophages lacked cell surface Fib, and in this respect differed markedly from peritoneal macrophages, although both populations of macrophages avidly ingested neutral red and had cytophilic IgG on their surfaces. It is not known whether this is due to a lack of available extracellular fibrinogen in pulmonary alveoli or to a deficiency of Fib receptors on the pulmonary macrophages. Alveolar macrophages also differ from peritoneal macrophages in other respects (10), including response to migration inhibition factor (7) and to chemotactic stimuli (e.g., serum) (34, 35), but there is no evidence at present that these phenomena are related to the lack of surface Fib.

Mononuclear cells with surface Fib can be detected in small numbers in the peripheral blood of guinea pigs and man. While the identity of these cells is not yet certain, they are most probably monocytes, and it is tempting to speculate that they are the precursors of the Fib-positive macrophages found in much greater frequency in the peritoneal cavity. It is also possible that macrophages acquire receptors for Fib in the course of their differentiation from monocytes in the peritoneal cavity. In any event, it is clear that by the production of plasminogen activator (29) and tissue factor (36) and by the binding of Fib on their

surface, certain mononuclear cells can interact with the clotting system at several levels. Further studies are necessary to reveal the full extent and biologic significance of this interaction.

Summary

The peritoneal cavity of guinea pigs proved to be a rich source of mononuclear cells (34–52%) with fibrinogen or fibrin (Fib) on their surface. The Fib was readily detected on the surface of viable cells in suspension by fluorescence microscopy using antisera to guinea pig fibrinogen. The fluorescent staining occurred either in a speckled distribution, similar to that of cytophilic IgG, or in a distinctive net-like pattern that probably represented fibrin formation on the cell surface. The binding of Fib to the cell surface required calcium, but not magnesium, in the medium and could occur *in vitro* during incubation in heparinized plasma that contained fibrinogen concentrations comparable to that in normal peritoneal fluid (0.58 mg/ml). Cell surface Fib was more susceptible to plasmin and trypsin digestion than surface cytophilic IgG. By morphologic and physiologic criteria, cells exhibiting surface Fib were chiefly, if not exclusively, macrophages. Granulocytes, erythrocytes, and lymphocytes from lymph node and thymus had no appreciable Fib. Cells with surface Fib were rarely observed among mononuclear cells prepared by Ficoll-Hypaque sedimentation of guinea pig and human blood (1.4 and 4.6%, respectively). Pulmonary alveolar macrophages, functionally distinct from peritoneal macrophages, lacked surface Fib (0.8%).

Polymerization of Fib on the surface of macrophages might participate in certain cell interactions, such as the adherence of peritoneal macrophages during the antigen-induced macrophage disappearance reactions. The unexpected finding of Fib binding to the surfaces of peritoneal macrophages raises the possibility of a biologically significant interaction between these cells and the clotting system.

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