# RAPID COMMUNICATIONS

## IDENTIFICATION OF MACROPHAGE EXTERNAL MEMBRANE PROTEINS AND THEIR POSSIBLE ROLE IN CELL ADHESION

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ABSTRACT

Starch-activated mouse peritoneal macrophages (STpMAC) plated on plastic demonstrate the adhesive properties typical for activated pMAC: attaching as round cells and, within 15 min, spreading out with marginal membrane ruffles. These attached STpMAC were labeled by lactoperoxidase-catalysed $^{125}$I surface iodination, sodium dodecyl-sulfate-lysed, and the lysates electrophoresed on polyacrylamide gels which were examined by autoradiography. The STpMAC morphological phenotype correlates with the labeling of a particular protein (195,000, estimated mol wt). Normal pMAC (NpMAC), from unstimulated mice, do not spread and do not display the 195,000 band. Both pMAC band patterns, including the 195,000 band, are relatively resistant to trypsin digestion, as is pMAC adhesion itself trypsin-resistant. Neither class of pMAC exhibits fibronectin (Cell Adhesion Factor, LETS protein) which is a component in the adhesive matrix of cells forming trypsin-sensitive monolayers. When pMAC are tested against antifibronectin antibody, these cells do not give immunofluorescent staining. In summary, two functions in pMAC adhesion, enzyme resistance and the ability to spread, appear related to molecular properties distinctive for pMAC surface protein.

KEY WORDS    macrophage · plasma membrane · cell adhesion · fibronectin · cell morphology

Cells of the mononuclear phagocyte system, macrophages, are functionally distinct from other adherent cell types in their interactions with artificial substrates (2, 3, 6, 12, 19, 22, 23). For example, peritoneal macrophages do not form continuous monolayers of confluent cells; rather, the cells remain as discrete units in culture, even when plated at high density. Once attached, these cells cannot be freed from glass or plastic by trypsin. They adhere to substrates either as round cells or as spread cells that are flat, motile, and exhibit peripheral ruffled membranes (2, 3, 5, 19). What is particularly notable is that the form of peritoneal macrophage adhesion can be a useful indica-tor of the physiological status of the whole population (2, 12). That is, macrophages, obtained from an animal undergoing an inflammatory reaction spread on substrates, exhibit high rates of metabolism and high levels of specific enzyme synthesis and secretion (2, 3, 12, 22, 23). Macrophages from an untreated animal plate as round cells and are relatively quiescent in their physiology.

Our immediate purpose has been to compare the pattern of cell surface labeling obtained by lactoperoxidase-catalysed 125-iodination of both physiologically activated and normal peritoneal macrophages. We have compared the patterns generated with those of other adherent cells and we have examined the qualitative relationship between trypsin resistance of adhesion with tryp-sin resistance of the iodine-labeled surface moie-ties. Although other kinds of differences are not-

able, we have limited our discussion here to the occurrence of certain high molecular weight proteins.

## MATERIALS AND METHODS

### Macrophages

Both normal (NpMAC) and activated (STpMAC) mouse peritoneal macrophages were obtained by standard methods. STpMAC were derived by peritoneal injection of 2% starch solution 3 days before harvest. Briefly, pMAC were collected by lavage of killed mice with 1-2 cycles of injected 5 ml N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-buffered Eagle's MEM, 5 U heparin/ml. Cells were kept on ice until washed, counted, and planted in MEM with 10% fetal calf serum. A rigorous protocol to establish the differential adhesion of pMAC from the peritoneal exudate was adopted: after inoculation, dishes were incubated for only 10 min at 37°C, then washed twice with cold saline lacking Ca and Mg. The cell population adherent after this procedure constituted the pMAC (see also reference 4 for characterization of these cells).

### Mouse Embryo Cells (MEC)

Third passage MEC were plated after brief trypsinization and cultured in 10% fetal calf serum-MEM.

### Iodination

Lactoperoxidases-catalysed iodination was performed essentially as described (7, 16) except that all solutions were at 0°-4°C. N or STpMAC (2-4 × $10^6$/plate) or confluent MEC adherent to 6-cm plastic dishes (Bio-Quest, BBL, and Falco, Products, Becton, Dickinson, and Co., Cockeysville, Md.) were washed three times with phosphate-buffered saline containing calcium and magnesium (PBS; Grand Island Biological Co., Grand Island, N.Y.), and 0.5 ml of PBS containing 0.9 mg/ml $\beta$-D-glucose and carrier-free $Na^{125}I$ (400 $\mu$Ci/ml) was added. 10 $\mu$l of an enzyme mixture containing 1 mg/ml lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) and 15 U/ml glucose oxidase (Sigma Cemical Co.) were added and the dishes were incubated for 10 min with occasional rocking. 5 ml of physiological phosphate-buffered iodide were added to stop the reaction; the dishes were washed twice with PBS and the cells lysed directly on the dish by the addition of 0.2 ml of 2% sodium dodecyl sulfate, 10% glycerol, 0.1 M tris-HCl, and 2 mM phenyl methyl sulfonyl fluoride, pH 6.8. The resultant viscous solution was pipetted from the dish and frozen until use.

As a second labeling strategy, whole peritoneal exudate cell populations were iodinated in suspension directly after recovery and washing. Labeled cells were then plated in serum-free PBS (37°C, 30 min), and the adhesive fraction was selected as before and then lysed.

It should be noted that, without serum, both the rate of attachment and the degree of spreading are reduced.

Labeling of erythrocytes (17), fibroblasts (15), and lymphocytes (20) by lactoperoxidase-catalysed iodination has been shown to be restricted to plasma membrane constituents. In the study of pMAC plasma membrane components, the addition of reagents at 0°-4°C for labeling has been done to eliminate the internalization of label.

### Polyacrylamide Gel Electrophoresis

Before electrophoresis, samples were boiled for 2 min with 100 mM dithiothreitol. Polyacrylamide gel electrophoresis (18) was performed in a slab gel (10) at an acrylamide monomer concentration of 7.5%. After completion of the electrophoresis, the slab gels were dried in vacuo and autoradiographed using Kodak X-ray film RP/R54 to locate the radioactively labeled components. For autoradiography, equal amounts of radioactivity were applied for each sample.

### Immunofluorescence

MEC or pMAC were plated on cover slips in 35-mm dishes at subconfluent cell densities. All cells were fixed in 10% formalin, and some were then made permeable with acetone:methanol, 7:3, −20°C. All cover slips were then pre-incubated with calf serum, incubated with rabbit antifibronectin, and stained with fluorescein-goat anti-rabbit IgG. Incubations were done at 37°C with PBS washes between each step. Antibody was prepared as described in reference 14. The pre-incubation in calf serum aided in the suppression of background staining due to nonspecific binding of immunoglobulins by pMAC, a problem that does not occur with MEC.

## RESULTS AND DISCUSSION

When first plated, populations of NpMAC from unstimulated animals and STpMAC from those stimulated were clearly distinguishable. NpMAC appeared to be smaller, uniformly refractile in the phase microscope, and round. The STpMAC appeared more vacuolated and, although initially round like NpMAC immediately after attachment, STpMAC rapidly spread flat on the plastic. Fig. 1 shows the N and STpMAC populations as prepared for subsequent iodination. Once spread, STpMAC were motile. The cytological distinctions of the STpMAC are also found in pMAC activated by other methods (2, 3, 12, 19).

In comparing the electrophoresis patterns of the adherent N and STpMAC (Fig. 2), it can be seen that a striking difference occurs in the prominent high molecular weight bands. STpMAC possess a major band absent or in relatively smaller
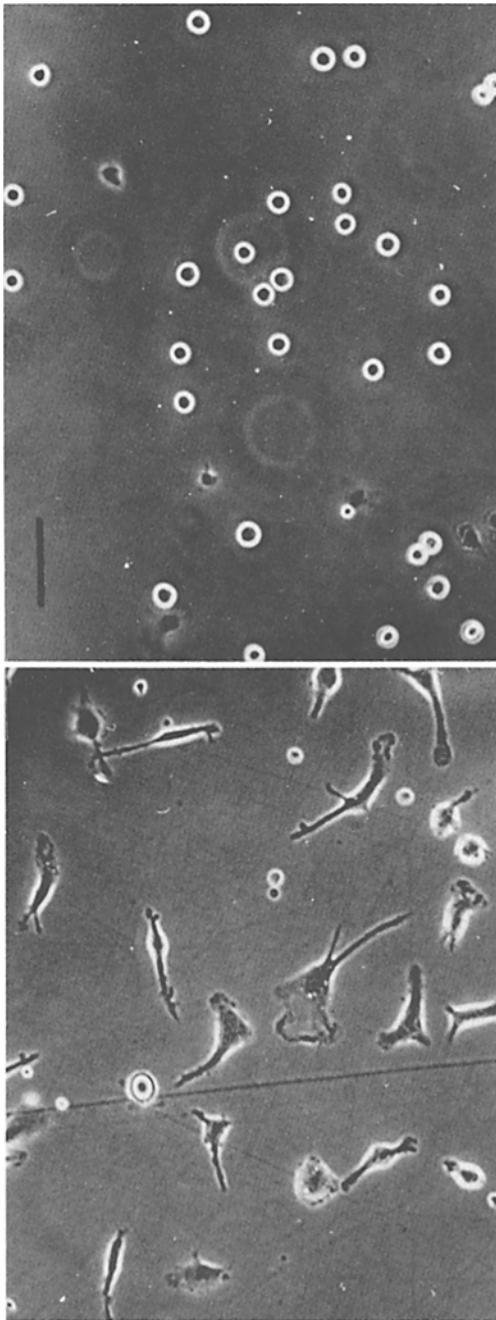
FIGURE 1 Morphologies of pMAC from unstimulated mice (top) or from mice stimulated by in vivo starch (bottom). Cells were photographed within 15 min of incubation at 37°C, after isolation and washing at 4°C, and before iodination. Phase-contrast, obj. ×16. Bar, 50 μm.
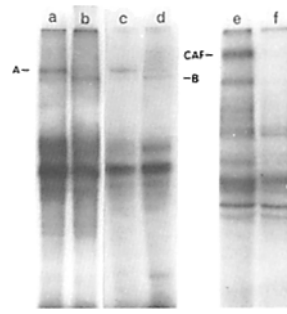


FIGURE 2 Autoradiograph of dithiothreitol-sodium dodecyl sulfate-7.5% polyacrylamide slab gel. Slots a, b, e: 125-iodinated band patterns of STpMAC, NpMAC, MEC, respectively. Slots c, d, f: STpMAC, NpMAC, and MEC labeled, then treated with trypsin (TPCK-treated, Worthington Biochemical Corp., Freehold, N.J.), 10 μg/ml/10 min at 20°C. Glucose oxidase/lacto-peroxidase-catalysed iodination was performed as usual except that solutions were at 4°C (7, 10, 15, 17). All samples were normalized for equal amounts of radioactivity. Fibronectin, band CAF; pMAC bands A and B.

amounts on NpMAC (Fig. 2a, A). Reciprocally, NpMAC display a major band either absent or available in relatively insignificant amounts on STpMAC (Fig. 2b, B). When run in comparison with molecular weight markers (not shown), A is of an estimated molecular weight of 195,000, and B of 180,000.

MEC grown to confluence and labeled show the major band pattern typical for many normal cells in culture (7, 15, 16, 25). These cells display a high molecular weight constituent (220,000–250,000) (Figure 2e, CAF) that has been shown to be reduced or absent from cell surfaces after transformation. And this protein (fibronectin) can effect the adhesiveness and spreading of normal or transformed cells (11, 13, 26). On the contrary, the attached and round NpMAC and the attached and spread STpMAC do not display this protein. This is confirmed by immunofluorescence examination using anti-fibronectin for surface or whole-cell staining. Neither NpMAC nor STpMAC bind the antibody. Fig. 3 shows that the external fibronectin meshwork found on MECs is absent on STpMAC.

One major functional difference between the adhesion of pMAC and MEC is related to trypsin sensitivity. MEC are readily suspended by trypsin, but appreciable numbers of pMAC are not. In addition, the constituents exposed on the surface of MEC, including fibronectin, are trypsin diges-
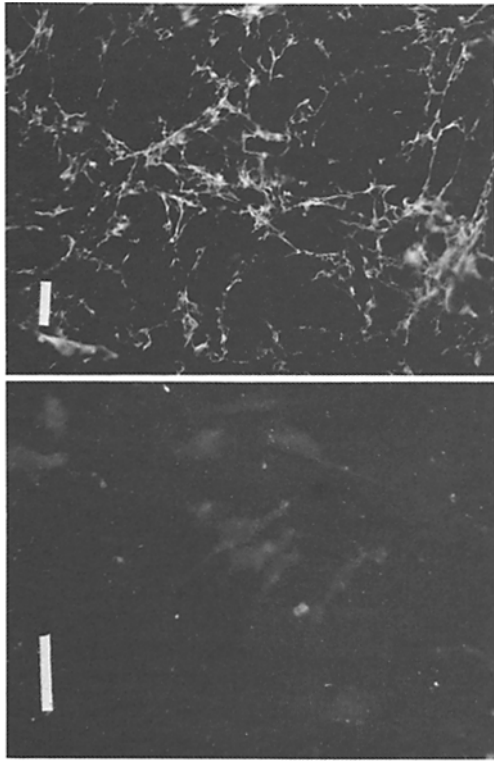
FIGURE 3 MECs show specific binding of anti-fibronectin with the typical surface and intercellular distribution of the antigen (top). STpMAC do not stain specifically, showing only background binding of immunoglobulins (bottom). Preparations were similar to those used for iodination and fixed in 10% formalin. UV-fluorescence, obj. ×25. Bars, 50 μm.
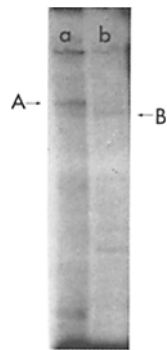


FIGURE 4 Radioiodination profiles of STpMAC (slot a) and NpMAC (slot b) labeled in serum-free conditions in vitro (see Materials and Methods). The reciprocal appearance of bands A and B is evident in these controls, although both the rate of attachment and degree of spreading are reduced.

tible (7, 16). The question was posed as to whether the trypsin-resistant attachment of pMAC for the plastic correlated with a similar resistance of surface constituents to trypsin digestion. To test the sensitivity of the major surface constituents to removal by trypsin, labeled plates of cells, before lysis, were treated with enzyme. Fig. 2c, d, and f show the electrophoresis patterns of STpMAC, NpMAC, and MECs, respectively, after both labeling and mild trypsinization. It is notable that, under conditions in which trypsin removes fibronectin, pMAC bands A and B are retained. Using other conditions, other workers have noted that a significant proportion of pMAC surface protein resists proteolysis (27).

The finding that pMAC surface components A and B clearly resist proteolysis to a degree that MEC proteins, e.g., fibronectin, do not is compatible with pMAC activity in vivo. For macrophages to retain functional adhesion and migratory capability at sites of inflammation or tissue remodeling, surface resistance to protease attack is a requirement. Macrophages synthesize and secrete many of the enzymes usually involved in the breakdown of extracellular matrix and solid tissues (3, 21, 24). The major bands A and B of pMAC adherent in vitro may be involved in the adhesion process. The initial indications of this are (a) the reciprocal appearance of A and B on ST and NpMAC, respectively, (b) the correlation of A and B with the degree of cell spreading, i.e., the morphologies of ST and NpMAC, and (c) the relative resistance to trypsin attack of A and B. Agents that can effect pMAC spreading include activated complement and coagulation factors and proteases (1, 19). In other work (reference 5 and Dienstman, Pearlstein, and Defendi, manuscript in preparation), we can demonstrate changes in labeling of A and B by changing pMAC spreading or attachment. Band A, however, is characteristic of the activated state of pMAC.

Bands A and B most likely reflect cell-generated membrane proteins and not contaminating components from fetal calf serum (FCS). When ST and NpMAC are iodinated using an alternative strategy (see Materials and Methods) without exposure to FCS, each band is still detected (Fig. 4). Of course, even iodination of A and B without exposure to FCS in vitro does not exclude the possibility that these cells acquire exogenous proteins during their life cycle in vivo. Exhaustive washing of pMAC does not alter the pattern of iodination, and the two bands resist trypsin treat-

ment. Plasma or serum contaminants usually are removed by this treatment (Pearlstein, unpublished observation).

Why pMAC do not display surface fibronectin found on MEC and other cell types that form trypsin-sensitive monolayers in culture is not clear. While it is possible that pMAC degrade this protein themselves (8, 9), we also have been unable to identify any of its antigenic determinants superficially, intracellularly, or in extracellular supernates (Fig. 3, and Dienstman, Pearlstein, and Defendi, manuscript in preparation). The apparent lack of this constituent on macrophage surfaces as well as on lymphoid and erythroid surfaces (18, 20) may represent a common property of blood-borne cells, i.e., the absence of fibronectin may be a feature of hematopoietic cells differentiated for release into the circulation.

## REFERENCES

1. BIANCO, C., A. EDEN, and Z. COHN. 1976. The induction of macrophage spreading: role of coagulation factors and the complement system. *J. Exp. Med.* **144:**1531-1544.
2. BLANDEN, R. 1968. Modification of macrophage function. *J. Reticuloendothel. Soc.* **5:**179-202.
3. CARR, I. 1970. The fine structure of the mammalian lymphoreticular system. *Int. Rev. Cytol.* **27:**283-348.
4. DIENSTMAN, S. R., and V. DEFENDI. 1978. Necessary and sufficient conditions for recruitment of macrophages into the cell cycle. *Exp. Cell Res.* **115:**191-199.
5. DIENSTMAN, S. R., E. PEARLSTEIN, and V. DEFENDI. 1978. The macrophage surface: specific constituents related to cell type and cell shape. *In* 10th Miami Winter Symposium. J Schultz and W. Whelan, editors. Academic Press, Inc., New York. In press.
6. DIENSTMAN, S. R., E. PEARLSTEIN, P. WEISZ, and V. DEFENDI. 1977.

Cell surface comparisons among normal, activated, and virus-transformed macrophages. *J. Cell Biol.* **75:**69a. (Abstr.)
7. HYNES, R. 1973. Alteration of cell surface proteins by viral transformation and by proteolysis. *Proc. Natl. Acad. Sci. U. S. A.* **70:**3170-3174.
8. HYNES, R. 1974. Role of surface alterations in cell transformation: the importance of proteases and surface proteins. *Cell.* **1:**147-156.
9. HYNES, R., and E. PEARLSTEIN. 1976. Investigations of the possible role of proteases in altering surface proteins of virally transformed hamster fibroblasts. *J. Supramol. Struc.* **4:**1-14.
10. LAEMMLI, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680-685.
11. MAUTNER, V., and R. HYNES. 1977. Surface distribution of LETS protein in relation to the cytoskeleton of normal and transformed cells. *J. Cell Biol.* **75:**743-768.
12. MORLAND, B., and G. KAPLAN. 1977. Macrophage activation in vivo and in vitro. *Exp. Cell Res.* **108:**279-288.
13. PEARLSTEIN, E. 1976. Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. *Nature (Lond.).* **262:**497-500.
14. PEARLSTEIN, E., and L. GOLD. 1978. The high molecular weight glycoprotein as a mediator of cell adhesion. *Ann. N. Y. Acad. Sci.* In Press.
15. PEARLSTEIN, E., R. HYNES, L. FRANKS, and V. HEMMINGS. 1976. Surface proteins and fibrinolytic activity of cultured mammalian cells. *Cancer Res.* **36:**1475-1480.
16. PEARLSTEIN, E., and W. WATERFIELD. 1974. Metabolic studies on $^{125}$I labeled baby hamster kidney cell plasma membrane. *Biochim. Biophys. Acta.* **363:**1-12.
17. PHILLIPS, D., and M. MORRISON. 1970. The arrangement of proteins in the human erythrocyte membrane. *Biochem. Biophys. Res. Commun.* **40:**284-289.
18. PHILLIPS, D., and M. MORRISON. 1971. Exposed proteins on the intact human erythrocyte. *Biochemistry.* **10:**1766-1771.
19. RABINOVITCH, M. 1975. Macrophage spreading in vitro. *In* Mononuclear Phagocytes in Infection, Immunity, and Pathology. R. van Furth, editor. Blackwell Scientific Publications, Oxford. 369.
20. TROWBRIDGE, I., P. RALPH, and M. BEVAN. 1975. Differences in the surface proteins of mouse B and T cells. *Proc. Natl. Acad. Sci. U. S. A.* **72:**157-161.
21. UNKELESS, J., S. GORDAN, and E. REICH. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139:**834-850.
22. VASALLI, J.-D., J. HAMILTON, and E. REICH. 1976. Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nucleotides. *Cell.* **8:**271-281.
23. WAHL, L., S. WAHL, S. MERGENHAGEN, and G. MARTIN. 1974. Collagenase production by endotoxin-activated macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **71:**3598-3601.
24. WERB, Z., and S. GORDON. 1974. Secretion of specific collagenase and elastase by stimulated macrophages. *J. Exp. Med.* **142:**346-377.
25. YAMADA, K., S. YAMADA, and I. PASTAN. 1977. Quantitation of a transformation-sensitive, adhesive cell surface glycoprotein. *J. Cell Biol.* **74:**649-654.
26. YAMADA, K., S. YAMADA, and I. PASTAN. 1976. CSP partially restores morphology adhesiveness, and contact inhibition of movement to transformed fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **73:**1217-1221.
27. YIN, H., C. BIANCO, and Z. COHN. 1977. Plasma membrane proteins of mouse peritoneal macrophages. *J. Cell Biol.* **75:**240a. *(Abstr.)*