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# Macrophage Interactions with Laminin: PMA Selectively Induces the Adherence and Spreading of Mouse Macrophages on a Laminin Substratum

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**Abstract.** The ability of thioglycollate (TG)-elicited mouse peritoneal macrophages to adhere to a laminin substratum has been studied. These cells do not adhere to laminin-coated (20  $\mu\text{g/ml}$ ) surfaces, but the addition of phorbol myristate acetate (PMA; 50 ng/ml) results in their rapid adherence and spreading on this substratum. TG-elicited and PMA-activated macrophages, however, can bind soluble laminin. Macrophages adhere to fibronectin-coated surfaces and tissue culture plastic without PMA stimulation, and PMA does not increase the number of cells that adhere to these surfaces. The predominant surface proteins that bind specifically to laminin-Sepharose exhibit an  $M_r$  of 67 and 36 kD, but the expression of these proteins does not increase after PMA stimulation. Laminin

receptor antibodies immunoprecipitate the 67-kD protein from radiolabeled surface lysates and are capable of blocking macrophage adherence to a laminin substratum. Indirect immunofluorescence microscopy indicates that PMA stimulation does not increase receptor expression, but that it may induce the aggregation of the receptor on the cell surface. PMA stimulation also promotes macrophage spreading and induces a reorganization of the actin cytoskeleton. Taken together, these data indicate the mechanism by which PMA promotes macrophage adherence to laminin does not involve increased 67-kD receptor surface expression, but that it is related to the changes in cytoskeletal and receptor surface organization that occur in response to PMA stimulation.

**U**NDERSTANDING the mechanisms used by cells to interact with laminin-containing structures is an area of intense investigation (for recent reviews see Martin and Timpl, 1987; von der Mark and Risse, 1987). The characterization of a specific laminin receptor (Rao et al., 1983; Malinoff and Wicha, 1983; Lesot et al., 1983), as well as other laminin-binding proteins (Smalheiser and Schwartz, 1987; Kleinman et al., 1988), and the identification of sequences within the laminin molecule itself that mediate cellular interactions (Graf et al., 1987) have provided an impetus for more molecular approaches to problems in this field. Despite these advances, little is known about the mechanisms that are involved in regulating the ability of cells to interact with laminin. Biological examples of such regulation are numerous. During embryonic development, temporal and spatial interactions with laminin have been reported (Ekblom et al., 1986). Transformed cells that acquire a metastatic phenotype exhibit an enhanced ability to adhere to laminin-containing structures such as basement membranes (Albini et al., 1986). Macrophages and other leukocytes, although studied less thoroughly in this regard, also interact with basement membranes and probably other extracellular matrices at specific times during their ontogeny and differentiation, as well as in response to appropriate physiological stimuli (Hoover et al., 1980; Harlan, 1985). In this case, defining

the receptor mechanisms used by macrophages to interact with basement membrane glycoproteins, and investigating the possibility that such mechanisms might be modulated by factors that control macrophage differentiation and activation are key areas that merit investigation. Information obtained from studies on macrophages is likely to be useful in understanding the mechanisms used by other cell types to regulate their ability to interact with laminin.

The capacity of different macrophage populations to adhere to extracellular matrix substrata *in vitro* has been studied previously (Giavazzi and Hart, 1983; Bohnsack et al., 1985). One theme that has emerged from this work is that both monocytes and tissue macrophages adhere preferentially to fibronectin-coated surfaces in comparison to laminin and other basement membrane glycoproteins. The underlying reasons for these differences in adherence have not been explored. In particular, the receptors used by macrophages to interact with laminin substrata have not been adequately defined. Moreover, the possibility that a given population of macrophages can be modulated to exhibit enhanced interactions with laminin-containing structures has not been critically examined. This latter point could be of importance, for example, in understanding how monocytes traverse basement membranes during their emigration from blood.

We report here on the regulation of macrophage-laminin

interactions in response to PMA and on the laminin-binding proteins that may be involved in these interactions.

## Materials and Methods

### Mice

Female C57/BL6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 7 wk of age and were used in all experiments.

### Macrophages

Thioglycollate (TG)<sup>1</sup>-elicited peritoneal macrophages were obtained as described previously (Mercurio et al., 1984) from mice that had been injected 3–4 d before they were killed with 1.5 ml of a 4% (wt/vol) solution of TG broth (Difco Laboratories, Inc., Detroit, MI). This procedure yielded 2–3 × 10<sup>7</sup> peritoneal cells, of which >90% were macrophages as determined by Wright–Giemsa staining.

### Adherence Assay

Multi-well (11.3 mm) tissue culture plates (Costar, Cambridge, MA) were coated with purified Engelbreth–Holm swarm tumor laminin (Gibco Laboratories, Grand Island, NY) by incubation with 5–20 µg/ml laminin in PBS overnight at 4°C. The laminin solution was filtered through a 0.45 µm Schleicher and Schuell, Inc. (Keene, NH) Uniflo filter unit before being added to the wells. In some experiments, plates were also coated with 10–20 µg/ml fibronectin (Biomedical Technologies Inc., Cambridge, MA) by the same procedure. After the overnight incubation, the wells were washed several times with PBS and 0.1 ml of MEM was then added to each well. TG-elicited macrophages, obtained as described above, were resuspended in MEM to yield a final concentration of 5 × 10<sup>5</sup> cells/ml and 0.1 ml of this suspension was added to each well and to wells that had not been coated with laminin. It should be noted that the addition of 5–10% heat-inactivated FCS, or 0.5–1% BSA, to the MEM did not alter the results obtained with MEM alone. PMA (Calbiochem-Behring Corp., La Jolla, CA) was dissolved in DMSO and aliquots were kept frozen at –80°C until use. To some of the wells in each assay, PMA (50 ng/ml) was added immediately after the addition of macrophages. Similar results were obtained, however, when the PMA was added several hours after the macrophages had been plated. After the addition of PMA, the plates were incubated at 37°C for 60 min. The wells were then washed three times with warm Hanks balanced salt solution (HBSS). The adherent cells were fixed in methanol and stained with a 10% (vol/vol) solution of Giemsa stain (Sigma Chemical Co., St. Louis, MO). The cells were examined using bright-field optics with a Diaphot microscope (Nikon Inc., Garden City, NY) equipped with a 16-mm square reticle. Using a 20× objective, the surface area of this grid was measured with a stage micrometer and determined to be 0.25 mm<sup>2</sup>. For each experiment, the number of cells in this area was determined twice by two different investigators (L. Shaw and A. Mercurio). In some experiments, the cells were examined and photographed in phase-contrast before washing.

Inhibition of adherence was investigated by preincubating the macrophages in suspension for 15 min at 4°C with laminin receptor antibodies. The cells were then plated in laminin-coated wells and the adherence assay was carried out as described above. The following antibodies were used for these inhibition experiments: a rabbit antisera produced against a laminin receptor fusion protein (Segui-Real et al., 1988), this same antisera that had been affinity purified using the isolated fusion protein, a preimmune rabbit serum, and normal rabbit serum. All of these antibodies were provided by Drs. B. Segui-Real and Y. Yamada at the National Institute of Dental Research, National Institutes of Health (Bethesda, MD; NIDR, NIH).

### Laminin Surface Binding

TG-elicited macrophages were resuspended in MEM/10% FCS and plated in 6-mm wells of a 96-well microtiter plate (Falcon Labware, Oxnard, CA) at a density of 3 × 10<sup>5</sup> cells/well. After adherence, the wells were washed with warm HBSS. Some of the wells were then incubated in the same medium containing PMA (50 ng/ml) for 30 min. After washing with warm HBSS, the plates were placed on ice and cold PBS containing 1% BSA, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, and 0.02% sodium azide was added to all of the wells (0.1 ml). All subsequent manipulations were carried out on ice in this

PBS solution. Laminin was added to some of the wells to yield a final concentration of 20 µg/ml. The plates were incubated for 90 min and then washed twice. Normal goat serum (Cappel Laboratories, Cochranville, PA) diluted 1:50 was added to all of the wells for 30 min. After washing twice, a rabbit antisera specific for laminin (Gibco Laboratories) was diluted 1:50 and added to the wells for 60 min. The cells were then washed and incubated in the presence of an <sup>125</sup>I-goat anti-rabbit F(ab)<sub>2</sub> fragment (1:100; DuPont Co., Wilmington, DE) for 60 min. After a final set of washings, the cells were lysed in 1 N NaOH. Aliquots of the lysates were removed and their radioactive content determined by gamma counting.

### Surface Labeling

TG-elicited macrophages were resuspended in MEM containing 10% heat-inactivated FCS and 10<sup>7</sup> cells were plated in 60-mm tissue culture dishes (Falcon Labware). The cells were allowed to adhere for either 3–4 h or overnight at 37°C, and nonadherent cells were then removed by vigorous washing with HBSS. The adherent cells were incubated for 30 min in MEM/10% FCS or in the same medium containing 50 ng/ml PMA. The cells were washed again with HBSS, and then PBS (2 ml) containing 20 mM D-glucose was added to each plate. The plates were placed immediately on ice for the lactoperoxidase-catalyzed iodination. The iodination was performed by the sequential addition of the following reagents to each plate: lactoperoxidase (125 µl of a 1 mg/ml solution; Boehringer Mannheim Diagnostics Inc., Houston, TX), <sup>125</sup>I (0.5 mCi of DuPont Co., NEN #033A) and glucose oxidase (100 µl of a 1 mg/ml solution; Boehringer Mannheim Diagnostics Inc.). The iodination was allowed to proceed for 45 min on ice. Subsequently, the plates were washed with PBS containing 5 mM potassium iodide and 0.02% sodium azide. The cells were then solubilized by the addition of 1 ml of lysis buffer containing 1% Triton X-100, 0.15 M NaCl, 2 mM CaCl<sub>2</sub> and MgCl<sub>2</sub>, 2 mM PMSF, and 1 µM aprotinin, leupeptin, and pepstatin in 0.01 M Tris buffer, pH 8.0. The cell lysates were centrifuged at 13,000 g for 5 min to remove nuclei and other debris. Aliquots of the lysates were precipitated with cold TCA to determine the amount of radioactivity that had been incorporated into protein. The lysates were kept frozen at –80°C until use.

### Laminin–Sephacrose Chromatography

Aliquots (0.2–0.3 ml) of the radiolabeled macrophage lysates were incubated in the presence of 35 µl of laminin–Sephacrose for 5 h at 4°C. The laminin–Sephacrose was a generous gift of Dr. Hynda Kleinman (NIDR, NIH) and was prepared by the conjugation of 5 mg of purified Engelbreth Holm–Swarm tumor laminin to 1 ml of Sepharose C4B. The laminin–Sephacrose beads were washed twice with 0.1% Triton X-100, 0.15 M NaCl, 2 mM CaCl<sub>2</sub> and MgCl<sub>2</sub> in 0.01 M Tris buffer, pH 8, twice with this same buffer containing 0.5 M NaCl, and once with 0.05 M Tris buffer, pH 6.8, and then resuspended in Laemmli sample buffer containing 5% 2-mercaptoethanol. The samples were incubated at 100°C for 5 min and resolved on SDS–polyacrylamide gels. The gels were then dried and exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

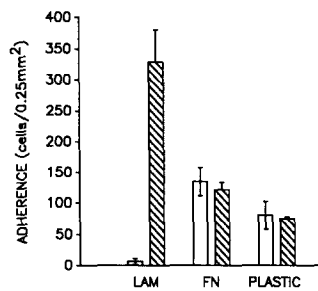
### Immunoprecipitations

Radiolabeled macrophage lysates, obtained as described above, were used for the immunoprecipitations. The equivalent of 3 × 10<sup>6</sup> macrophages were initially “precleared” with normal rabbit serum and then protein A–agarose (Boehringer Mannheim Diagnostics Inc.). The precleared lysates were incubated overnight at 4°C in the presence of 5 µl of antibody specific for the laminin receptor. Antigen–antibody complexes were recovered by the addition of 30 µl of protein A–agarose. After incubation for 2 h at 4°C with agitation, the beads were washed as described above. Laemmli sample buffer containing 5% 2-mercaptoethanol was added to the washed beads and the beads were incubated at 100°C for 5 min. The samples were then resolved by SDS–PAGE.

### Immunofluorescence Microscopy

TG-elicited macrophages were resuspended in MEM/10% FCS and plated on glass coverslips (12 × 12 mm; Bradford Scientific Inc., Epping, NH) at a density of 2.5 × 10<sup>4</sup> cells/ml. Some coverslips were coated with laminin (20 µg/ml) as described above. After adherence, the coverslips were either fixed immediately or washed with warm HBSS, incubated in MEM/10% FCS containing PMA (50 ng/ml) for 30 min, and then fixed. The macrophages were fixed in formaldehyde (3.7%; Sigma Chemical Co.) for 15 min at room temperature.

1. Abbreviation used in this paper: TG, thioglycollate.



**Figure 1.** Effect of PMA on macrophage adherence to laminin, fibronectin, and plastic. Tissue culture wells (11.3 mm diam) were coated overnight with murine laminin (20  $\mu$ g/ml) or bovine plasma fibronectin (20  $\mu$ g/ml). Freshly isolated TG-elicited macrophages were resuspended in MEM and added to the wells at a concentration of  $5 \times 10^4$  cells/well. PMA (50 ng/ml) was added to one-half of the protein-coated wells. The tissue culture plates were incubated at 37°C for 60 min. Nonadherent cells were removed by washing and adherent cells were counted by microscopic examination as described in Materials and Methods. The data reported in this figure were obtained from one experiment performed in triplicate, and similar results were obtained in eight other experiments. Error bars, SD; open boxes, control; hatched boxes, PMA; LAM, laminin; FN, fibronectin.

Fixed macrophages were washed in PBS and then incubated in PBS containing mouse F<sub>c</sub> fragments (50  $\mu$ g/ml; Jackson Laboratories) for 30 min at 37°C. BSA (1%) was added to the PBS used in the immunofluorescent studies. After washing in PBS, the coverslips were incubated for 1 h at 37°C

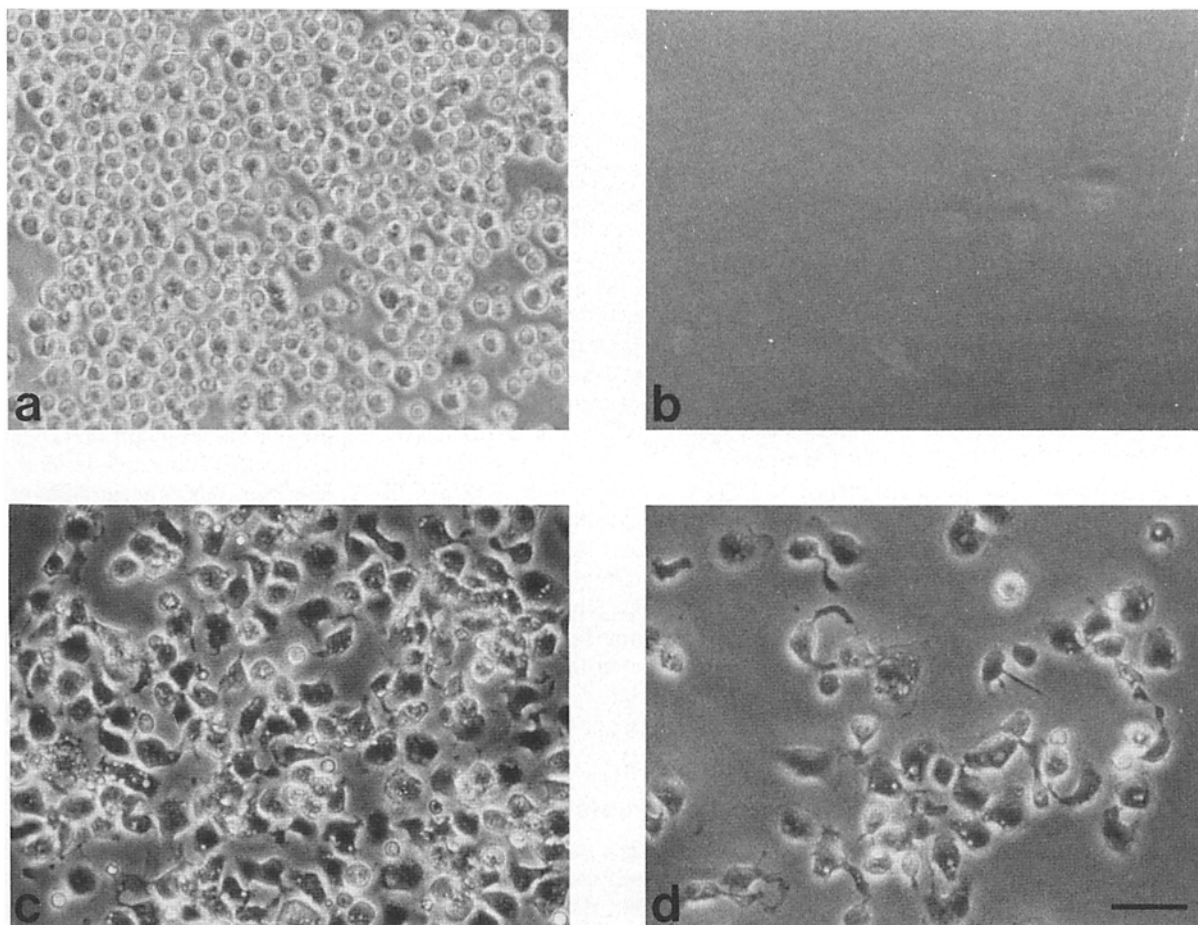
in the presence of PBS containing a 1:20 dilution of either laminin receptor antibodies or preimmune sera. The coverslips were washed in PBS and then incubated in PBS containing a 1:30 dilution of a fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories) for 1 h at 37°C. After washing in PBS, the coverslips were mounted in glycerol gelatin (Sigma Chemical Co.) and examined with a Lasersharp scanning confocal microscope (model MRC-500; Bio-Rad Laboratories, Richmond, CA) using an argon-ion laser operating at 488-nm wavelength as the excitation source (White et al., 1987).

## Results

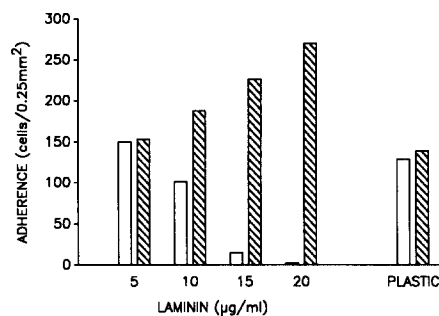
### Macrophage Adherence to Extracellular Matrix Glycoproteins

The ability of freshly isolated TG-elicited macrophages to adhere to tissue culture plastic was compared to their ability to adhere to plastic that had been coated with either laminin or fibronectin. TG-elicited macrophages adhere avidly to plastic surfaces, and as shown in Fig. 1, these cells also adhered well to fibronectin. In marked contrast, TG-macrophages exhibited little, if any, adherence on laminin-coated surfaces (Fig. 1). Macrophages added to wells coated with laminin remained in suspension (Fig. 2 *a*) and they were viable for at least several hours.

As shown in Fig. 3, this laminin adherence phenomenon



**Figure 2.** Macrophage adherence to laminin-coated surfaces. Freshly isolated TG-elicited macrophages ( $5 \times 10^4$ ) were added to laminin-coated tissue culture wells (20  $\mu$ g/ml) either in the absence (*a* and *b*) or presence (*c* and *d*) of PMA. After incubation at 37°C for 30 min, the wells were photographed in phase-contrast either immediately (*a* and *c*) or after being washed three times with warm HBSS (*b* and *d*). Bar, 12.5  $\mu$ m.



**Figure 3.** Effect of laminin concentration on macrophage adherence. Tissue culture wells were coated overnight with concentrations of laminin ranging from 5 to 20 µg/ml. The ability of TG-elicited macrophages to adhere to such wells was assessed by the procedure outlined in the legend to Fig. 1 and described in Materials and Methods. The data shown in this figure for adherence to uncoated tissue culture wells (*PLASTIC*) were obtained by the same procedure.

was a function of the laminin concentration used to coat the tissue culture wells. Minimal adherence was observed at a laminin concentration of 15–20 µg/ml. With decreasing laminin concentrations, a dose-dependent increase in adherence was observed, presumably the result of adherence to plastic sites that had not been coated with laminin (Fig. 3). Countercoating the wells with BSA was not done routinely because we found that macrophages exhibited some adherence to plastic surfaces coated with BSA alone.

#### ***PMA Stimulation Promotes Macrophage Adherence and Spreading on a Laminin Substratum***

The addition of PMA (50 ng/ml) to TG-elicited macrophages that had been plated in laminin-coated wells resulted in their rapid adherence and spreading on this substratum (Figs. 1 and 2). Adherence was evident within 5–10 min after the addition of PMA and was maximal within 20 min. As shown in the photomicrographs presented in Fig. 2, PMA stimulation induced not only adherence but also macrophage spreading on a laminin substratum.

The data presented in Fig. 1 indicate that the number of macrophages that adhered to laminin in the presence of PMA was far greater than the number that adhered to either plastic or fibronectin. Also, PMA stimulation did not increase the amount of adherence on plastic and fibronectin (Fig. 1), although it did increase cell spreading on these other substrata.

Cycloheximide was used to determine if *de novo* protein synthesis is required for the PMA-stimulated adherence of macrophages to laminin. When macrophages were preincubated in the presence of 75 µg/ml of cycloheximide before their addition to laminin-coated wells, no decrease in adherence was observed in comparison to control macrophages (data not shown).

#### ***Role of the Cytoskeleton in Macrophage Adherence to Laminin***

To assess the cytoskeletal requirements for macrophage adherence to laminin, the effects of colchicine and cytochalasin B on laminin adherence were tested and compared to their effects on macrophage adherence to plastic (Table I). In

**Table I.** Effects of Inhibitors of Cytoskeletal Proteins on Macrophage Adherence to Laminin and Plastic

Condition	Adherence (%)	
	Experiment 1	Experiment 2
<b>Laminin</b>		
PMA	100	100
Colchicine	27.1	24.2
Cytochalasin B	0	0
<b>Plastic</b>		
Control	100	100
Colchicine	51.0	41.6
Cytochalasin B	48.5	51.2
PMA	100	100
Colchicine	96.4	76.0
Cytochalasin B	71.6	71.9

Cytoskeletal requirements for macrophage adherence to laminin. The adherence assay outlined in Fig. 1 and described in Materials and Methods was carried out with macrophages plated on either laminin or tissue culture plastic. For laminin adherence, the assay was performed in the presence of PMA (50 ng/ml); and for plastic adherence, the assay was performed both in the absence or presence of PMA. Colchicine and cytochalasin B ( $10^{-5}$ M) were added to the microtiter wells at the same time as PMA, or, in the case of plastic adherence, at the same time as the macrophages. The adherence assay was allowed to proceed for 60 min. The number of cells that adhered in the absence of these inhibitors was considered 100% adherence and the other values listed in this table are the adherence values (%) observed in the presence of these inhibitors relative to the control values. Similar results were obtained when the inhibitors were added 10 min after the addition of PMA. The results reported in this table were obtained from two independent experiments.

general, macrophage adherence to laminin was much more sensitive to these cytoskeletal inhibitors than adherence to plastic. Colchicine ( $10^{-5}$ M), a microtubule inhibitor (Kirschner, 1978), reduced PMA-mediated adherence to laminin to 25% of the adherence observed in the absence of this drug (Table I). This same concentration of colchicine inhibited macrophage adherence to plastic by only 50% and it was even less inhibitory of plastic adherence in the presence of PMA (Table I). The effects of the microfilament inhibitor cytochalasin B (Weihsing, 1976; Lin et al., 1980) on laminin adherence were even more pronounced. At a concentration of  $10^{-5}$ M, this drug completely blocked adherence to laminin in the presence of PMA. However, this concentration of cytochalasin B inhibited adherence to plastic by only 50%, and, as with colchicine, it exhibited less of an inhibitory effect on adherence to plastic in the presence of PMA (Table I). Similar results were obtained with cytochalasin D (data not shown). The addition of these inhibitors several minutes after the addition of PMA did not alter the results shown in Table I. This suggests that these inhibitors do not affect PMA action on macrophages.

#### ***PMA Does Not Augment Binding of Soluble Laminin to Macrophages***

The ability of TG-elicited macrophages to bind soluble laminin, both before and after PMA stimulation, was examined. For this purpose, macrophages were incubated sequentially in the presence of laminin, a rabbit anti-murine laminin IgG, and an  $^{125}$ I-labeled second antibody as described in Materials and Methods. As shown in Table II, both TG-elicited and PMA-stimulated macrophages were able to bind exogenous

**Table II. Binding of Soluble Laminin to Macrophage Surfaces**

Condition	Control	PMA
	<i>cpm bound/3 × 10<sup>5</sup> cells</i>	
LAM + Anti-LAM + <sup>125</sup> I-2nd antibody	16,054 ± 924	16,353 ± 1,881
Anti-LAM + <sup>125</sup> I-2nd antibody	8,779 ± 1,240	8,773 ± 690
LAM + <sup>125</sup> I-2nd antibody	2,106 ± 288	2,219 ± 186

Adherent TG-elicited and PMA-activated macrophages were incubated sequentially with laminin (LAM; 20 µg/ml), a rabbit antisera specific for laminin (Anti-LAM; 1:50), and an <sup>125</sup>I-goat anti-rabbit F(ab)<sub>2</sub> fragment (<sup>125</sup>I-2nd antibody; 1:100). Some wells were incubated without either laminin or the laminin-specific antisera to determine the specificity of binding. The numbers shown are the cpm (mean ± SEM) present in the cell lysates and represent two independent experiments performed in triplicate.

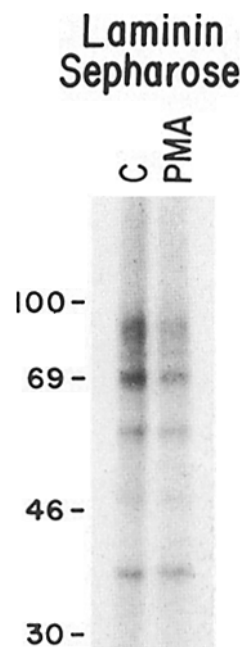
laminin specifically. However, no difference in the amount of soluble laminin bound was evident between these two macrophage populations. Macrophages incubated in the absence of either laminin or anti-laminin exhibited a significant reduction of bound radiolabeled second antibody (Table II).

### Identification of Macrophage Laminin-binding Proteins

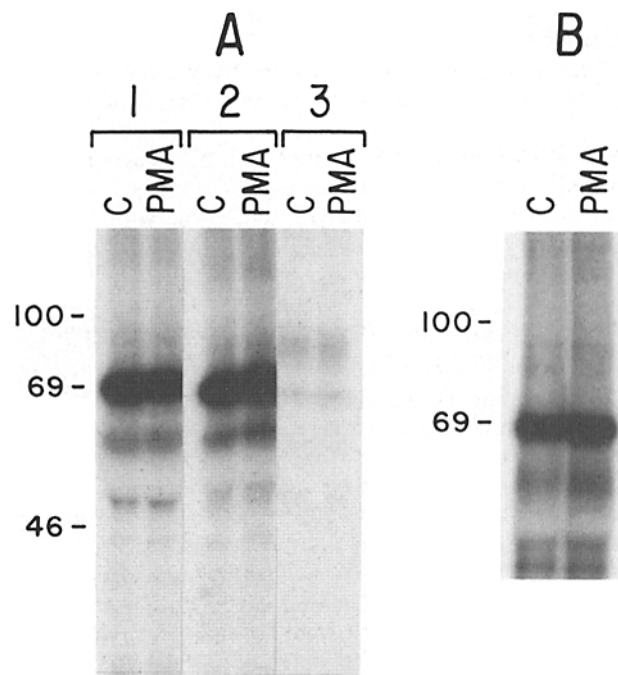
To obtain information on the surface proteins that bind laminin, detergent lysates obtained from <sup>125</sup>I-surface-labeled populations of both TG-elicited and PMA-stimulated macrophages were incubated in the presence of laminin-Sepharose beads and analyzed by SDS-PAGE and autoradiography. The predominant proteins that bound specifically to laminin-Sepharose exhibited an *M<sub>r</sub>* of 67 and 36 kD (Fig. 4). Bands were also evident at 80 and 55 kD (Fig. 4). The amount of radiolabeled protein that bound to laminin-Sepharose did not appear to differ between control and PMA-stimulated macrophages (Fig. 4).

Antibodies against the purified 67-kD laminin receptor (Wewer et al., 1987) were used to immunoprecipitate detergent lysates obtained from <sup>125</sup>I-surface-labeled macrophages. As shown in Fig. 5 A (lane 1), these antibodies specifically precipitated a 67-kD protein from macrophage lysates. No other major surface proteins were precipitated by

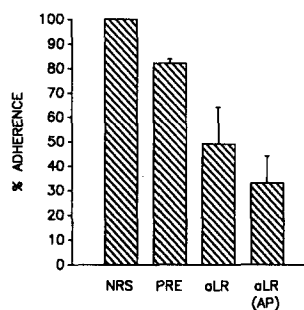
these antibodies. Antibodies that were generated against a laminin receptor fusion protein that was obtained by bacterial expression of a receptor cDNA (Segui-Real et al., 1988) also precipitated the 67-kD protein. However, they did not precipitate any proteins in the 30-kD range (Fig. 5 A, lane 2), even though these antibodies react with both a 67-kD and a 32-kD protein on Western blots of tumor cell extracts (Segui-Real et al., 1988). It is interesting to note that, as with the laminin-Sepharose data shown in Fig. 4, no increase in the surface labeling of the 67-kD receptor was evident after PMA stimulation (Fig. 5 A). Similar results were obtained when the macrophages were PMA stimulated and surface labeled in suspension (Fig. 5 B). Together, these data indicate



**Figure 4.** Laminin-Sepharose chromatography of <sup>125</sup>I-surface-labeled macrophage lysates. Radiolabeled detergent lysates, containing the equivalent of 3 × 10<sup>6</sup> TG-elicited and PMA-activated macrophages, were incubated in the presence of laminin-Sepharose. After washing with a series of buffers as described in Materials and Methods, the bound proteins were resolved by 10% SDS-PAGE under reducing conditions. The autoradiograph shown is representative of the results obtained in several independent experiments.



**Figure 5.** Immunoprecipitations of <sup>125</sup>I-surface-labeled macrophages with antisera specific for the laminin receptor. (A) Adherent TG-elicited and PMA-activated macrophages were surface iodinated and lysates were immunoprecipitated with rabbit antibodies specific for a 67-kD laminin receptor (lane 1; Wewer et al., 1987), rabbit antibodies produced against a laminin receptor fusion protein (lane 2; Segui-Real et al., 1988), and normal rabbit serum (lane 3). (B) Macrophages were surface iodinated in suspension and lysates were immunoprecipitated with the same rabbit antibodies used in lane 1. Immunoprecipitates were resolved by 7.5% SDS-PAGE under reducing conditions. C, control.



**Figure 6.** Inhibition of macrophage adherence to a laminin substratum with receptor antibodies. TG-elicited macrophages were incubated in suspension with the following antisera before their addition to laminin-coated wells: normal rabbit serum (NRS; 1:10), a preimmune rabbit serum (PRE; 1:10), a rabbit antisera produced against a laminin receptor fusion protein

(aLR; 1:10), and this same antisera which was affinity purified using the isolated fusion protein (aLR[AP]; 1  $\mu$ g/ml). After addition to the wells, PMA (50 ng/ml) was added and the adherence assays were continued as described in Materials and Methods. The data shown are the mean values ( $\pm$ SEM) obtained in three separate experiments.

that macrophages express a 67-kD laminin receptor, as well as other laminin-binding proteins on their surface, that these proteins are present on both TG-elicited and PMA-stimulated macrophages before they adhere to a substratum, and that PMA stimulation does not increase their surface labeling.

#### **Antibodies That Recognize a 67-kD Laminin Receptor Inhibit Macrophage Adherence to a Laminin Substratum**

The fusion protein antibodies that precipitated the 67-kD receptor from surface-labeled lysates (Fig. 5 A) were tested for their ability to inhibit the PMA-stimulated adherence of macrophages to laminin. Antisera against this protein, at a dilution of 1:10, were able to inhibit adherence by 50% in comparison to normal rabbit serum (Fig. 6). Adherence was inhibited to an even greater extent (70%) when affinity-purified antibodies were used at a concentration of 1  $\mu$ g/ml (Fig. 6). In contrast, preimmune serum only slightly inhibited adherence.

#### **Macrophage Adherence to Laminin Is Distinct From Fibronectin Adherence**

As shown in Fig. 1, unstimulated TG-elicited macrophages adhere avidly to fibronectin but not to laminin. This observation suggests that these two types of adherence are distinct and that they are regulated by different mechanisms. This difference is also supported by the observation that the RGDS peptide found in fibronectin and many other adhesion proteins (Pierschbacher and Ruoslahti, 1984) did not inhibit macrophage adherence to laminin, even at a concentration of 2 mg/ml (data not shown). Similarly, antibodies to a mammalian fibronectin receptor (Brown and Juliano, 1986), which do block macrophage adherence to fibronectin-coated surfaces (Barlozzari, T., and A. Mercurio, submitted for publication) did not inhibit macrophage adherence to laminin (data not shown).

#### **Immunofluorescence Microscopy**

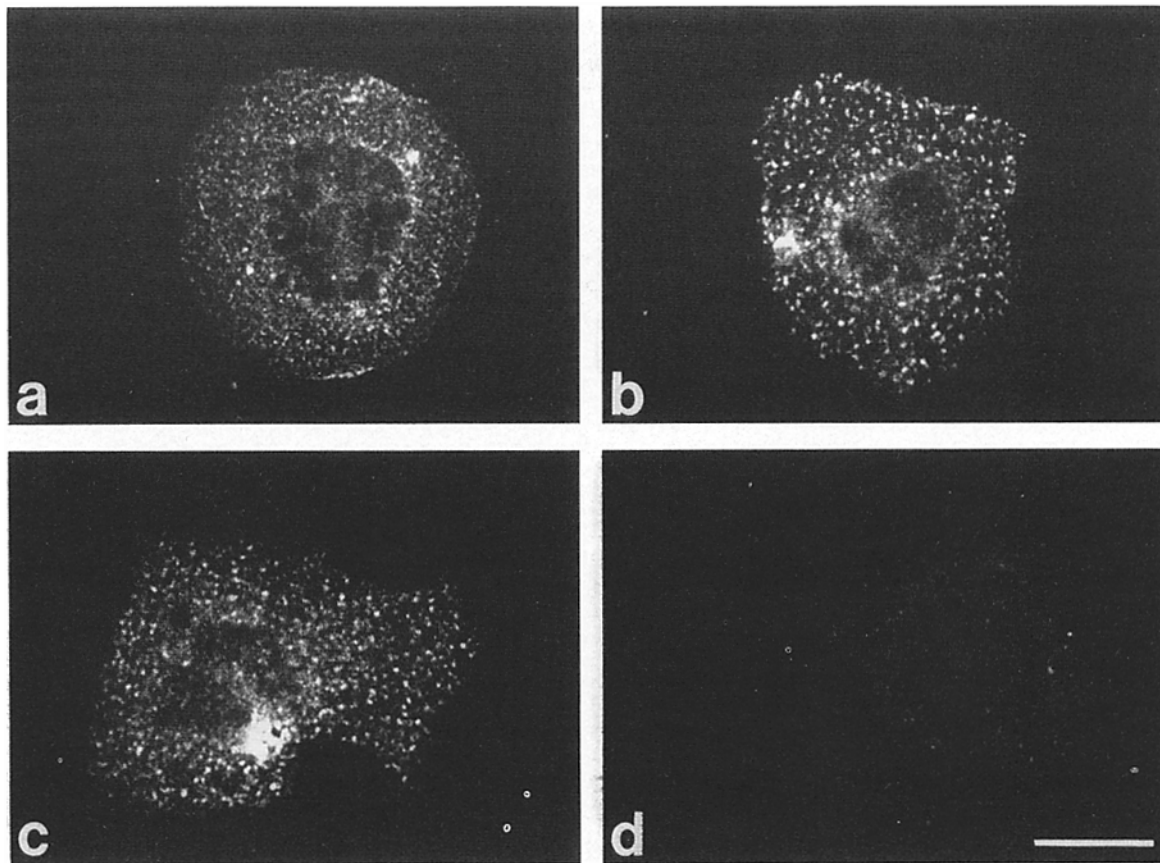
Indirect immunofluorescence microscopy studies were performed to examine the cell surface localization of the laminin receptor, and to examine the possibility that PMA alters receptor localization. The surface staining pattern of the receptor obtained by scanning confocal microscopy is shown

in Fig. 7. It is evident that the receptor is present on the surfaces of both TG-elicited and PMA-stimulated macrophages and that significant differences in the amount of surface staining are not apparent after PMA stimulation (Fig. 7). This observation corroborates both the surface labeling and soluble laminin binding data described above. These images do suggest, however, that PMA stimulation may result in a more clustered or aggregated pattern of receptor staining in comparison to unstimulated cells. This clustered pattern of staining was observed for PMA-stimulated macrophages that were plated on both glass and laminin (Fig. 7, b and c). Nonstimulated macrophages that were relatively well spread did not exhibit a clustered pattern of receptor staining (Fig. 7 a). The images shown in Fig. 7 were obtained using an antibody that was generated against the purified 67-kD laminin receptor (Wewer et al., 1987). Similar staining patterns were obtained with antibodies generated against the receptor fusion protein (Segui-Real et al., 1988; data not shown).

#### **Discussion**

A major problem in cell biology is understanding the mechanisms used by cells to interact with extracellular matrices (Martin and Timpl, 1987; von der Mark and Risse, 1987; Buck and Horwitz, 1987; Liotta et al., 1986; Mercurio, 1988). Within this context, we examined the ability of mouse macrophages to interact with a laminin substratum and tested the possibility that this interaction can be regulated by factors that are known to influence macrophage function. The results obtained demonstrate that the ability of TG-elicited macrophages to adhere and spread on a laminin substratum is not a constitutive function of these cells, but that it can be induced rapidly by PMA stimulation. In contrast, macrophages adhered avidly to fibronectin and tissue culture plastic without prior stimulation, and, in fact, PMA did not augment adherence to these other substrata. Thus, macrophage interactions with different adhesive substrata are probably independent events, and, as discussed below, they are probably mediated by distinct biochemical mechanisms.

The results presented here indicate that macrophage adherence to a laminin substratum is a receptor-mediated event. A cell surface receptor for laminin was identified initially on both tumor cells (Rao et al., 1983; Malinoff and Wicha, 1983) and striated muscle cells (Lesot et al., 1983). Subsequent reports have shown it to be present on a number of cell types including macrophages (Huard et al., 1986). This receptor exhibits an  $M_r$  of 67 kD on SDS-PAGE reducing gels, and it has been shown to mediate some of the known cellular interactions with laminin (Martin and Timpl, 1987; von der Mark and Risse, 1987; Liotta et al., 1986). The recent report that a full-length cDNA clone of the receptor encodes a protein of  $M_r$  32 kD (Segui-Real et al., 1988) is intriguing. Antibodies prepared against a fusion protein expressed by this clone react with both a 67-kD and a 32-kD protein on Western blots of tumor cell extracts (Segui-Real et al., 1988), suggesting that, at the least, these two proteins share common epitopes. These antibodies specifically inhibited the PMA-stimulated adherence of macrophages to a laminin substratum, but they immunoprecipitated only the 67-kD protein from surface-labeled macrophages. Thus, these observations indicate that the 67-kD receptor is involved in the PMA-stimulated adherence of macrophages to



**Figure 7.** Immunofluorescent localization of laminin receptor on formaldehyde-fixed, TG-elicited (*a*) and PMA-activated (*b*, *c*, and *d*) macrophages. Cells were plated on either glass (*a*, *b*, and *d*) or laminin-coated (*c*) coverslips and after fixing were incubated in the presence of antibodies to a 67-kD laminin receptor (*a*, *b*, and *c*) or a preimmune rabbit serum (*d*), and then a fluorescein-conjugated goat anti-rabbit IgG as described in Materials and Methods. Note the clustered pattern of staining that is apparent after PMA activation. The images shown are 0.2- $\mu$ M sections obtained by scanning confocal microscopy as described in Materials and Methods. Bar, 10  $\mu$ m.

laminin. However, other macrophage surface proteins, particularly the 36-kD protein, bound to laminin-Sephadex. We cannot discount, therefore, the possible contribution of these or other surface molecules to the adherence and spreading of macrophages on a laminin substratum.

Although laminin receptor antibodies inhibited the PMA-stimulated adherence of macrophages to laminin, PMA stimulation did not increase the expression of the 67-kD receptor or other laminin-binding proteins on the cell surface. In fact, laminin receptor was present on the surfaces of both TG-elicited and PMA-stimulated macrophages maintained in suspension. In addition, the observation that both of these macrophage populations bound soluble laminin to the same extent supports the conclusion that the laminin receptor is on the cell surface before PMA stimulation. Taken together, the data argue against the possibility that PMA-stimulated adherence of macrophages to laminin results from an increased expression of laminin receptors on the macrophage surface. Our results on macrophages are therefore distinct from the report that PMA stimulation of human neutrophils results in a massive translocation of laminin receptors from an intracellular compartment to the cell surface (Yoon et al., 1987).

The mechanism by which PMA promotes macrophage adherence and spreading on a laminin substratum is likely to be more subtle than an increase in receptor surface expres-

sion. Although the specific mechanisms that are involved in this adherence remain to be elucidated, several possibilities exist based on the data presented in this paper. The ability of unstimulated macrophages to spread is limited, and in these cells sufficient surface area may not be available to initiate adherence and spreading. However, plasma membrane movement promoted by PMA (Phaire-Washington et al., 1980*a,b*) may generate adequate surface area to initiate such adherence and spreading on a laminin substratum. In addition, the possible aggregation of the laminin receptor into clusters on the cell surface after PMA stimulation could enhance its ability to promote adherence and cell spreading. Receptor oligomerization has been proposed as a mechanism for activation of the epidermal growth factor receptor on cell surfaces (Cochet et al., 1988) and evidence for ligand-induced clustering of the laminin receptor has been reported previously (Cody and Wicha, 1986). The possibility also exists that PMA induces the association of laminin receptors with specific cytoskeletal proteins. For example, the recent report that PMA promotes the specific association of integrin with talin in human lymphocytes supports this notion (Burn et al., 1988). These possibilities are not mutually exclusive and they may represent different manifestations of the response of macrophages to PMA stimulation.

Determining the initial, primary target(s) of PMA action

on macrophages within the context of laminin adherence is a problem of considerable importance. PMA is a potent activator of protein kinase C (Parker et al., 1986; Castagna et al., 1982; Niedel et al., 1983), and the possibility that PMA induces the phosphorylation of proteins that are involved, either directly or indirectly, in laminin adherence is strong. In this connection, we have been unable to demonstrate that PMA induces phosphorylation of the 67-kD laminin receptor or other macrophage laminin binding proteins (Shaw, L. M., and A. M. Mercurio, unpublished observations). However, other macrophage proteins, particularly cytoskeletal components, are likely to be phosphorylated in response to PMA (e.g., Pontremoli et al., 1987) and this type of modification may initiate a cascade of events that results in laminin adherence.

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