

# Phorbol Ester-Induced Promyelocytic Leukemia Cell Adhesion to Marrow Stromal Cells Involves Fibronectin Specific $\alpha 5\beta 1$ Integrin Receptors

V. MARTIN-THOUVENIN, M.C. GENDRON, F. HOGERVORST, C.G. FIGDOR, AND M. LANOTTE\*

INSERM, U301, Institute of Hematology, Centre G. Hayem, Hôpital Saint-Louis, 75010 Paris, France (V.M.-T., M.C.G., M.L.); Department of Immunohematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (F.H.), and Division of Immunology, The Netherlands Cancer Institute (C.G.F.), 1066 CX Amsterdam, The Netherlands

The human promyelocytic cell line NB4 exhibited a weak adhesion capacity for bone marrow-derived stromal cells and their extracellular matrices (5–15% of adherent cells). Adhesion was enhanced by pulse-treatment of cells with phorbol-ester (PMA  $10^{-7}$  M). Adhesion was induced within minutes, was fibronectin-specific, and affected up to 100% of the treated cells. This biological response to PMA resulted from the activation of protein kinase C (PKC), since PKC inhibitors (staurosporine, sphingosine, CGP 41251, and calphostin C) prevented the phenomenon. Phenotypical analysis of integrin receptor expression (particularly FN receptors VLA-4 and VLA-5) at the membrane of untreated or PMA-treated cells revealed that PMA induced no significant modification of the level of expression of these receptors. However, inhibition studies carried out with anti-VLA monoclonal antibodies demonstrated that the FN-specific adhesion triggered by PKC involved the  $\alpha 5\beta 1$  FN-specific receptors (VLA-5). We showed that the binding of NB4 cells to fibronectin was RGD-dependent. PMA-induced adhesion was not correlated to phosphorylation of the VLA-5 receptor. These findings may partially explain the malignant behaviour of these cells: The loss of their capacity to adhere to stromal cells may arrest differentiation and explain the large number of leukemic cells in the circulation. © 1992 Wiley-Liss, Inc.

The cellular microenvironment plays a critical role in the development of the hematopoietic moiety of the marrow by the production of soluble factors and an extracellular matrix (ECM) (Dexter et al., 1977a,b; Tavassoli and Takahashi, 1982; Kamenov and Longenecker, 1985; reviewed by Torok-Storb, 1988). Roles for ECM in the regulation of proliferation, differentiation, and cell migration have been described in the development of a great number of tissues (Thiery et al., 1989). In marrow, it has been proposed that ECM contributes to cell survival, cell renewal, and cell egress as well as the expression of specific cell function by binding regulatory molecules (Gordon et al., 1988). Fibronectin (FN), hemonectin, collagens, and laminins are molecules that promote growth, differentiation, anchoring, spreading, and migration of hemopoietic cells. The interactions between hematopoietic cells and ECM components are mediated by specific receptors that associate a common  $\beta$  subunit ( $\beta 1$ ,  $\beta 2$ , or  $\beta 3$ ) to different  $\alpha$  chains and belong to the integrin family (Hynes, 1987). Among these integrins present on many hemopoietic cells, the  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  FN receptors (Ruoslahti, 1988; Hemler, 1990; Hynes, 1990) have potentially important roles in ECM-dependent progenitor cell differentiation

and maturation. The expression of adhesion molecules is correlated to the capacity of progenitor cells to interact with stromal cells and varies according to their lineage and/or their stage of differentiation (Coulombel et al., 1988), including alterations related to malignancy (Caligaris-Cappio et al., 1989).

Hemopoietic cells at the immature stages of development are confined to the marrow tissue in close association with stromal cells. This feature is verified in Dexter cultures (Dexter et al., 1977a) where these cells are preferentially nested in the hemopoietic foci of the adherent stromal cell layer. In vivo a massive marrow egress of immature blasts and promyelocytic cells is indeed observed in some pathological conditions, and particularly in leukemia. The lack of interaction of leukemic cells with stromal cells might be due to some constitutive or functional defects in the adhesion effector system. Numerous reports have proposed that re-

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\*To whom reprint requests/correspondence should be addressed.

ceptor-mediated cell adhesion is regulated by protein kinases (Hirst et al., 1986) and, obviously, linking the loss of ECM adhesion of leukemic cells to both a characterized intracellular signal pathway and a membrane adhesion receptor complex should help to define the defect of leukemic cells.

The NB4 cell line, the first human promyelocytic leukemia cell line (M3) (Lanotte et al., 1991) with the specific t(15;17) chromosomal translocation (de Thé et al., 1990) furnished a unique in vitro model for cellular and molecular studies. In this work, we demonstrate that NB4 cells, only a minor proportion of which bound to stromal cell ECM or fibronectin, were rapidly induced to adhere in response to PMA. We show that adhesion of NB4 cells to FN was mediated by the  $\alpha 5 \beta 1$  integrin receptor and required PKC activation.

## MATERIALS AND METHODS

### Chemicals and reagents

Fibronectin was purified from human plasma (Sigma-Chimie or IMEDEx, Lyon, France). Collagens (I, III, IV, and V), laminin, staurosporine, sphingosine, PMA, PDBu, PMA-methyl-ether, phorbol, 4 $\alpha$ -phorbol, A 23187, PGE<sub>1</sub>, PGE<sub>2</sub>, PGF<sub>1</sub> $\alpha$ , PGF<sub>2</sub> $\alpha$ , PGA<sub>1</sub>, PGD<sub>2</sub>, IBMX, isoproterenol, 8-CPT-cAMP, 8-Cl-cAMP, W7, RGDS, and RGEs peptides were all purchased from Sigma. Phorbols were prepared as 2 mM stock solutions in dimethylsulfoxide (DMSO) and diluted in RPMI before each experiment. Cholera toxin (CT) was furnished by the Institut Mérieux. (Lyon, France), CGP 41251 was given by Ciba-Geigy (Basel, Switzerland). Calphostin C and microcystin were gifts from S.O. Doskeland (Bergen, Norway). Calphostin C was photoactivated by fluorescent light as described by Bruns et al. (1991). Monoclonal antibodies (MoAbs) against  $\beta 1$ (K20),  $\alpha 2$ (G19),  $\alpha 4$ (HP2/1),  $\alpha 5$ (SAM1), and  $\alpha 6$ (GoH3) chains of integrins were provided by Immunotech (Luminy, France). AIB2 anti- $\beta 1$  rat monoclonal antibody was used for inhibition of adhesion studies. Ultrosor HY medium supplement was furnished by IBF (France) and BCA reagents by Pierce (Netherlands).

### Cell culture and cell treatment with PMA

The promyelocytic cell line NB4 (Lanotte et al., 1991) was grown in RPMI medium with 1% (v/v) Ultrosor HY, in a humidified 5% CO<sub>2</sub>/air incubator, at 37°C. Exponential cell growth was obtained by culturing cells between 5.10<sup>4</sup> and 5.10<sup>5</sup> cells per ml. The doubling time was 48 h. Cells were washed in RPMI medium and incubated in culture medium (10<sup>6</sup>/ml) with PMA (10<sup>-7</sup> M) for 60 min at 37°C. Cells were then washed twice before analysis.

### Adhesion to human stromal cells and their extracellular matrices

Stromal cells were obtained by trypsinization of the adherent layer derived from normal human bone marrow cell culture (Lanotte et al., 1981). Stromal cells were cultured in 24-well plates (3.10<sup>4</sup>/well) for 7 days at 37°C, until they became confluent. Cultures were then washed three times in serum-free RPMI medium before the adhesion assay.

ECM were prepared from confluent stromal cell cultures in 24-well plates. Cells were washed once in phosphate-buffered saline (PBS) then, 0.5 ml aliquots of 0.5% (w/v) Triton X100 in PBS were added to each well as described by Vlodavsky et al. (1980). Plates were incubated under slow agitation for 1 h at room temperature. Wells were then washed five times with PBS before use.

NB4 cells (2.10<sup>5</sup>/ml) were incubated with 1  $\mu$ Ci/ml of <sup>125</sup>I-5-Iododeoxyuridine (sp. act. 2,000 Ci/mmol; Amersham-France) for 4 h at 37°C. They were then washed three times in RPMI and resuspended at 1.6  $\times$  10<sup>6</sup>/ml in RPMI supplemented with 1% Ultrosor HY and 1% BSA, with or without 10<sup>-7</sup> M PMA. In each well, 300  $\mu$ l aliquots of radiolabeled-cell suspensions were added containing either stromal cells or ECM and incubated for 2 h at 37°C. Radioactivity in each well, evaluated using a gamma counter (LKB), represented the total cpm. Nonadherent cells were discarded and wells were washed three times with RPMI medium. Adherent cells were solubilized with a 0.5 M NaOH, 1% SDS solution and counted.

### Adhesion assay to coated surfaces

Culture plate wells (96 well plates) were coated for 3–4 h at 37°C with 50  $\mu$ l of PBS containing 50  $\mu$ g/ml of either human fibronectin or other substrata (bovine serum albumin, BSA; human laminin; human collagens). Wells were then washed with PBS and 200  $\mu$ l of a 3% (w/v) BSA solution was added to each well to saturate nonspecific adsorption sites on the plastic surface. Plates were incubated for 1 h at 37°C then washed three times in PBS.

Exponentially growing NB4 cells were pelleted, washed once in fresh medium, and resuspended at 10<sup>6</sup> cells/ml, in medium supplemented with Ultrosor HY. To each well were added 100  $\mu$ l aliquots of cell suspension; cells were allowed to adhere to the substrata for 2 h at 37°C. Unattached cells were subsequently removed by washing three times with PBS. Adherence of cells was quantified according to the method of Tuszynski and Murphy (1990), based on the colorimetric evaluation of cellular proteins in each well. Briefly, 200  $\mu$ l of bicinchoninic acid (BCA) reagent were added to each well and the plates were incubated for 30 min at 60°C. The optical density was then read at 570 nm.

To determine the inhibition of adhesion to fibronectin, NB4 cells were preincubated in suspension either with protein kinase C (PKC) inhibitors, with synthetic peptides or with antibodies for 30–60 min at 37°C; cells were then added to fibronectin-coated wells. When cells were simultaneously treated with PMA and a PKC inhibitor, the incubation with the PKC inhibitor preceded by 30 min the PMA treatment and by 45 min the addition of cells to FN-coated wells. For the experiments with calphostin C, the incubation was carried out for 2 h in an incubator with a fluorescent light source to photoactivate calphostin C. Data in adhesion assays represent triplicate measurements from at least two separate experiments. Adherent cells are expressed as the percent of the total cell number in the assay. Inhibition of adhesion is expressed as a percentage of the control (maximal adhesion).

### Radiolabeling and integrin immunoprecipitation experiments

NB4 cells were grown in RPMI with 2% HY. For  $^{125}\text{I}$  labeling of membrane proteins,  $30 \times 10^6$  cells were incubated for 20 min at room temperature with 1 mCi Na  $^{125}\text{I}$  (Amersham) using the iodogen method. They were then washed three times and lysed. To measure phosphorylation,  $30 \times 10^6$  cells were washed three times in phosphate-free medium. Cells were resuspended at  $2 \times 10^6/\text{ml}$  in the same medium with 2% HY and incubated with 1 mCi  $^{32}\text{P}$ -orthophosphate (Amersham) for 2 h at  $37^\circ\text{C}$ . DMSO ( $1/10^4$  diluted) or PMA ( $2 \times 10^{-7}$  M) were added for 20 min at the end of the incubation time. Thereafter cells were washed and lysed.

Labeled cells were resuspended in 500  $\mu\text{l}$  of ice-cold lysis buffer containing 10 mM triethanolamine, 150 mM NaCl, 1% NP40, 5 mM  $\text{Mg}^{++}$ , 2 mM sodium metavanadate, 1 mM PMSF, 20  $\mu\text{g}/\text{ml}$  leupeptin, 1 mM TLCK, 0.2 mM trifluoperazin, 1  $\mu\text{M}$  microcystin at pH 7.8, for 45 min at  $0-4^\circ\text{C}$ . Cell lysates were spun down at 13,500 rpm for 15 min at  $4^\circ\text{C}$  and supernatants were precleared by incubating them with 100  $\mu\text{l}$  of protein G-Sepharose precoated with normal mouse serum overnight at  $4^\circ\text{C}$ . The precleared lysates were then incubated with 100  $\mu\text{l}$  of protein G-Sepharose coated with anti- $\alpha 5$  antibody for 2 h at  $4^\circ\text{C}$ . Beads were then pelleted and washed six times in lysis buffer. Immunoprecipitates were solubilized with 100  $\mu\text{l}$  of SDS-sample buffer for 5 min at  $100^\circ\text{C}$ . Samples were electrophoresed on 7.5% SDS-PAGE according to Laemmli (1970).

### Cell sorter analysis

NB4 cells were pretreated or not with  $10^{-7}$  M PMA for 1 h at  $37^\circ\text{C}$ . Cells ( $2 \times 10^7/\text{ml}$ ) were centrifuged and resuspended in PBS with 0.5% BSA, at  $4^\circ\text{C}$ . Subsequent steps were performed at this temperature; 50  $\mu\text{l}$  aliquots of NB4 suspensions were added to 20  $\mu\text{l}$  of MoAbs (previously mentioned) in Eppendorf tubes. After a 30-min incubation, cells were washed twice with a BSA-containing PBS solution, by centrifugation at 2,700 rpm for 5 min. Cell pellets were resuspended in 100  $\mu\text{l}$  of a 1/30 dilution of a FITC-labeled goat antimouse antibody (Nordic Immunology) and incubated for 30 min. After two washings, cells were resuspended in 1 ml PBS and analyzed by flow cytometry on an Ortho 50 H flow cytometer.

## RESULTS AND DISCUSSION

### PMA modulates leukemic cell adhesion to marrow stromal cells and ECM

It is well established that hemopoietic progenitors, immature myeloblastic or promyelocytic cells are tightly associated with marrow stromal cells in the marrow as well as to in vitro cultured stromal cells (Dexter et al., 1977a; Coulombel et al., 1988, reviewed by Storok-Storb, 1988). By contrast, leukemic cells at the same stages show marrow egress that could be favoured by loose interactions with stromal cells. In this work, using NB4 leukemic cells in an in vitro assay, we found that only 10% and 7% of these cells adhered to stromal cells and to extracellular matrices, respectively

TABLE 1. NB4 cell adhesion to human marrow stromal cells and their ECM<sup>1</sup>

	NB4 adherent cells (% of total cpm)	
	PMA	
	-	+
Stromal cells	$10.0 \pm 1.5$	$33.5 \pm 2$
ECM	$7.0 \pm 1.5$	$38.0 \pm 2$

<sup>1</sup>Radio-labeled NB4 cells treated or not with PMA ( $10^{-7}$  M) were added to stromal cells or ECM preparations and allowed to adhere for 2 h at  $37^\circ\text{C}$ . Results are expressed as a percentage of total cpm in each well. Each value represents the mean of quadruplicate assays and standard deviations.

(Table 1). We wondered whether these low scores reflected some constitutive or functional adhesion defects. PMA, a potent activator of the PKC intracellular messenger effector system, added to the NB4 cell culture rapidly raised the percent adherent cells to 33% on stromal cells and 38% on ECM (Table 1). The percentages of adherent cells remained relatively low (no more than 38%). This may be explained by the fact that cells adhere to a specific substrate, which is the limiting parameter in the assay. Indeed, we found that nonadherent cells from the first exposure attached to a similar stromal cell layer in a second assay, with the same proportions, thus demonstrating that all PMA-treated cells gained the capacity to adhere to microenvironmental cells (not shown). These data posed the question of the mechanism responsible for PMA-induced leukemic cell adhesion and the biochemical features of the receptor-ligand complex which is the ultimate target of the PMA signal.

### PMA-induced adhesion of leukemic cells to ECM is fibronectin-specific and involves the activation of PKC

We tested the ability of nontreated or PMA-treated cells to adhere to different substrata that are known components of the ECM: laminin, fibronectin, collagens I, III, IV, and V. We observed that nontreated cells did not adhere to collagens IV and V and presented only a weak adhesion to laminin, collagen I, collagen III, and fibronectin (Fig. 1). Controls with noncoated or BSA-coated plastic surfaces were negative. When cells were incubated with PMA, adhesion to FN was enhanced by 5–8-fold when compared to control without PMA. This response was restricted to FN: adhesion to other substrata was not enhanced by PMA treatment. NB4 cell suspension cultures consisted of spherical, nonadherent cells; 2 h after PMA treatment about 50% of the adherent cell population showed cytoplasmic elongation (pseudopodia). Prolonged incubation in the presence of PMA (3 h or more) increased this fraction and resulted in the spreading of cells on the substrata.

We wondered if other substances acting on the different signal transduction pathways could restore a physiological state of adhesion to FN in the same manner as PMA. The cAMP elevating agents (CT, IBMX, isoproterenol, PGE1 and PGE2) and the cAMP analogs (8-CPT-cAMP and 8-Cl-cAMP) did not modify the adhesion of NB4 cells (Table 2). Other prostaglandins such as PGF1 $\alpha$ , PGF2 $\alpha$ , PGA1, and PGD2 were also ineffective in inducing adhesion. PMA ( $10^{-7}$  M), PDBu ( $10^{-6}$

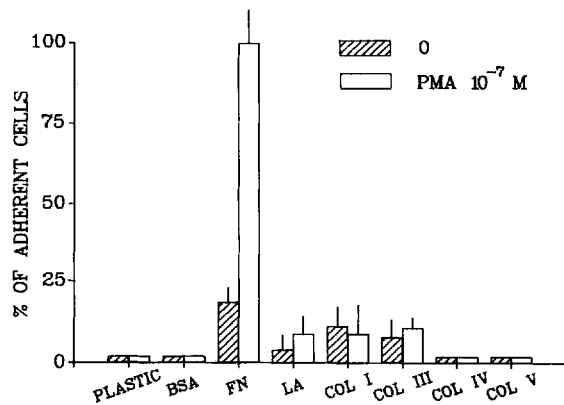


Fig. 1. Adhesion of NB4 cells to different substrata. Substrata were prepared by coating tissue culture plates for 4 h at 37°C with 2.5  $\mu$ g/well of the indicated proteins and saturating nonspecific binding sites with BSA (see Materials and Methods). Cells treated or not with PMA ( $10^{-7}$  M) were allowed to adhere to the substrata for 2 h at 37°C. Cell adhesion was estimated using the spectrophotometric BCA assay.

TABLE 2. Effects of various compounds triggering the different signal transduction pathways on the FN-adhesion of NB4 cells

Reagents	Molarity <sup>1</sup> (M)	Induction of cell adhesion <sup>2</sup>
cAMP-elevating agents		
CT	$10^{-8}$	—
IBMX	$10^{-4}$	—
Isoproterenol	$10^{-5}$	—
PGE1	$10^{-4}$	—
PGE2	$10^{-4}$	—
cAMP analogs		
8-CPT-cAMP	$10^{-4}$	—
8-Cl-cAMP	$10^{-6}$	—
phorbols		
PMA	$10^{-7}$	+++
PDBu	$10^{-6}$	+++
PMA-Methyl-Ether	$2.10^{-6}$	+++
Phorbol	$2.10^{-6}$	—
4 $\alpha$ -Phorbol	$2.10^{-6}$	—

<sup>1</sup>Highest concentration tested.

<sup>2</sup>Compared to control without reagent (i.e., 5–15% of adherent cells/ (–): 0–15%; (+): 15–30%; (++) : 30–70%; (+++) : 70–100%).

M), and PMA-methyl-ether ( $2.10^{-6}$  M) induced an increase in the adhesion of NB4 cells to FN (from 5–15% to 70–100% adherent cells). However PMA was the most potent phorbol to increase adhesion. Nontumor promoter phorbols (phorbol and 4 $\alpha$ -phorbol) were inefficient.

NB4 cells were incubated for 30 min with increasing doses of PMA ( $10^{-10}$  M to  $10^{-6}$  M), at 37°C (Fig. 2A). Maximal adhesion was obtained with  $10^{-7}$  M PMA (90–100% of adherent cells). PMA-induced NB4 cell adhesion to FN followed a rapid kinetic. A maximal adhesion was obtained 15 min after addition of PMA (Fig. 2B) and persisted for at least 360 min. Moreover, we found (data not shown) that a pulse of PMA, followed by washing off PMA, was enough to fully induce adhesion to FN. It suggests that adhesion of NB4 cells could account for a direct modification of the adhesion effector complex, rather than a transcriptional regulation. Adhesion to FN-coated surface was inhibited by blocking the membrane remodeling: adhesion decreased by 66% in the presence of 10 mM sodium azide

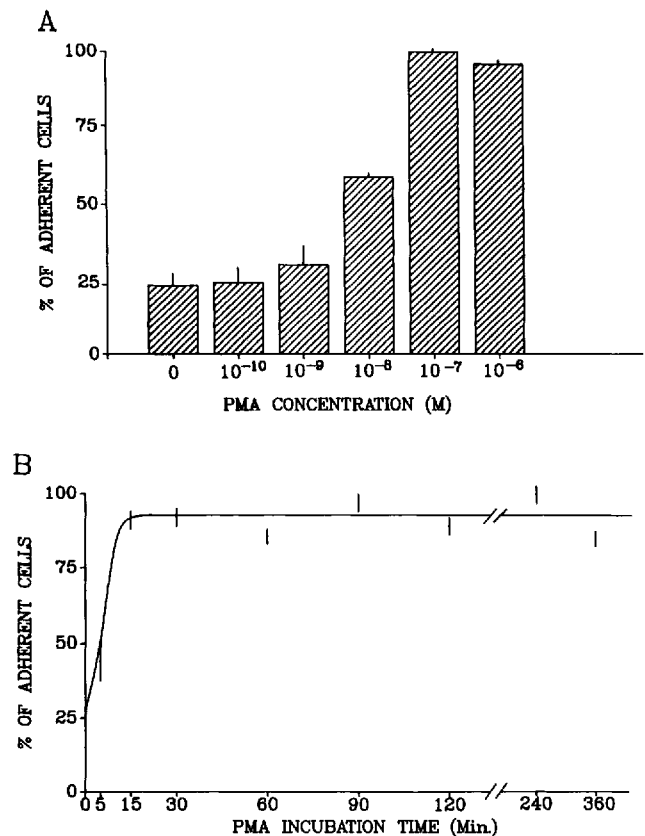


Fig. 2. Dose-response and kinetic of adhesion of PMA-treated cells to fibronectin-coated wells. A. NB4 cells ( $10^6$ /ml) treated with different concentrations of PMA ( $10^{-10}$  M– $10^{-6}$  M) were allowed to attach to fibronectin-coated plates for 2 h at 37°C. The number of adherent cells was determined by the BCA assay (the curve is calibrated using the linear correlation between the absorbance at 570 nm and the number of attached cells observed in this assay, not shown). B. Plates were coated with fibronectin (50  $\mu$ g/ml solution). NB4 cells were treated with PMA ( $10^{-7}$  M) and added to the wells. Plates were immediately centrifuged for 1 min at 1,200 rpm and incubated at 37°C for various periods of time (5, 15, 30, 60, 90, 120, 240, and 360 min of incubation). Reaction was stopped by removing nonattached cells as described in Materials and Methods. Adherent cells were measured using the BCA assay.

and this was not due to cell death. In addition, the induction of adhesion by PMA was blocked when plates were incubated at 4°C (not shown). These data suggested an energy-dependent process in adhesion.

As PMA is a potent activator of PKC, we wondered whether the induction of adhesion could be inhibited by specific PKC inhibitors. Cells were either preincubated for 30 min with staurosporine ( $10^{-9}$  M to  $10^{-6}$  M) or sphingosine ( $10^{-7}$  M to  $10^{-4}$  M) before addition of PMA ( $10^{-7}$  M) or co-incubated with PMA and CGP41251 ( $10^{-10}$  M to  $10^{-5}$  M) or calphostin C ( $5.10^{-9}$  M to  $10^{-6}$  M). All these compounds inhibited adhesion in a dose-dependent manner (Table 3). CGP 41251 and calphostin C showed higher specificity for PKC compared to staurosporine or sphingosine (Meyer et al., 1989; Bruns et al., 1991). W7, a specific inhibitor of calmodulin-dependent protein kinase did not modulate the NB4 cell adhesion to FN. All these compounds had no effect on cell viability for the 2-h incubation time, even at the

TABLE 3. Effects of different protein kinase inhibitors on the PMA-induced adhesion of NB4 cells to FN<sup>1</sup>

Inhibitors	Molarity (M)	% of inhibition of adhesion	IC50 (M)
Staurosporine	10 <sup>-8</sup>	0	1.65.10 <sup>-7</sup>
	10 <sup>-7</sup>	25	
	10 <sup>-6</sup>	90	
Sphingosine	10 <sup>-6</sup>	0	6.3.10 <sup>-6</sup>
	10 <sup>-5</sup>	90	
CGP41251	10 <sup>-9</sup>	0	8.5.10 <sup>-8</sup>
	10 <sup>-7</sup>	54	
	10 <sup>-6</sup>	87	
Calphostin C	2.10 <sup>-8</sup>	8	3.6.10 <sup>-7</sup>
	10 <sup>-7</sup>	23	
	5.10 <sup>-7</sup>	69	
W7	10 <sup>-6</sup> to 5.10 <sup>-12</sup>	0	

<sup>1</sup>Cells were either preincubated with serial dilutions of an inhibitory compound (staurosporine or sphingosine) before the addition of PMA, or simultaneously incubated with an inhibitory compound (CGP 41251, Calphostin C or W7) and PMA. Adhesion was carried out for 2 h at 37°C.

TABLE 4. NB4 cell surface expression of VLA integrin subunits analyzed by flow cytometry<sup>1</sup>

Monoclonal antibody	Integrin subunit specificity	Positive cells (% of total)	
		PMA	
		-	+
K 20	β1	85.5	90.1
GI 9	α2	2.5	0.2
HP2/1	α4	84.1	88.3
SAM 1	α5	85.0	88.3
GoH 3	α6	6.3	1.2

<sup>1</sup>Untreated or PMA-treated cells were incubated with primary monoclonal antibodies directed against a specific integrin subunit, then with fluorescein isothiocyanate-labeled goat antimouse IgG. Results expressed as percentage of positive cells are representative values from two separate experiments.

highest doses tested. These results strongly suggested that the effects of PMA were mediated by protein kinase C activation.

#### VLA-5 specific FN-receptors are involved in PMA-induced leukemic cell adhesion

PMA-induced adhesion was also blocked when cells were incubated in medium with 1 mM EDTA (94% inhibition, not shown). This result supported the idea of a divalent cation-dependent mechanism resembling integrin-mediated adhesion. We examined the possibility of involvement of VLA-integrin receptors in PMA-induced adhesion. One hypothesis would be that PMA changes the expression pattern of receptors involved in recognition of ECM components and especially FN as it has been described for U937 cells (Ferreira et al., 1991). The expression of CD29, CDw49b, CDw49d, Cw49e, and CDw49f molecules, which correspond to β1, α2, α4, α5, and α6 chains of integrins, was analyzed. CD29, CDw49d, and CDw49e (β1, α4, and α5) were found to be expressed on NB4 cells (Table 4); other antigens (α2 and α6) were not detected. The results showed that α4β1 (VLA-4) and α5β1 (VLA-5) receptors are constitutively expressed on NB4 cells and that PMA did not modulate either the percentage of positive cells or the level of membrane expression of these integrins (Fig. 3). The absence of expression of other integrins (α2β1, α6β1) persists after PMA treatment. This suggests that PMA-induced adhesion could be explained

rather by a structural modification and/or activation of a FN-specific integrin receptor, than by an increase in its membrane expression.

We determined whether the expressed receptors (α4β1 and α5β1) were involved in PMA-induced adhesion. Gi9 (anti-α2) and HP2/1 (anti-α4) MoAbs did not affect NB4 adhesion to FN (Fig. 4). A slight inhibitory effect (13% inhibition) was observed with GoH3 (anti-α6) MoAb. By contrast, AIIIB2 (anti-β1) MoAb and SAM1 (anti-α5) MoAbs inhibited attachment of NB4 cells to FN (87% and 80% inhibition, respectively). This inhibitory effect was not significantly increased (90%) when the anti-β1 MoAb was simultaneously added to the anti-α5 MoAb. These results support the conclusion that adhesion of NB4 cells to FN observed after PMA treatment was mediated by the receptor α5β1 (VLA-5) and that the other FN-specific receptor VLA-4 was not involved in this interaction. The primary cell binding site of FN has been assigned to the sequence Arg-Gly-Asp (RGD) localized within the central binding domain of the molecule and is recognized with high affinity by the α5β1 integrin (Hynes, 1987; Ruoslahti, 1987). Competition experiments demonstrated that the synthetic RGDS peptide inhibited NB4 cells adhesion to FN with an ID<sub>50</sub> of ~ 5.8 mM (Fig. 5). The control peptide RGEs did not prevent cell adhesion. It confirmed that PMA-induced adhesion to FN is mediated by the α5β1 integrin, a conclusion that is in agreement with previous work showing that VLA-5 is a specific FN-receptor involving the RGD site in a number of cell systems (Ruoslahti and Pieerschbacher, 1987; Ruoslahti et al., 1988).

#### PKC-dependent activation of α5β1 receptor is not due to its phosphorylation

Considering the kinetic of response to PMA (Fig. 2B), it was unlikely that PMA acted at the gene transcription level to promote adhesion, as it has already been described in other cell systems (Goldstein et al., 1990). Cell sorter analyses proved that adhesion did not result from a modulation of the expression of integrin receptors at the membrane surface (de novo synthesis or redistribution of cryptic receptors). The activation of an integrin-receptor complex constitutively expressed at the cell surface, as already described in other hemopoietic cells (Symington et al., 1989; Ylanne et al., 1990; Van Kooyk et al., 1991), seems more likely. We questioned whether stimulation of PKC by PMA resulted in an altered pattern of phosphorylation of the α5β1 receptor. A modulation of phosphorylation of integrins (β2 integrins or α6β1) induced by PMA treatment has already been observed in mononuclear cells and in macrophages by Chatila et al. (1989) and Shaw et al. (1990). As seen in Figure 6, two bands were observed after immunoprecipitation of iodinated cells, corresponding to the α5 chain (160 kDa) and the β1 chain (135 kDa) of the receptor but none of them were constitutively phosphorylated in NB4 cells and PMA treatment did not induce their phosphorylation. To definitively exclude the possibility of phosphatase activity in lysates, the highly potent phosphatase inhibitor microcystin (1 μM) was added to the panel of phosphatase inhibitors classically used (see methods). In conclusion, VLA-5 seems to be a very poor substrate for PKC since no phosphoryla-

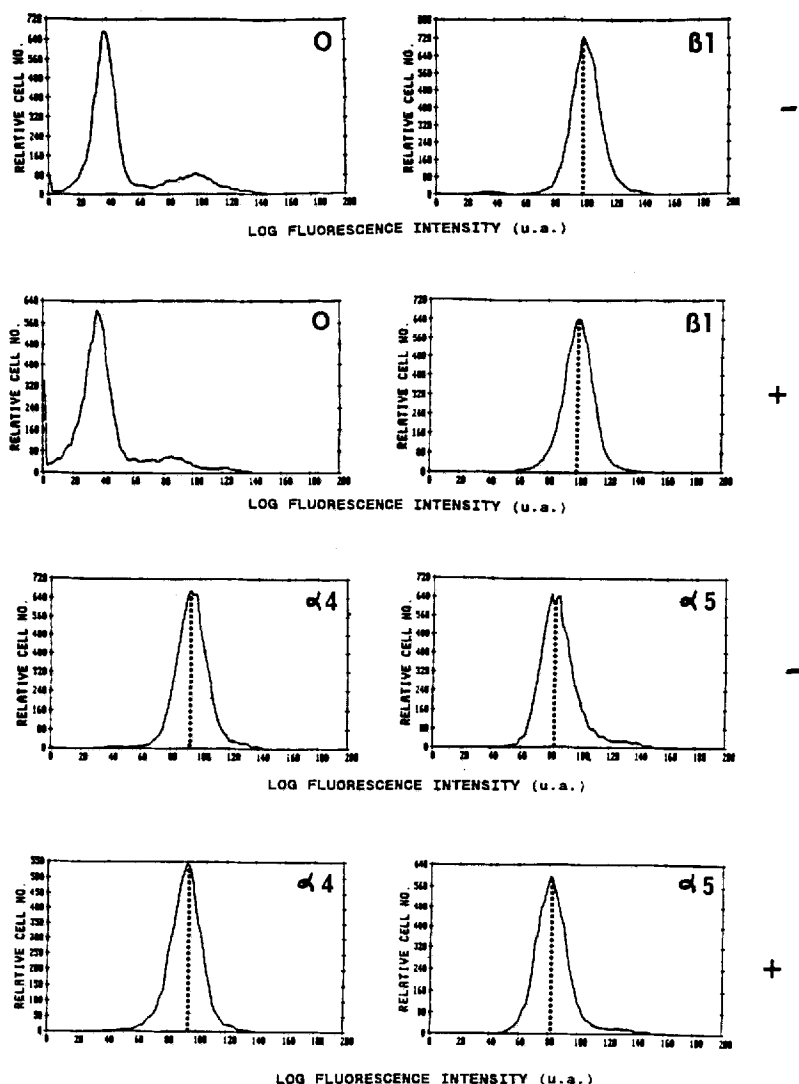


Fig. 3. Flow cytometry analysis of the expression of  $\beta 1$ ,  $\alpha 4$ , and  $\alpha 5$  integrin subunits on NB4 cell surface treated or not with PMA. NB4 cells were incubated for 30 min at 4°C with a primary antibody (K20, HP2/1 or SAM1), washed twice, then labeled with a FITC-conjugated goat antimouse Ig G for 30 min at 30°C.

tion was detected, and thus confirmed the observation made by Danilov and Juliano (1990).

These experiments show that PMA modulation of adhesion probably does not involve phosphorylation of the receptor itself. PKC-dependent adhesion could involve the phosphorylation of other cellular proteins that interact directly (cytoskeletal proteins) or indirectly with the FN receptor complex during the adhesion process. Previous reports, in other biological systems, suggest a regulation of  $\beta 1$  integrin function by modification of molecular avidity (Neugebauer and Reichardt, 1991) in relation to conformational changes. Components of cell membrane associated to integrins, like lipids (ganglioside GD<sub>3</sub> for  $\alpha 5\beta 1$  (Stallcup et al., 1989); IMF-1 lipid (Wright et al., 1990, 1992) for complement receptor type 3 (Wright et al., 1989), could also participate in transient allosteric changes sufficient to explain increased functional activity. Ferreira et al. (1991) showed that FN receptors of PMA treated U937 cells

had different molecular masses than those of resting cells. Differences in FN receptor sialylation have also been described in K562 leukemic cells after PMA treatment (Van de Water et al., 1988; Symington et al., 1989). It is unlikely that such glycosylation or sialylation changes are involved in the NB4 adhesion to FN since PMA treatment of NB4 cells is short (2 h) compared to PMA exposure (at least 38 h) in the cases of U937 or K562 cells. Other mechanisms of regulation of VLA-5 activation such as protein dephosphorylation or intracellular calcium changes are not excluded.

## CONCLUSIONS

Numerous reports have suggested that integrins are involved in cell migration and metastatic tumor invasion (Liotta, 1986). Moreover, the increased or decreased expression of integrins was found associated to the enhanced capacity of cells to become tumorigenic. In the hematopoietic system, the adhesive capacity of

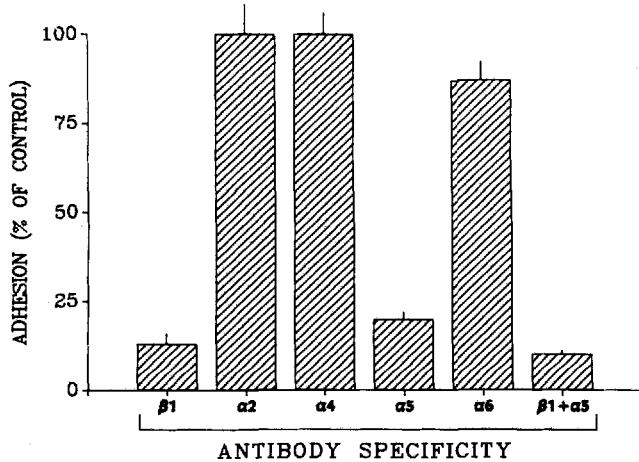


Fig. 4. Inhibition of PMA-treated NB4 cells adhesion to fibronectin by anti-integrin MoAbs. PMA-treated cells were preincubated for 30 min at 37°C with the following MoAbs (1/100 dilution) before their addition to fibronectin-coated wells: A1B2 (anti-β1 chain), G19 (anti-α2 chain), HP2/1 (anti-α4 chain), SAM1 (anti-α5 chain), and GoH3 (anti-α6 chain). Results are expressed as a percentage of adherent cells in controls (wells without MoAbs).

progenitor cells regulates proliferation and differentiation (Torok-Storb, 1988) and influences the response to growth factors (Gordon, 1988). Abnormal response to growth factors and maturation defects are features of leukemia generally associated to undue egress of immature cells from the marrow. In this work, we show that PKC activation can restore a physiological state of adhesion of promyelocytic cells to stromal cells or their ECM through a specific RGD-dependent binding to FN. This interaction resulted from a PKC driven intracellular mechanism that operated on the VLA-5 specific FN receptor. The increase in adhesion does not result from a modulation of the expression of VLA-5 receptors at the membrane surface or from an activation of this receptor consequent to its phosphorylation. In this work, we have defined a promising model to study the synergistic effects of FN-dependent adhesion and growth factor or maturation inducer signals on leukemic cell differentiation.

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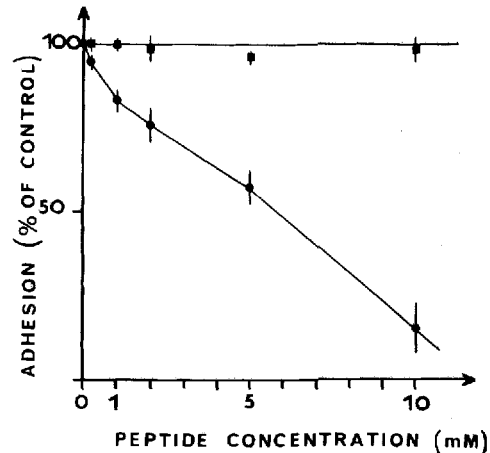


Fig. 5. Inhibition of PMA-treated NB4 cell adhesion to FN by RGDS peptide. Cells were incubated with RGDS peptide (●). RGES peptide (■) was used as a negative control. Adhesion on FN coated wells (coating concentration of 25 µg/ml) was carried out for 2 h at 37°C. Each point is the mean of triplicate experiments.

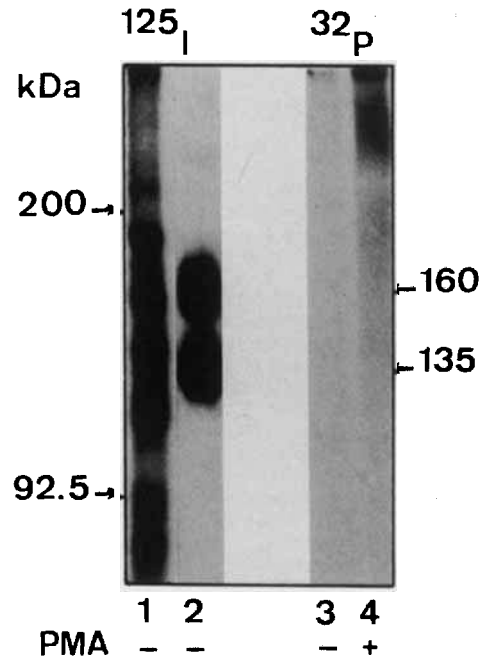


Fig. 6. Immunoprecipitation of α5β1 integrin. Surface iodinated NB4 cells (lanes 1, 2) or <sup>32</sup>P-labeled untreated (lane 3) or PMA-treated (lane 4) cells were lysed and immunoprecipitated. Lysates (lane 1) were incubated with normal mouse serum coupled to protein G-Sepharose beads overnight, then with anti-α5-protein G-Sepharose beads. Bound proteins were solubilized by SDS-containing sample buffer and resolved by 7.5% SDS-PAGE and visualized by autoradiography.

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