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A LEUKOCYTE LIPID UP-REGULATES THE AVIDITY OF LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN-1

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Summary: An acidic lipid termed leukocyte adhesion lipid (LAL) was isolated from PMA stimulated lymphoid and myeloid cell lines HL60, Jurkat, K562 and U937 but not from unstimulated cells or PMA treated Cos7 cells. LAL treated peripheral blood leukocytes (PBL) adhered strongly to IL-1β activated human umbilical vein endothelial cells (HUVEC), and the interaction could be inhibited by antibodies to intercellular adhesion molecule (ICAM-1) or lymphocyte function-associated antigen-1 (LFA-1). Leukocytes treated with LAL maintained the high avidity state of LFA-1 for at least 1 hr whereas the avidity of LFA-1 in PMA treated cells declined after 30 min. LAL was stable to heat (100°C, 10 min), alkaline phosphatase and proteinase K treatments. Chemical analysis suggested that LAL contained unsaturated lipids. Our findings provide evidence for the involvement of lipids in LFA-1 activation.

It has been established that in resting leukocytes, the heterodimeric β_2 integrin, LFA-1, does not (or only weakly) interact with its ligand, ICAM-1 (1). Upon cellular activation by phorbol esters (2) or cross linking of surface molecules such as CD3 (3), there is a transient and rapid increase in the avidity of LFA-1, subsequently leading to ligand binding. Although the detailed mechanism for activating LFA-1 has not been elucidated, it is believed to involve multiple cellular signal transduction events (4).

Lipids have been shown to be involved in the regulation of cell adhesion events. By depriving macrophages of essential fatty acids, which affects sphingomyelin synthesis, spreading and adherence were impaired (5, 6). Fibrinogen binding to αIIbβ3 is also

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ABBREVIATIONS:

HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IMF-1, integrin modulating factor-1; LAL, leukocyte adhesion lipids; LFA-1, lymphocyte function associated-antigen-1.

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modulated by exogenous addition of phosphatidic acid and lysophosphatidic acid (7). Only one lipid fraction, an acidic lipid termed integrin modulating factor 1 (IMF-1) that was purified from activated neutrophils (8), was reported to up-regulate β2 integrin function. IMF-1 has been shown to regulate Mac-1 binding to iC3b and modulated ICAM-1/LFA-1 dependent cell adhesion. We report here the isolation of an acidic lipid fraction from lymphocytic (Jurkat and K562), histiocytic (U937) and monocytic (HL60) cell lines after stimulation with PMA. This lipid fraction which we named leukocyte adhesion lipids (LAL), up-regulates LFA-1 avidity. Human PBL treated with LAL bind to IL-1 activated HUVEC in an ICAM-1/LFA-1 dependent manner. Our findings provide evidence for the involvement of lipids in LFA-1 activation and cell adhesion.

MATERIALS AND METHODS

Cells and cell culture: The fibroblastic Cos7, promyelocytic HL60, T-leukemic Jurkat, erythroleukemic K562 and histiocytic U937 cells were obtained from the American Type Culture Collection (Rockville, MD). Jurkat, K562 and U937 cells were maintained in RPMI medium supplemented with antibiotics and 10% FCS and cultured at 37°C with 5% CO_2. The HL-60 cells were similarly maintained except that 20% FCS was used. HUVEC (Clonetics, San Diego, CA) were maintained in M199 supplemented with antibiotics, growth factors and 20% FCS.

Antibodies, chemicals, enzymes and reagents: Purified monoclonal anti-ICAM-1 antibody 84H10 was obtained from AMAC (Westbrook, ME). The hybridoma TS1/22 (anti-CD11a) was obtained from the American Type Culture Collection (Rockville, MD) and maintained in protein free hybridoma medium. Hybridoma culture supernatant was filtered and used without dilution. FITC-conjugated goat anti-mouse antibody was obtained from Becton Dickinson (San Jose, CA). Purified monoclonal anti-CD3 antibody OKT3 was obtained from Ortho Biotech (Raritan, NJ). General chemicals, spray reagents (orcinol ferric chloride, rhodamine B, α-cyclodextrin, cupric acetate, 1,6-diphenyl-2,3,5-hexatriene and phosphomolybdic acid) and organic solvents were obtained from Sigma (St. Louis, MO), BDH (Poole, England) or Fisher (Fairlawn, NJ). Calf intestine alkaline phosphatase was obtained from New England Biolab (Beverly, CA). Proteinase K was obtained from Stratagene (La Jolla, CA).

LAL extraction: All glassware was prewashed in chloroform:methanol, 1:1 and dried at 120°C before use. Cells were harvested and resuspended at 10^7/ml in PBS and prewarmed to 37°C. PMA (50ng/ml) was added to the cell suspension and incubated at 37°C for 20 min. The suspension was diluted with cold PBS and cells were pelleted by centrifugation at 4°C for 10 min at 800 xg. The pelleted cells were washed twice in cold PBS and lysed in chloroform:methanol:H_2O, 10:10:1 (10^8 cells/ml). The cell lysate was vigorously extracted for 36 hr with 2 solvent changes. Acidic lipids were prepared by ion exchange and C18 reverse phase chromatography as described (8), dissolved in chloroform:methanol, 2:1 and applied to a prewashed silica G TLC plate (Whatman, Maidstone, England). The plate was developed in chloroform:methanol:water, 65:25:4. A strip of the plate was cut off, air-dried and stained with iodine. Visible stained spots were marked. The regions in the remainder of the plate corresponding to the marked spots

were scraped off and extracted with chloroform:methanol, 2:1 for 16hr. The solvent was evaporated and the lipids were dissolved in methanol. The activity of the LAL was measured by its ability to activate resting human PBL to adhere to IL-1β-treated HUVEC. Typically, lipids extracted from 20 cells were used to stimulate one PBL and this amount of lipids was arbitrarily defined as 1 active unit. LAL was stored under nitrogen at 4°C.

Liquid chromatography: For HPLC, samples of PMA or LAL in ethanol were analyzed using Beckman's System Gold (San Ramon, CA). The samples were passed through a Beckman Ultrasphere XL C18 column (dp 3μ, 4.6mm x 7cm) eluted with isocratic 80% acetonitrile and detected spectrophotometrically at 230nm.

Cell adhesion assay: Preparation of ICAM-1$^+$ monolayer by treating HUVEC with IL-1 (9) and fluorescein labelling of PBL with 5-carboxyfluorescein diacetate (10) (Molecular Probes, Eugene, OR) were carried out as described. PBL were isolated using Ficoll-paque (Pharmacia, Uppsala, Sweden) according to the vendor's instructions. Lipid samples dissolved in methanol or antibodies were added to the wells in triplicates and made up to 50 μl with RPMI 1640 medium containing 5% FCS. Fifty microliters of the fluorescein-labelled PBL ($2x10^5$ cells) were dispensed into each well. Control PBL that required PMA treatment were preincubated with 50 ng/ml of PMA at 37°C for 20 min. PBL were allowed to adhere at 37°C for 30 min. Unbound cells were removed by 3 successive washings with PBS (11). Bound cells were quantitated by measuring the fluorescence of each well in a fluorescence concentration analyzer (Idexx, Westbrook, ME). Data were normalized to total fluorescence of each well and expressed as percent cells bound.

RESULTS AND DISCUSSION

Acidic lipids isolated from PMA-treated cells were further fractionated by TLC using the solvent system chloroform:methanol:water, 65:25:4. Seven bands were identified after iodine staining (Fig. 1a). To determine whether any of these lipid bands enhanced the adhesion of PBL, PBL were incubated with the lipid sample extracted from the various bands on the TLC plate for 20 min at room temperature. These PBL were allowed to adhere to a HUVEC monolayer which had been treated with IL-1β to enhance ICAM-1 expression. Only one lipid fraction stimulated PBL adhesion which could be inhibited by anti-ICAM-1 or anti-LFA-1 antibodies (Fig. 1b). This lipid fraction which exhibited an Rf value of 0.969 in the running solvent system, was designated as LAL. TLC analysis indicated that the Rf of LAL was very similar to ceramides and linoleic acid (Fig. 1a). Pretreatment of LAL with alkaline phosphatase, proteinase K, or boiling for 5 min did not affect its LFA-1 modulating activity. Flow cytometric analysis showed that the LAL did not affect LFA-1 expression on PBL (data not shown) suggesting that LAL stimulated PBL adhesion by up-regulating LFA-1 avidity rather than affecting LFA-1 expression.

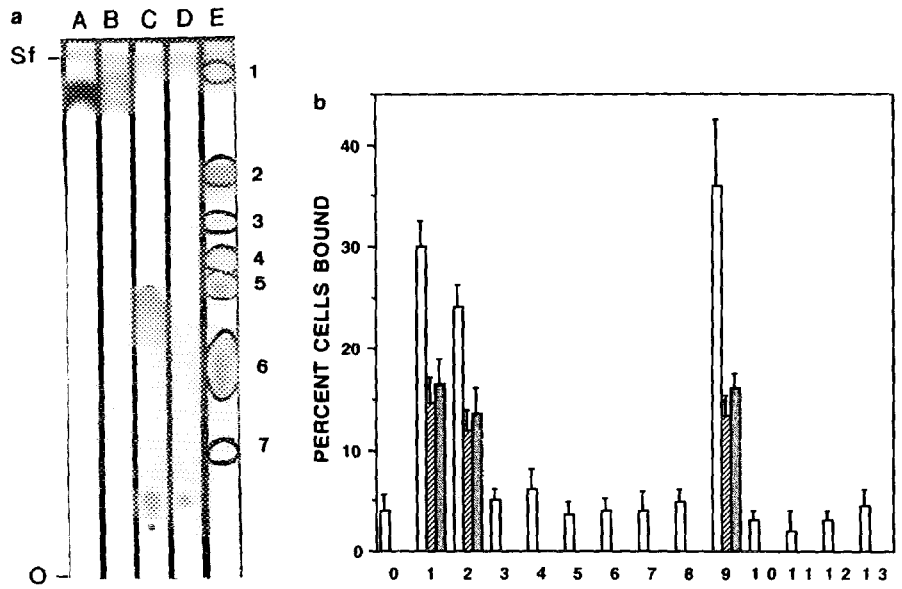
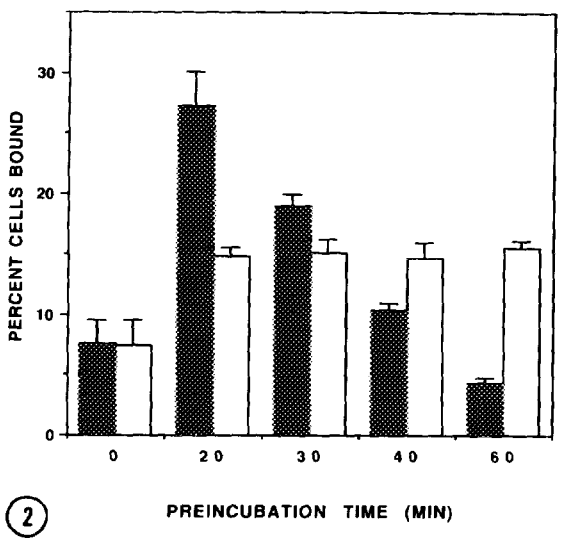


Figure 1a. TLC for acidic lipids isolated from PMA treated leukocytes. About $1x10^7$ units of acidic lipids isolated from PMA treated leukocytes (lane E) were applied onto a Silica G TLC plate which was then developed and stained as described in Materials and Methods. Visible stained spots in lane E were marked and numbered 1-7. Lipid standards linoleic acid, palmitic acid, ceramides and sphingosines were included as controls (lanes A-D). O and Sf represent the origin and solvent front respectively.

Figure 1b. LAL stimulates ICAM-1/LFA-1-dependent adhesion of PBL to IL-1β activated HUVEC. PBL were incubated with acidic lipids extracted from PMA stimulated PBL (lane 1), extracts from TLC bands 1-7 of acidic lipids isolated from PMA stimulated PBL (see Fig. 1a) (lanes 2-8) (~$1x10^4$ units/well), a blank region on the TLC plate (lane 0), 50ng/ml PMA (lane 9), acidic lipids isolated from unstimulated PBL (lane 10), acidic lipids isolated from PBL that were lysed prior to PMA stimulation (lane 11), acidic lipids isolated from PMA stimulated Cos7 cells (lane 12) and PMA that was subjected to the lipid extraction protocol (lane 13) and allowed to adhere to IL-1β treated HUVEC in the absence (open bars) or presence of anti-LFA-1 antibody, TS1/22 (hatched bar) and anti-ICAM-1 antibody, 84H10 (shaded bars). Data are mean values of three wells and error bars represent standard deviations. Multiple batches of lipids extracted from PMA stimulated leukocytes gave similar TLC and activity profiles.

Since LAL was extracted from PMA treated cells and PMA is known to stimulate PBL adhesion, it was crucial to rule out the possibility that the activity was due to PMA contamination of the LAL preparations. The stimulating effect of LAL on LFA-1 avidity was maintained even after 60 min of pretreatment. The effect of PMA on adhesion, on the contrary, declined with increasing pretreatment time and little stimulation was observed after 60 min of PMA pretreatment (Fig. 2). This provided evidence that the adhesion of PBL stimulated with LAL preparations was not simply due to PMA contamination. It is not



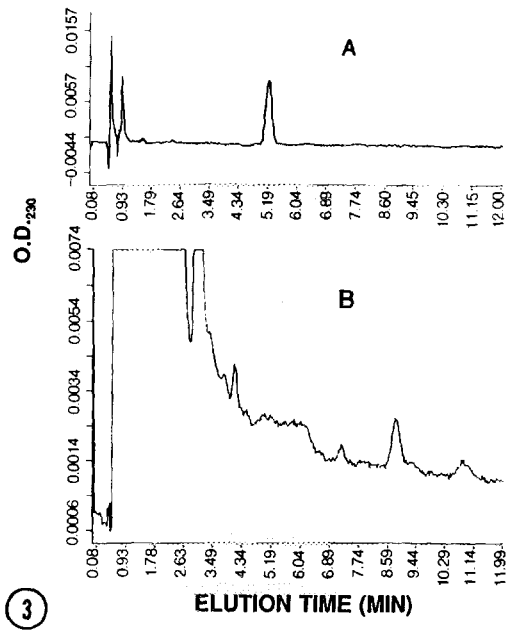


Figure 2. LAL induced PBL adhesion to IL-1β activated HUVEC is independent of the incubation time with PBL prior to adhesion assay. LAL ($1x10^4$units/well) (open bars) or PMA (50ng/ml) (closed bars) were added to PBL and incubated for different time periods prior to the adhesion assay. The percentages of adherent cells were measured. Data are mean values of three measurements and error bars represent standard deviations. Data obtained from PBL of a single individual are shown.

Figure 3. LAL does not contain significant PMA contamination. PMA (25ng) (Panel A) and LAL (~$5x10^7$ units) (Panel B) were analyzed by C18 reverse phase HPLC. The lipids were eluted with 80% isocratic acetonitrile and detected spectrophotometrically at 230nm.

clear why LAL, a product of PMA stimulation, maintained LFA-1 activation better than PMA. A possible explanation is that PMA stimulates a cascade of events leading to the generation of LAL. One (or some) of these reactions may be negatively regulated resulting in the termination of LAL synthesis and/or enhancement of LAL degradation. Exogenous addition of LAL to the cells by-passed the cellular regulatory mechanisms and was able to maintain the LFA-1 avidity. Thus, LAL probably represents a downstream event involved in the activation of LFA-1.

No detectable LAL activity could be extracted from unstimulated leukocytes, or when the leukocytes were first lysed in chloroform:methanol:water, 10:10:1 before PMA addition (Fig. 1b). Similarly, no LAL activity could be detected when Cos7 cells were treated with PMA or when a solution of PMA was subjected to the same extraction

protocol (Fig.1b). Finally, when LAL samples were subjected to C18 reverse phase HPLC analysis, no detectable species that resembled PMA in the elution profile could be detected (Fig. 3). These results excluded significant PMA contamination in the LAL preparations and identified LAL as a distinct biomolecule(s) that modulated LFA-1 avidity. The fact that LAL could be isolated only from PMA treated leukocytes but not fibroblasts suggested that its production was under stringent cell type specific regulatory control.

In order to characterize LAL, chemical analyses were performed using reagents such as orcinol ferric chloride, rhodamine B, α-cyclodextrin, cupric acetate, 1,6-diphenyl-2,3,5-hexatriene and phosphomolybdic acid. All these reagents reacted with LAL (data not shown) suggesting that LAL contains unsaturated lipid molecule(s). Thirty six known lipids were also tested for their abilities to stimulate PBL adhesion. None of the lipids when used alone, stimulated PBL adhesion (Table 1) even though some of the lipids such as ceramides and oleic acid had similar Rf values in the TLC analysis. However, when the lipids were tested in the presence of ionomycin and dioctylglycerol (12), linoleic acid (18:2), oleic acid (18:1) and palmitelaidic acid (16:1) stimulated adhesion of PBL to HUVEC (Fig. 4). The involvement of LFA-1 in the adhesion was determined by inhibiting

Table 1. Lipids that were tested for the ability to modulate LFA-1 avidity[1]

ceramides	arachidonic acid
lysophosphatidylcholine decanoyl	linolelaidic acid
lysophosphatidylcholine ethanolamine	linolenic acid
lysophosphatidylcholine myristoyl	γ-linolenic acid
lysophosphatidylcholine palmitoyl	myristoleic acid
lysophosphatidylcholine stearoyl	nervonic acid
lysophosphatidyl serine	palmitoleic acid
N-palmitoyl sphingomyelin	palmitelaidic acid
N-oleyl-D-sphingomyelin	palmitoleic acid
N-palmitoyl-D-sphingomyelin	petroselinic acid
N-(10-[1-pyrene]-decanoyl)sphingomyelin	recinelaidic acid
D-sphingosine	stearic acid
N-palmitoyl-D-sphingosine	9-tetradecynoic acid
hydroxymyristic acid	phosphatidic acid
hydroxypalmitic acid	phosphatidic acid dimyristoyl
hydroxystearic acid	phosphatidic acid dipalmitoyl
oleic acid	phosphatidic acid distearoyl
palmitelaidic acid	linoleic acid

[1]Lipids dissolved in methanol or ethanol were incubated with PBL at a final concentration of 25μM in the absence or presence of 50μM dioctylglycerol and 0.5μM ionomycin at 37°C for 20 min prior to the adhesion assay.

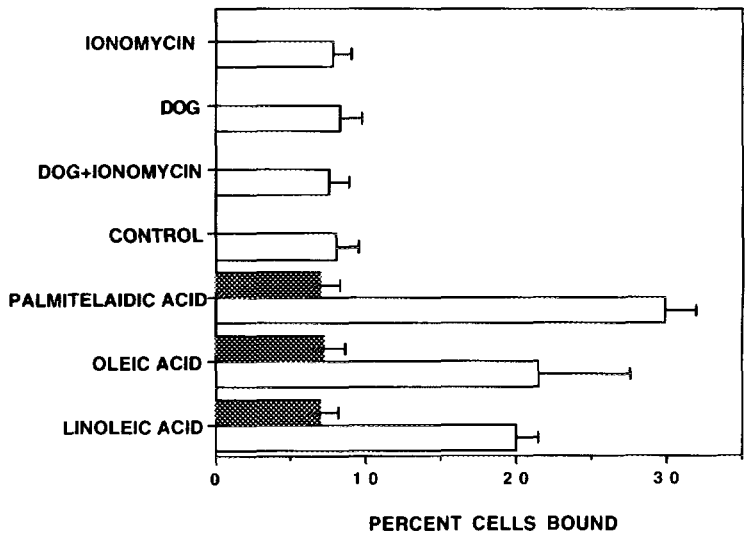


Figure 4. Three unsaturated fatty acids stimulated PBL adhesion to IL-1β treated HUVEC. Fatty acids (25μM), ionomycin (0.5μM) and dioctylglycerol (DOG, 50μM) were mixed with PBL and incubated at 37°C for 20 min prior to adhesion assay in the absence (open bars) or presence of the anti-LFA-1 antibody, TS1/22 (hatched bars). Data are mean values of three measurements and error bars represent standard deviations. Similar results were obtained in two experiments using PBL from two different individuals.

adhesion with an anti-LFA-1 antibody TS1/22. It is of interest to note that these three lipids are unsaturated fatty acids that contain a double bond at C-9 of the molecule. It is possible that LAL contains related unsaturated fatty acid(s) to account for its LFA-1 modulating activity.

We have isolated an acidic unsaturated lipid, LAL, from PMA treated cell lines of lymphoid or myeloid lineage which can modulate LFA-1 avidity. The exact mechanism for LAL to up-regulate LFA-1 avidity and the molecular composition of LAL are not clear. LAL may up-regulate LFA-1 avidity directly or indirectly by stimulating the production of other intermediates that would ultimately up-regulate LFA-1 avidity. Our data and that of others (8) define the involvement of lipids in the regulation of LFA- 1 avidity.

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