

Induction of Tyrosine Phosphorylation During ICAM-3 and LFA-1-mediated Intercellular Adhesion, and Its Regulation by the CD45 Tyrosine Phosphatase

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Abstract. Intercellular adhesion molecule (ICAM)-3, a recently described counter-receptor for the lymphocyte function-associated antigen (LFA)-1 integrin, appears to play an important role in the initial phase of immune response. We have previously described the involvement of ICAM-3 in the regulation of LFA-1/ICAM-1-dependent cell-cell interaction of T lymphoblasts. In this study, we further investigated the functional role of ICAM-3 in other leukocyte cell-cell interactions as well as the molecular mechanisms regulating these processes. We have found that ICAM-3 is also able to mediate LFA-1/ICAM-1-independent cell aggregation of the leukemic JM T cell line and the LFA-1/CD18-deficient HAFSA B cell line. The ICAM-3-induced cell aggregation of JM and HAFSA cells was not affected by the addition of blocking mAb specific for a number of cell adhesion molecules such as CD11a/CD18, ICAM-1 (CD54), CD2, LFA-3 (CD58), very late antigen α 4 (CD49d), and very late antigen β 1 (CD29). Interestingly, some mAb against the leukocyte tyrosine phosphatase CD45

were able to inhibit this interaction. Moreover, they also prevented the aggregation induced on JM T cells by the proaggregatory anti-LFA-1 α NKI-L16 mAb. In addition, inhibitors of tyrosine kinase activity also abolished ICAM-3 and LFA-1-mediated cell aggregation. The induction of tyrosine phosphorylation through ICAM-3 and LFA-1 antigens was studied by immunofluorescence, and it was found that tyrosine-phosphorylated proteins were preferentially located at intercellular boundaries upon the induction of cell aggregation by either anti-ICAM-3 or anti-LFA-1 α mAb. Western blot analysis revealed that the engagement of ICAM-3 or LFA-1 with activating mAb enhanced tyrosine phosphorylation of polypeptides of 125, 70, and 38 kD on JM cells. This phenomenon was inhibited by preincubation of JM cells with those anti-CD45 mAb that prevented cell aggregation. Altogether these results indicate that CD45 tyrosine phosphatase plays a relevant role in the regulation of both intracellular signaling and cell adhesion induced through ICAM-3 and β 2 integrins.

LEUKOCYTE β 2 integrins (lymphocyte function-associated antigen [LFA]-1, Mac-1, and p150,95) (Hynes, R. O., 1992) are one of the most important families of adhesion molecules involved in immune response. Three counter-receptors for LFA-1, which belong to the immunoglobulin superfamily, have been described: intercellular adhesion molecule (ICAM)-1, a widespread and cytokine-inducible molecule; ICAM-2, constitutively expressed in several cell types but noninducible; and ICAM-3, that has recently been characterized (de Fougères and Springer, 1992). ICAM-3 (CD50) contains five Ig domains and it is

structurally homologous to ICAM-1 and -2 (Fawcett et al., 1992; Vazeux et al., 1992; de Fougères et al., 1993). ICAM-3 expression is restricted to the leukocyte cell lineage, and its presence on resting T cells points out to a role for this antigen in the initial phases of immune response (de Fougères and Springer, 1992; Acevedo et al., 1993). Recently, we have reported the ability of ICAM-3 to regulate both the LFA-1/ICAM-1-dependent homotypic aggregation of T lymphoblasts and the affinity of LFA-1 for ICAM-1 (Campanero et al., 1993). Moreover, ICAM-3 induces T lymphocyte activation, expression of the activation antigens CD25 and CD69, and T cell proliferation (Campanero et al., 1993; Hernández-Caselles et al., 1993).

The functional involvement of the integrin LFA-1 in adhesion events during the immune response has been well demonstrated. LFA-1 plays a role in T cell activation facilitating cell-cell interactions (Springer, T.A., 1990). In this regard, LFA-1 can be considered not only as one of the most

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1. *Abbreviations used in this paper:* ICAM, intercellular adhesion molecules; IL, interleukin; LFA, lymphocyte function-associated antigen; PHA, phytohaemagglutinin; RT, room temperature; VLA, very late antigen.

important leukocyte adhesion molecules but also as an accessory coactivation receptor for antigen-driven T lymphocyte-mediated responses. Accordingly, LFA-1 participates in the induction of $[Ca^{2+}]_i$ changes, DNA synthesis, and interleukin (IL)-2 production by peripheral blood T cells (Pardi et al., 1989; Wacholtz et al., 1989; Van Seventer et al., 1991; Hernández-Caselles et al., 1993). In addition, it has recently been reported that $\beta 2$ integrin engagement triggers actin polymerization and phosphatidylinositol triphosphate formation in nonadherent human neutrophils (Löfgren et al., 1993).

The triggering of tyrosine protein phosphorylation upon the engagement of TcR/CD3 complex or other accessory molecules has previously been described (Hsi et al., 1989; Veillette et al., 1989). On the other hand, the tyrosine phosphatase CD45 has also been involved in T cell activation (Bernabeu et al., 1987; Ledbetter et al., 1988; Kiener et al., 1989; Pingel and Thomas, 1989; Koretzky et al., 1990, 1991; Samelson et al., 1990; Volarevic et al., 1990; Marvel et al., 1991). Phosphotyrosine signaling has also been described to play an important role in $\beta 1$ and $\beta 3$ integrin-mediated cellular aggregation (Lipfert et al., 1992; Sánchez-Mateos et al., 1993). Nevertheless, the induction of protein tyrosine phosphorylation through $\beta 2$ integrins or their ligands had not been investigated.

We report herein the existence of an alternative ICAM-3-mediated intercellular adhesion pathway in human leukocytes that is independent of LFA-1/ICAM-1. This homotypic aggregation can be regulated by anti-CD45 tyrosine phosphatase mAb. We also demonstrated that this regulatory effect is related to the triggering of protein tyrosine phosphorylation through either ICAM-3 or LFA-1. The ICAM-3 and LFA-1-mediated enhancement of tyrosine phosphorylation is modulated by the CD45 tyrosine phosphatase.

Materials and Methods

Cells and Cell Lines

Human T leukemic JM and Jurkat cell lines were grown in RPMI 1640 medium (Whittaker Labs., Walkersville, MD) supplemented with 5% FCS (Seromed, Biokrom, Berlin, Germany), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin (Seromed). The LFA-1/CD18-deficient HAFSA B cell line has been described (López-Rodríguez et al., 1993).

Human T lymphoblasts were obtained from peripheral blood mononuclear cells by treatment with phytohemagglutinin (PHA) at 5 μ g/ml for 48 h. Then, cells were washed and cultured in RPMI 1640 (Whittaker Labs.) containing 10% FCS, and 50 U/ml IL-2. T lymphoblasts cultured by 7–12 d were typically used in the experiments.

Mouse 300-19 pre-B cells lines transfected with the pZipneo plasmid vector or with cDNAs coding for different CD45 isoforms containing either the constant region of CD45 alone or in combination with different protein regions encoded by the variable exons (ABC, AB, BC, and B isoforms) were kindly provided by Dr. Michel Streuli (Dana Farber Cancer Institute, Boston, MA) and grown as described (Streuli et al., 1988).

Reagents

Genistein was purchased from Sigma Chem. Co. (St. Louis, MO). Herby-mycin A and tyrphostin 25 were purchased from Calbiochem (La Jolla, CA).

Monoclonal Antibodies

Anti-ICAM-3 HP2/19 and TP1/25; anti-LFA-1 α TP1/40 and NKI-L16; anti- $\beta 2$ Lia3/2; anti-CD3 SPV-T3b; anti-ICAM-1 RRI/1; anti-very late antigen (VLA)-4 HP2/1; anti-VLA- $\beta 1$ TS2/16; anti-CD2 TS2/18; anti-LFA-3 TS2/9; and anti-CD43 TP1/36 mAb have been described (Sánchez-Madrid

et al., 1982, 1986; Hemler et al., 1984; Spits et al., 1985; Rothlein et al., 1986; Keizer et al., 1988; Campanero et al., 1991, 1993). The anti-CD45 TP1/41 mAb was obtained in our laboratory from a fusion with splenocytes from mice immunized with activated human T lymphocytes and its precise specificity is described in this report. The other anti-CD45 mAb used in this study have been previously described (Pulido et al., 1988, 1989; Zapata et al., 1994). The anti-CD45RO UCHL.1 mAb was kindly provided by Dr. P. Beverley (Imperial Cancer Research Fund, London, U.K.). mAb were purified from ascites fluid using affinity chromatography on protein A-Sepharose columns (Pharmacia Fine Chemicals, Uppsala, Sweden). The anti-phosphotyrosine 4G10 and Py20 mAb were purchased from Upstate Biotechnology (Lake Placid, NY) and ICN Biochemicals (Cleveland, OH), respectively.

Aggregation Assays

Homotypic cell aggregation assays were performed as previously described (Campanero et al., 1990). Briefly, 10^5 cells/well were incubated in complete medium in flat-bottomed 96-well plates (Costar, Cambridge, MA) in the presence of mAb (1 μ g/ml), and cells were allowed to settle at 37°C and 5% CO₂ atmosphere. Aggregation was then determined at different periods of time by direct visualization of the plate with an inverted microscope and counting free cells in at least five randomly chosen fields of 0.025 mm², using a special grid under the plate. The assays were performed by duplicate. Results were expressed as percent of aggregated cells. For inhibition assays, cells were pretreated with different mAb for 10 min at room temperature (RT) before the addition of the inducing mAb.

Immunoprecipitation

Cells were ¹²⁵I-radiolabeled, lysed, and immunoprecipitated with different monoclonal antibodies as previously described (Sánchez-Madrid et al., 1983). Samples were subjected to SDS-7% PAGE under nonreducing conditions.

Cytofluorometry Analysis

Fluorescence flow cytometry analysis was performed on a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA). Cells were incubated at 4°C with 100 μ l hybridoma culture supernatant, followed by washing and labeling with and FITC-labeled goat anti-mouse Ig (Dakopatts, Copenhagen, Denmark). Data were collected in a logarithmic scale and the percentage of positive cells was determined by subtracting the background fluorescence given by the negative control mouse myeloma P3X63.

Immunofluorescence Staining

JM cells were incubated in flat-bottomed, 24-well microtiter plates (Costar) at 2×10^6 cells/ml in a final volume of 500 μ l of complete medium. mAb were added at a final concentration of 1 μ g/ml and cells were allowed to settle in an incubator at 37°C and 5% CO₂ atmosphere. After the induction of aggregation, the cells were fixed with 3.7% formaldehyde in PBS for 10 min RT and rinsed in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NaN₃). To directly visualize the mAb-inducing cell aggregation, 1:50 dilution of the FITC-labeled rabbit F(ab)₂ anti-mouse IgG (Pierce Chemical Co., Rockford, IL) was added. In order to detect tyrosine-phosphorylated proteins, cells were fixed and permeabilized with 0.2% Triton X-100. Then cell aggregates were incubated with biotinylated anti-phosphotyrosine Py20 mAb (ICN Biochemicals, Inc., Costa Mesa, CA) at a final concentration of 1 μ g/ml. The cells were washed and incubated with an 1:1,000 dilution of TRITC-avidin D (Vector, Burlingame, CA), then with an 1:100 dilution of anti-avidin D-biotin (Vector), and again with an 1:1,000 dilution of TRITC-avidin D (Vector). Cells were observed using a Nikon Labophot-2 photomicroscope with a 60 \times oil immersion objective and photographed on TMAX 400 film (Eastman Kodak Co., Rochester, NY) processed to 800–1600 ASA with TMAX developer (Eastman Kodak Co.).

Western Blot Analysis

JM cells ($5-10 \times 10^6$) were incubated in culture medium in presence of activating mAb for 5 min on ice bath. In some experiments, the cells were pretreated with different anti-CD45 mAb for 1 min at RT. A sheep anti-mouse Ig (Sigma Chem. Co.) at 20 μ g/ml was used as cross-linker during the time indicated. After stimulation, cells were lysed by adding a buffer containing 137 mM NaCl, 20 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, 1% NP-40, 150 μ M sodium orthovanadate, 1 μ g/ml

leupeptin, and 1 mM PMSF during 15 min on ice, and then centrifuged. Lysates were incubated with the anti-phosphotyrosine Py72 mAb at 5 μ g/sample and immunocomplexes were then isolated by addition of 187.1 anti-mouse kappa chain mAb and protein A-Sepharose. After washing, phosphoproteins were specifically eluted by 20 mM phenylphosphate incubation. Then samples were subjected to SDS-8% PAGE under reducing conditions and electrotransferred onto Immobilon-P membrane (Millipore, Bedford, MA) in Tris-Glycine-Methanol as buffer, for 12 h at 0.2 A, 50 V at 4°C. After blocking the membrane with 10% BSA in TBS (20 mM Tris, pH 7.5, 150 mM NaCl), protein bands were visualized by incubation with an ¹²⁵I anti-phosphotyrosine 4G10 mAb (Upstate Biotechnology, Inc., Lake Placid, NY), about 10⁶ cpm/ml during 2 h. Membranes were exposed to AGFA Curix film, and developed after 48 h.

Results

Homotypic Cell Aggregation Induced by Anti-ICAM-3 mAb Involves LFA-1/ICAM-1-dependent and -independent Pathways

To ascertain the role of ICAM-3 in leukocyte intercellular interactions, we studied the ability of anti-ICAM-3 mAb to induce intercellular adhesion in normal T lymphoblasts, leukemic JM T cells, and HAFSA B cells which are deficient for β 2 integrins. As shown in Fig. 1, the anti-ICAM-3 HP2/19 mAb was capable to induce cell aggregation of the three different cell types (Fig. 1, *top*). In contrast, the anti-ICAM-3 TP1/25 mAb, that recognizes a different epitope than HP2/19 (Campanero et al., 1993), did not aggregate these cells (Fig. 1, *bottom*).

We have previously reported that the ICAM-3-induced cell aggregation of T lymphoblasts is LFA-1/ICAM-1 dependent (Campanero et al., 1993). Since HAFSA cells do not express LFA-1, the only counter-receptor described for ICAM-3, it is possible that other adhesion molecular pathways could be involved in the intercellular interaction triggered by ICAM-3. Therefore, we tested several blocking mAb directed to adhesion receptors involved in leukocyte interactions, including CD2, LFA-3, LFA-1, ICAM-1, VLA α 4, and VLA β 1. As shown in Table I, cell aggregation induced by the anti-ICAM-3 HP2/19 mAb in JM and HAFSA cells was inhibited with another anti-ICAM-3 mAb, but not with mAb against any of the other adhesion molecules explored. In T lymphoblasts, the induced cell aggregation was also inhibited by mAb anti-ICAM-1 and anti-LFA-1 (Table I), as

Table I. Inhibition of ICAM-3-induced Cell Aggregation by Different mAb

mAb	Specificity	Cell Types (% aggregation)		
		JM	HAFSA	T blasts
—	—	73	80	51
TS2/16	VLA- β 1	81	87	58
HP2/1	VLA- α 4	76	78	N.D.
Lia3/2	LFA-1 β	63	82	12
TP1/40	LFA-1 α	50	82	8
RR1/1	ICAM-1	65	79	1
TP1/25	ICAM-3	14	47	17
TS2/18	CD2	66	83	38
TS2/9	LFA-3	70	76	45

JM cells were preincubated with the different mAb indicated prior to the addition of the proaggregatory anti-ICAM-3 HP2/19 mAb. Cell aggregation was quantified as described under Materials and Methods at 2 h, 30 min, and 5 h for JM, HAFSA, and T blasts, respectively. Arithmetic mean of six independent experiments performed in duplicate is shown. SD was less than 10%.

previously described (Campanero et al., 1993). Anti-CD2, anti-LFA-3, anti-VLA α 4, and anti-VLA β 1 mAb showed no inhibitory effect on any of the different cell types tested (Table I).

Altogether, these data indicated the existence of two different pathways involved in homotypic lymphocyte aggregation triggered through ICAM-3, including both LFA-1/ICAM-1-dependent and -independent interactions.

The CD45 Tyrosine Phosphatase Regulates ICAM-3-induced Intercellular Adhesion on JM Cells

To identify the molecules involved in the regulation of ICAM-3-mediated homotypic cell aggregation, a wide number of mAb of different specificities was screened by their ability to inhibit the anti-ICAM-3-triggered cell aggregation of JM cells. Interestingly, one mAb, termed TP1/41, was able to abrogate this intercellular adhesion phenomenon (Fig. 2 B). Anti-ICAM-3 TP1/25 and anti-LFA-1 α TP1/40 mAb were included as positive and negative control for inhibition (Fig. 2 C and D, respectively). The viability of cells was not affected after treatment with these mAb as assessed by trypan blue exclusion (data not shown).

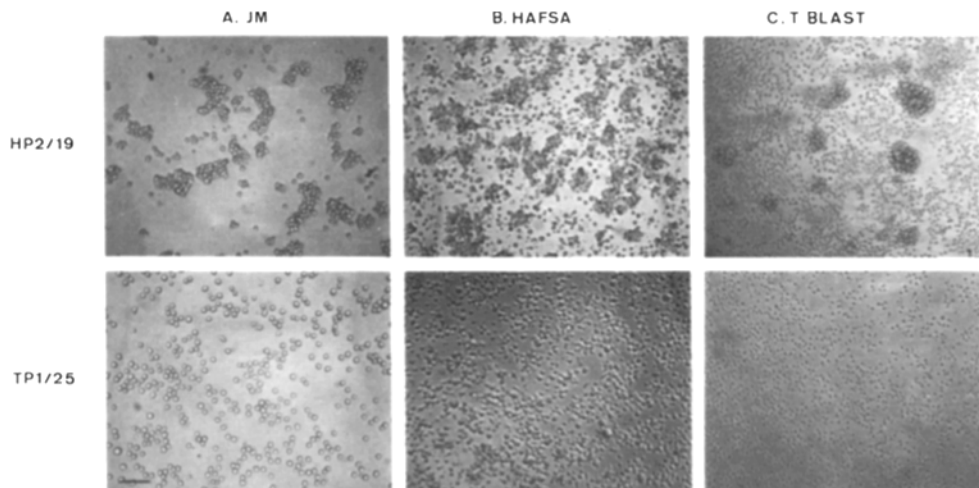


Figure 1. Induction of homotypic aggregation by anti-ICAM-3 mAb in different lymphoid cells. JM, HAFSA cells, and T lymphoblasts (A, B, and C, respectively) were incubated with 1 μ g/ml of either HP2/19 or TP1/25 anti-ICAM-3 mAb. Cell aggregation was determined at 2 h, 30 min, and 5 h for the three different cell types, respectively. A representative out of 10 independent experiments is shown. Bar, 150 μ m.

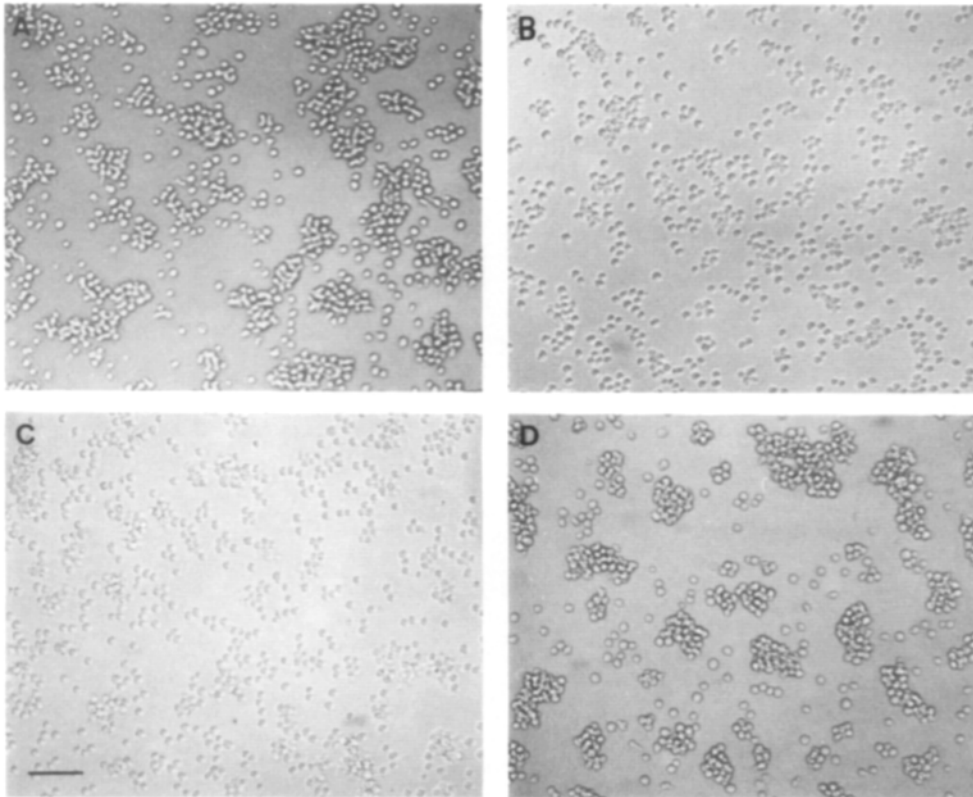


Figure 2. CD45 tyrosine phosphatase regulates ICAM-3-mediated cell aggregation on JM cells. JM cells were preincubated with none (A), 1 $\mu\text{g/ml}$ anti-CD45 TP1/41 mAb (B), 1:10 dilution supernatant anti-ICAM-3 TP1/25 mAb (C), or anti-LFA-1 α TP1/40 mAb (D), before addition of the proaggregatory anti-ICAM-3 HP2/19 mAb used at 1 $\mu\text{g/ml}$. Aggregation was determined at 2 h. A representative out of five independent experiments is shown. Bar, 150 μm .

The specificity of the TP1/41 mAb was investigated by immunoprecipitation assays from ^{125}I -labeled Jurkat cell lysates. The pattern of polypeptides precipitated by the TP1/41 mAb (Fig. 3, lanes 1 and 2) was identical to that obtained with the anti-CD45 D3/9 mAb (Fig. 3, lane 3). The specificity of the TP1/41 mAb was further demonstrated by analyzing its reactivity with cells transfected with cDNAs

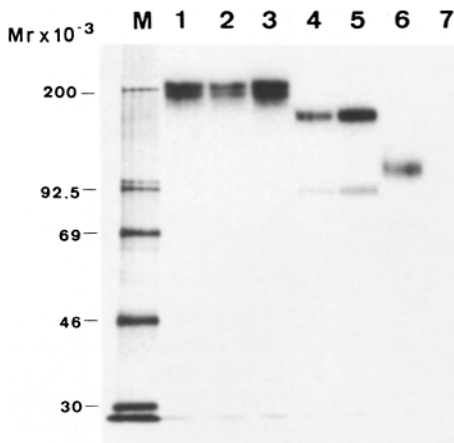


Figure 3. Immunoprecipitation analysis with TP1/41 mAb. Jurkat cells were radioiodinated, and the cell lysates were immunoprecipitated with different mAb: TP1/41 hybridoma culture supernatant (lane 1); TP1/41 purified mAb (lane 2); anti-CD45 D3/9 mAb (lane 3); anti-LFA-1 α TP1/40 mAb (lane 4); anti-LFA-1 β Lia 3/2 mAb (lane 5); anti-ICAM-3 TP1/25 mAb (lane 6); and P3X63 as negative control (lane 7). Note the identical pattern immunoprecipitated by TP1/41 and D3/9 mAb. Molecular mass markers are shown on the left (M).

encoding for the different isoforms of the CD45 antigen. As shown in Table II, the TP1/41 mAb recognized cells transfected with any isoform of CD45 but not the mock-transfected cells. These data demonstrated that the specificity of the TP1/41 mAb was coincident with that of conventional anti-CD45 mAb.

The ability of other anti-CD45 mAbs to inhibit ICAM-3-triggered homotypic cell aggregation was also tested. The conventional anti-CD45 D3/9 and HP2/23 mAb and the anti-CD45RB RP2/21 mAb were also able to inhibit the aggregation of JM cells (Table III). In contrast, other conventional anti-CD45 mAb and mAb that recognize other isoforms of CD45 did not exert any inhibitory effect (Table III, and data not shown).

Table II. Reactivity of TP1/41 mAb with CD45-transfected Cells

mAb	CD	% positive cells						
		ABC	AB	BC	B	O	-	
D3/9	CD45	67	99	51	73	99	1	
UHL-1	CD45RO	2	1	3	3	93	0	
MC5/2	CD45RB	53	98	56	98	4	1	
RP1/11	CD45RA	94	99	4	3	4	1	
TP1/41	CD45	63	98	47	66	97	0	

Mouse 300-19 pre-B cells transfected with CD45 cDNA coding for different isoforms of CD45 were assayed for the reactivity with several anti-CD45 mAb recognizing the distinct isoforms of this antigen. Cytofluorometric analysis was performed as described under Materials and Methods. Note that the TP1/41 mAb reacts with all the transfected cells, and thus corresponds to a conventional anti-CD45 mAb.

Table III. Effects of Different Anti-CD45 mAb on ICAM-3-induced Aggregation of JM Cells

mAb	Specificity	% cell aggregation
—	—	78
TP1/41	CD45	7
D3/9	CD45	12
HP2/23	CD45	18
RP1/10	CD45	65
RP2/16	CD45	71
RP1/11	CD45RA	74
RP2/7	CD45RA	78
RP2/23	CD45RB	62
RP2/21	CD45RB	13
MC5/2	CD45RB	69
UCHL-1	CD45RO	77

JM cells were preincubated with different anti-CD45 mAb for 10 min at RT before the addition of the proaggregatory anti-ICAM-3 HP2/19 mAb. Cell aggregation was quantified after 2 h. Arithmetic mean of three independent experiments performed by duplicate is shown. SD was less than 10%.

β2 Integrin-induced JM T Cell Aggregation Is Also Regulated through CD45 Phosphatase

We next investigated whether anti-CD45 TP1/41 mAb also regulates JM cellular aggregation induced through antigens

different from ICAM-3. To this end, the proaggregatory anti-LFA-1α NKI-L16 mAb was used to induce aggregation of JM cells. As shown in Fig. 4, the anti-CD45 TP1/41 mAb was able to inhibit this intercellular adhesion pathway. A similar blocking effect of the TP1/41 mAb was observed on the cell aggregation triggered by the anti-β2 KIM127 mAb (Robinson et al., 1992) (data not shown). In contrast, other cellular aggregation pathways including that triggered by the anti-CD43 TP1/36 mAb were unaffected by the anti-CD45 TP1/41 mAb (Fig. 4).

Tyrosine Phosphorylation Is Induced in Cell-Cell Contacts upon ICAM-3- and LFA-1-triggered JM Cell Aggregation

The CD45 molecule displays tyrosine phosphatase activity in its cytoplasmatic tail (Trowbridge, 1991). The results shown above, indicating the inhibitory effects of anti-CD45 mAb on ICAM-3- and LFA-1-triggered JM cell aggregation might be related to this enzymatic activity. Therefore, we investigated the possibility that the induction of JM cell aggregation through ICAM-3 and LFA-1 antigens could correlate with triggering of protein tyrosine phosphorylation. In this regard, the presence of tyrosine-phosphorylated proteins at intercellular contacts on cell aggregates induced through

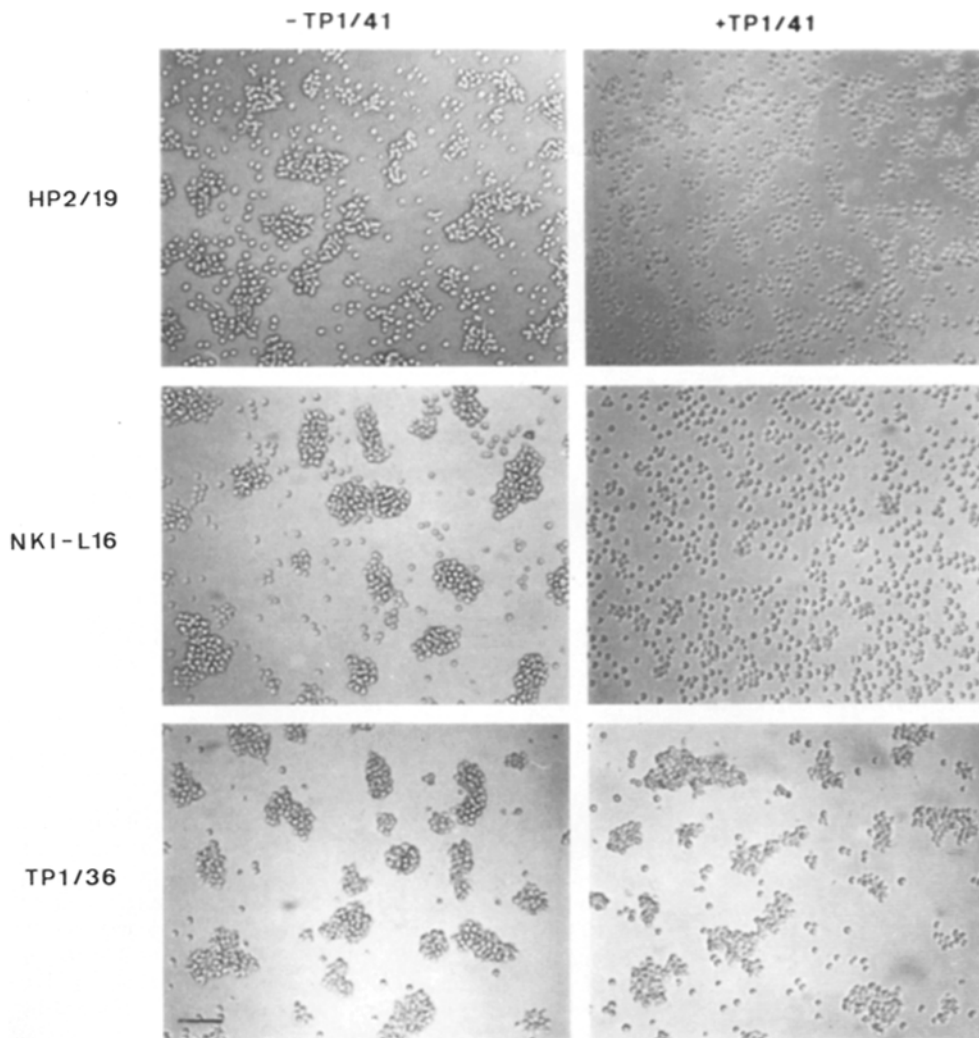


Figure 4. LFA-1-mediated JM cell aggregation is also regulated by the CD45 tyrosine phosphatase. JM cells were preincubated with either none or 1 μg/ml TP1/41 mAb. Then, cell aggregation was induced by treatment with anti-ICAM-3 HP2/19, anti-LFA-1α NKI-L16, or anti-CD43 TP1/36 mAb for 2 h. A representative out of four independent experiments is shown. Bar, 150 μm.

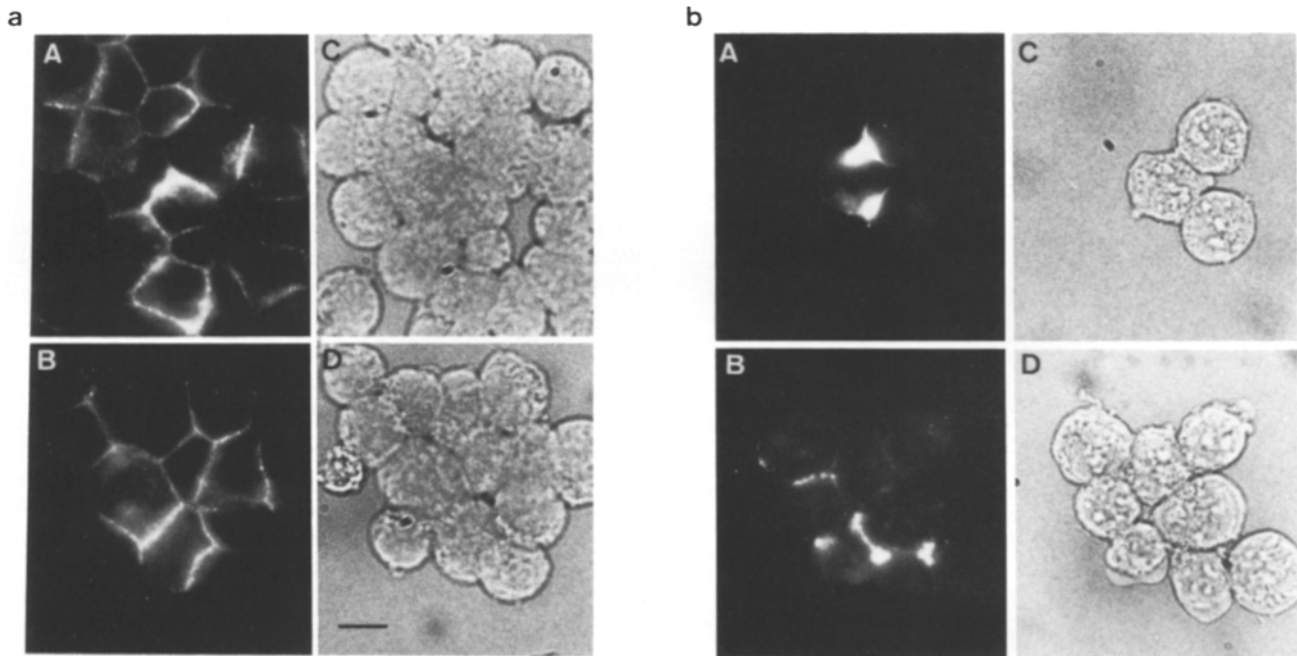


Figure 5. Tyrosine phosphorylation is induced in cell-cell contact areas upon induction of JM cell aggregation through ICAM-3 and LFA-1. Aggregation of JM cells was induced by anti-ICAM-3 HP2/19 (A and C) or anti-LFA-1 α NKI-L16 (B and D) mAb. Tyrosine phosphorylation (a) or ICAM-3 and LFA-1 antigen localization (b) was determined by immunofluorescence as described under Materials and Methods. Bar, 25 μ m.

ICAM-3 or LFA-1 α NKI-L16 mAb was easily detected by immunofluorescence (Fig. 5 a, A and B, respectively). The ICAM-3 and LFA-1 antigens were also detected in cell-cell boundaries, as assessed by using the HP2/19 and the NKI-L16 mAb (Fig. 5 b, A and B, respectively). These data suggest a direct involvement of these molecules in the triggering of tyrosine phosphorylation. Moreover, similar results were obtained when cell aggregation was studied on normal T lymphoblasts (data not shown). Although tyrosine phosphorylation was induced on both JM T cells and T lymphoblasts upon ICAM-3 or LFA-1 mediated aggregation, no regulatory effect of anti-CD45 mAb was observed on T blasts, thus reinforcing the existence of different intracellular signaling pathways in these two cell types.

ICAM-3- and LFA-1-mediated homotypic aggregation was inhibited by pretreatment of JM cells with the tyrosine-kinase inhibitors herbimycin A, tyrphostin 25, and genistein in a dose-dependent manner (Fig. 6). In contrast, the CD43-mediated aggregation was almost unaffected (Fig. 6). Altogether these results indicate that protein tyrosine phosphorylation is an important intracellular signaling event during ICAM-3- and LFA-1-triggered homotypic cell aggregation, and suggest a role for the tyrosine phosphatase activity of CD45 in regulating these processes.

CD45 Regulates the Tyrosine Phosphorylation Induced by Engagement of ICAM-3 or LFA-1 on JM T Cells

Western blot studies were performed to investigate the changes in the tyrosine phosphorylation protein pattern upon cell treatment with proaggregatory anti-ICAM-3 and anti-LFA-1 mAb. As shown in Fig. 7 A, the engagement of ICAM-3 or LFA-1 molecules with the HP2/19 and NKI-L16 mAb, respectively, induced the enhancement of tyrosine phosphory-

lation of several polypeptides of 125, 70, and 38 kD. The kinetics of this effect was very rapid, beginning after 1 min and declining after 15 min of either ICAM-3 or LFA-1 crosslinking (Fig. 7 A). The phosphorylation pattern induced through LFA-1 or ICAM-3 resembled that triggered through the CD3/TcR complex but at lower degree (Fig. 7 A). Preincubation

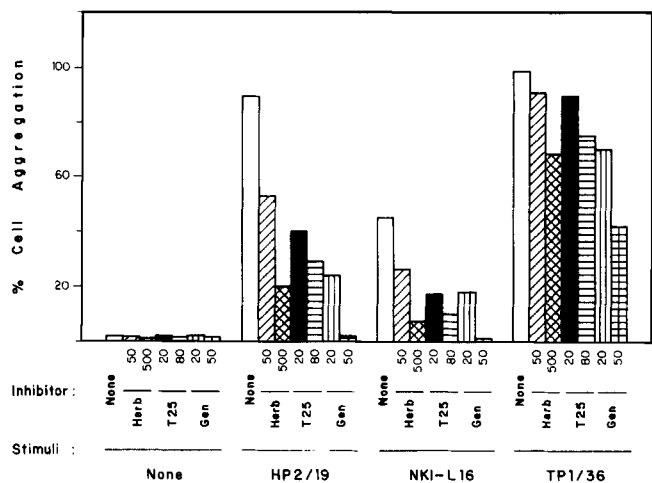


Figure 6. Homotypic JM cell aggregation induced by anti-ICAM-3 and anti-LFA-1 mAb is blocked by tyrosine kinase inhibitors. JM cells were preincubated with different doses of herbimycin A (*Herb*) or tyrphostin 25 (*T25*) for 24 h, or genistein (*Gen*) for 30 min at 37°C. Then, cell aggregation was induced by incubation with 1 μ g/ml of anti-ICAM-3 HP2/19, anti-LFA-1 α NKI-L16, or anti-CD43 TP1/36 mAb. Aggregation was quantified at 3 h. The arithmetic mean of five independent experiments performed by duplicate is shown. SD was less than 10%.

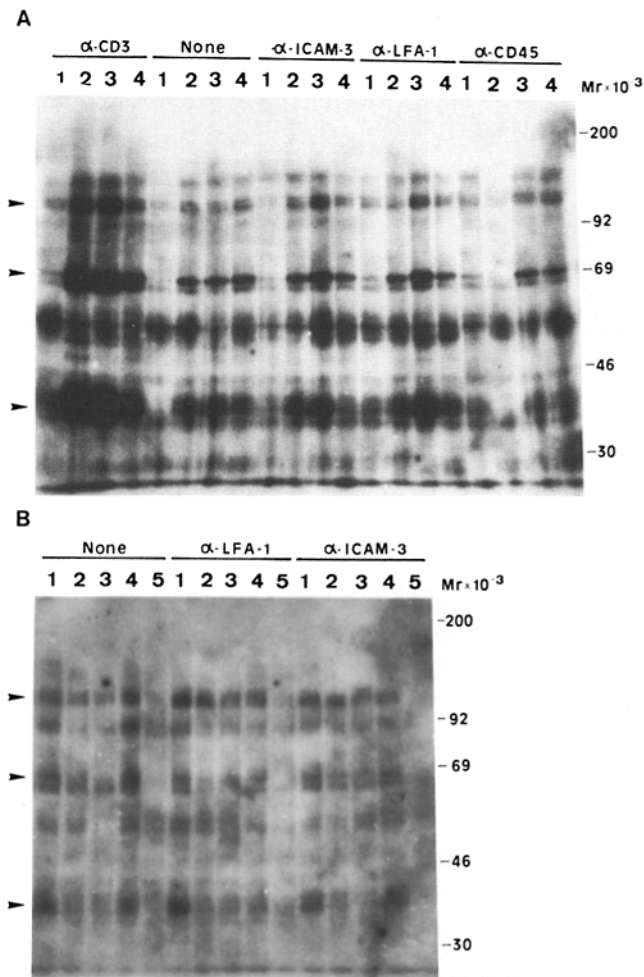


Figure 7. Western blot analysis of phosphotyrosine proteins upon engagement of ICAM-3 or LFA-1 antigens on JM cells. Effect of anti-CD45 mAb. (A) JM cells were incubated with 10 $\mu\text{g}/\text{ml}$ of either anti-CD3, medium alone, anti-ICAM-3 HP2/19, anti-LFA-1 NKI-L16, or anti-CD45 TPI/41 mAb for 5 min on ice and then sheep anti-mouse Ig at 20 $\mu\text{g}/\text{ml}$ was added during different times: 0, 1, 5, and 15 min at 37°C (lanes 1–4, respectively). Phosphoproteins were analyzed as described under Materials and Methods. Note the enhanced intensity of bands corresponding to 125, 70, and 38 kD when anti-CD3, HP2/19, or NKI-L16 were used as stimulus. A representative out of six independent experiments is shown. In some analysis, the phosphoproteins of 38 kD were resolved into two bands. (B) JM cells were preincubated with medium alone or 10 $\mu\text{g}/\text{ml}$ of either TPI/41, D3/9, or RPI/10 mAb (lanes 1–4, respectively) 1 min at RT before addition of either medium alone or 1 $\mu\text{g}/\text{ml}$ anti-ICAM-3 HP2/19, or anti-LFA-1 α NKI-L16 mAb for 5 min on ice. Then, sheep anti-mouse Ig was added during 3 min at 37°C, and phosphoproteins were analyzed as described under Materials and Methods. Note that preincubation with the anti-CD45 TPI/41 or D3/9 mAb inhibited the induction of phosphotyrosine of polypeptides of 125, 70, and 38 kD with no modifications of other bands. The anti-CD45 RPI/10 exerted no effect. Preincubation of JM cells with 50 $\mu\text{g}/\text{ml}$ of genistein for 30 min at 37°C before addition of the different stimuli is also included (lane 5). A representative experiment out of four independent ones is shown.

of JM cells with the tyrosine kinase inhibitor genistein abrogated the induction of tyrosine phosphorylation through these adhesion molecules (Fig. 7 B, lane 5).

To further explore the role of CD45 in the regulation of ICAM-3- and LFA-1-triggered intracellular signaling, we performed the induction of tyrosine phosphorylation in the presence of different regulatory and nonregulatory anti-CD45 mAb. As observed in Fig. 7 B, preincubation with TPI/41 or D3/9 but not with the functionally irrelevant RPI/10 mAb prevented the tyrosine phosphorylation of the 125, 70, and 38 kD polypeptides induced by ICAM-3 or LFA-1 engagement. Moreover, the dephosphorylation effect induced by TPI/41 was abolished by preincubation of JM cells with the tyrosine phosphatase inhibitor phenylarsine oxide (data not shown).

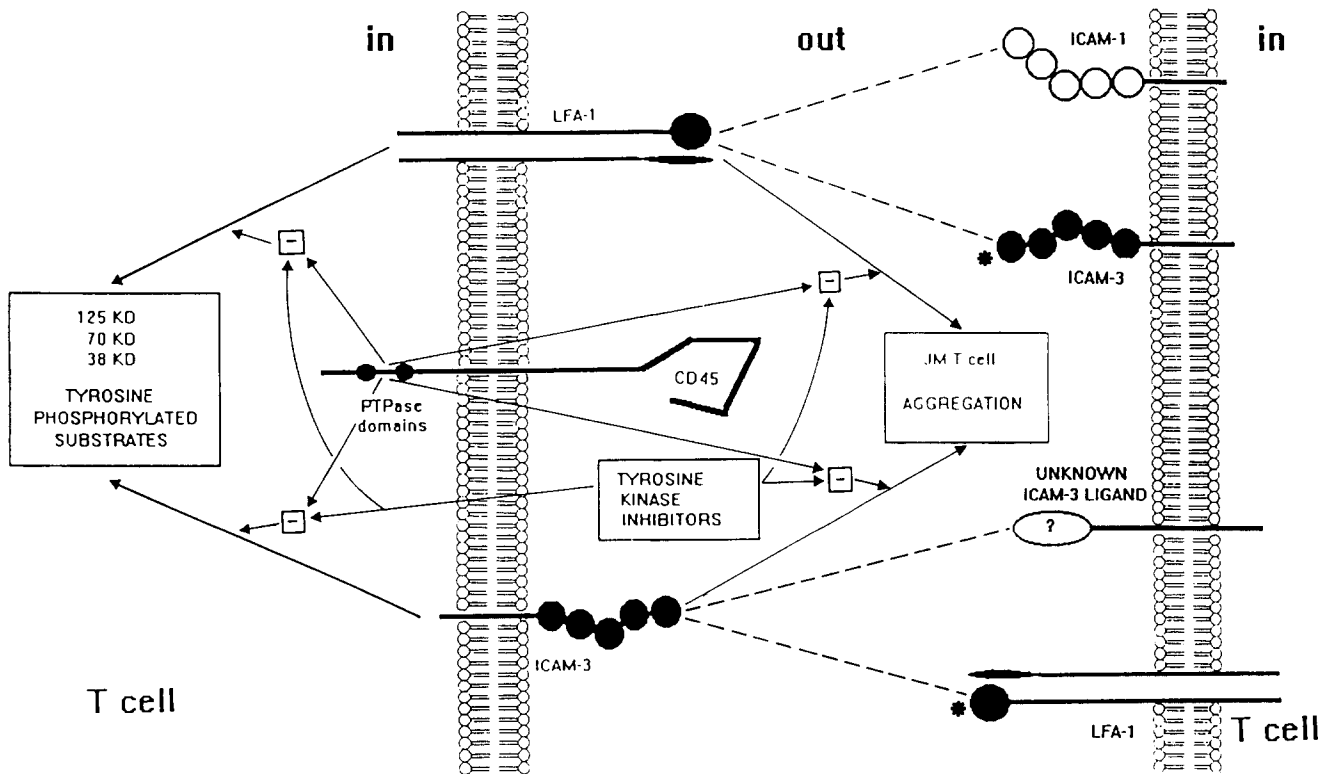
Altogether, these data indicate that ICAM-3 and LFA-1 trigger specific tyrosine phosphorylation of several cellular polypeptides, and that the CD45 tyrosine phosphatase regulates this intracellular signaling pathway.

Discussion

In this report, we describe the existence of an alternative pathway of anti-ICAM-3-induced lymphocyte homotypic aggregation which is LFA-1/ICAM-1 independent. This pathway, as well as that induced through LFA-1, triggers tyrosine phosphorylation, and is regulated by the CD45 tyrosine phosphatase. The diagram shown in Scheme 1 represents the possible events involved in these intracellular signaling pathways.

We have previously reported that the aggregation of T lymphoblasts triggered by ICAM-3 involves the activation of the LFA-1/ICAM-1 pathway (Campanero et al., 1993). We have shown herein that anti-ICAM-3 can induce cell aggregation of both LFA-1⁺ cells and β 2-deficient (LFA-1⁻) HAFSA cells, thus indicating that this molecule triggers an LFA-1/ICAM-1-independent cell adhesion pathway. The ICAM-3-mediated cell aggregation of LFA-1⁺ JM T or LFA-1⁻ HAFSA B cells was not affected by cell pretreatment with mAb against different molecules involved in intercellular interactions. Only an anti-ICAM-3 mAb recognizing a different epitope was able to inhibit this interaction. Cross-inhibitory effects of mAb directed to different epitopes on the same molecule have already been reported for other antigens such as LFA-1, VLA α 4, or VLA β 1 (Keizer et al., 1988; Puelido et al., 1991; Campanero et al., 1992). Several mechanisms may account for the LFA-1-independent ICAM-3-induced homotypic cell aggregation including the existence of other ligands for ICAM-3, or the triggering of intracellular signals through ICAM-3 that would activate other adhesion molecules.

When searching for molecules involved in the regulation of the LFA-1-independent ICAM-3-induced cell aggregation, we found that the TPI/41 mAb inhibited this process. Both immunoprecipitation analysis and binding assays with transfected cells revealed that this mAb is directed against the CD45 antigen. We have also demonstrated that this mAb, as well as other anti-CD45 mAb are able to inhibit the homotypic cell aggregation induced through either ICAM-3 or the LFA-1 integrin. In contrast, other intercellular adhesion pathways, as that induced by anti-CD43 mAb, remained unaffected, thus indicating that CD45 has a specific regula-



Scheme 1. Representation of the possible involvement of CD45 tyrosine phosphatase in ICAM-3- and LFA-1-mediated T cell aggregation and intracellular signaling. The molecular interactions likely involved in this homotypic cell aggregation are also represented (*dotted lines*). The putative interaction between LFA-1 and ICAM-3 (*) in this system could be a transient one as recently reported for T blasts (Campanero et al., 1993), since no blocking effects of anti-LFA-1 mAb are detected in anti-ICAM-3-triggered JM cell aggregation.

tory role in the aggregation events mediated by ICAM-3 and LFA-1. A minor inhibitory effect of these anti-CD45 mAb was observed in VLA-4-mediated homotypic cell aggregation (A.G. Arroyo, unpublished observations).

Since CD45 is a pan-leukocyte glycoprotein that contains two consensus domains of tyrosine-phosphatase in its cytoplasmic tail (Trowbridge, 1991), we next investigated the role of tyrosine phosphorylation in ICAM-3 and LFA-1 intracellular signaling. Our results on ICAM-3- and LFA-1-mediated signaling by both immunofluorescence staining and Western blot analysis, indicate an increase of tyrosine phosphorylation of several proteins upon LFA-1 or ICAM-3 engagement. The induction of tyrosine phosphorylation of different proteins by the engagement of other integrin members from $\beta 1$ and $\beta 3$ subfamilies have recently been described (Burrige et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992; Lipfert et al., 1992; Juliano and Haskill, 1993). Moreover, it was recently reported that $\alpha 2\beta 1$ integrin activation can result in tyrosine phosphorylation of 47–52 kD proteins as well as in activation of a signaling pathway involving p21^{ras} (Kapron-Bras et al., 1993). However, the induction of tyrosine phosphorylation by $\beta 2$ leukocyte integrins or their ligands had not previously been investigated. The functional significance of this signaling pathway in leukocyte intercellular adhesion is reinforced by the inhibition of ICAM-3- and LFA-1-mediated homotypic aggregation by specific tyrosine kinase inhibitors (Scheme 1).

The nature of the 125, 70, and 38 kD proteins that become phosphorylated upon engagement of ICAM-3 or LFA-1 re-

mains to be determined. Possible candidates of similar molecular masses would include proteins known to be phosphorylated through $\beta 1$ or $\beta 3$ integrins such as p125^{lak} (125 kD) or paxillin (70 kD) (Burrige et al., 1992), or those which are phosphorylated after activation through the CD3/TcR complex such as PLC γ (135 kD), the recently described ZAP-70 (70 kD), and different members of MAP kinase family (about 40 kD) (Nel et al., 1990*a,b*; Park et al., 1991; Secrist et al., 1991; Weiss et al., 1991; Chan et al., 1992; Whitehurst et al., 1992). It would also be very interesting to investigate the putative tyrosine-kinases involved in this signaling pathway, but these issues deserve further research.

Regarding the mechanism accounting for the inhibitory effect of different anti-CD45 mAb on anti-ICAM-3- and anti-LFA-1-induced JM T cell aggregation, it may involve a modulation of CD45 tyrosine phosphatase activity by engagement of CD45 with mAb, as suggested by a decrease in tyrosine phosphorylation of different polypeptides in Western blot analysis. This might cause dimerization of the receptor that could regulate tyrosine phosphatase function by sequestration or dephosphorylation of tyrosine phosphatase domains, as it has been recently described (Desai et al., 1993). Interestingly, two of the blocking anti-CD45 mAb, D3/9 and HP2/23, have been previously found that inhibit PHA-induced T lymphocyte proliferation (Bernabeu et al., 1987). Recently, the regulatory role of CD45 mAb in LFA-1-independent/tyrosine kinase-dependent B cell aggregation, and the inhibitory effect of these mAb on calcium

mobilization induced through LFA-1 in NK cells have been described (Poggi et al., 1993; Wagner et al., 1993). The tyrosine kinases likely involved in this regulation are not known yet. In this regard, it has been reported the activation of *lck* and *lyn* kinases in T lymphocytes by CD45-mediated dephosphorylation of tyrosine 505 and 531, respectively (Shiroo et al., 1992; Hurley et al., 1993). Moreover, CD45 tyrosine phosphatase is also able to regulate activation of MAP kinase (Anderson et al., 1990; Nel et al., 1991). The role that these kinases could play in ICAM-3- and LFA-1-triggered signaling deserves further research.

In summary, we have provided data demonstrating the existence of LFA-1/ICAM-1-independent homotypic cell aggregation induced by anti-ICAM-3 mAb in different cell lines. Remarkably, this interaction could be regulated by the CD45 tyrosine phosphatase, and this fact is related to the ability of ICAM-3 and LFA-1 antigens to induce tyrosine phosphorylation of different cellular substrates (Scheme 1).

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