B-Cell Homotypic Adhesion Through Exon-A Restricted Epitopes of CD45 Involves LFA-1/ICAM-1, ICAM-3 Interactions, and Induces Coclustering of CD45 and LFA-1

By Juan M. Zapata, Miguel R. Campanero, Mónica Marazuela, Francisco Sánchez-Madrid, and Manuel O. de Landázuri

Lymphocyte interactions with other leukocytes and other cell types, as well as with components of the extracellular matrix, are one of the key steps in the immune response. Three novel monoclonal antibodies (MoAbs) have been produced and selected for their ability to induce intercellular adhesion in B cells. These three MoAbs immunoprecipitated a polypeptide of 220 kD, displaying specific phosphotyrosine phosphatase activity that has been identified as CD45. These MoAbs recognize epitopes located on the alternative spliced exon-A-encoded region of CD45. These epitopes are of polypeptidic nature, but they can be masked by addition of carbohydrate during CD45 biosynthesis. Interestingly enough, CD45 epitopes recognized by these MoAbs appeared to be selectively expressed on both peripheral blood and tonsillar B lymphocytes as well as on peripheral blood natural killer (NK) cells. CD45-mediated intercellular adhesion was abrogated upon incubation with anti-leukocyte function-associated antigen 1 (anti-LFA-1), intercellular cell adhesion molecule 1 (ICAM-1), and ICAM-3 MoAbs, thus indicating that this phenomenon involved both LFA-1/ICAM-1 and LFA-1/ICAM-3 cell adhesion pathways. Moreover, CD45-mediated cell aggregation was also inhibited by preincubation with some conventional anti-CD45 MoAbs. Interestingly, the triggering of cell aggregation through CD45 induced membrane surface relocation of CD45 and LFA-1 molecules, with both of them colocalizing at cell-cell contact areas of B-cell aggregates. Studies with inhibitors of both phosphotyrosine phosphatase and tyrosine kinase activities suggest that CD45 phosphotyrosine phosphatase activity could be involved in CD45-mediated cell aggregation. Taken together, these results support the notion that CD45 is a key molecule in the regulation of LFA-1-mediated cell-cell interactions. © 1995 by The American Society of Hematology.

CD45 IS A MEMBRANE glycoprotein with intrinsic phosphotyrosine phosphatase activity that is expressed specifically on nucleated hematopoietic cells. Several isoforms of this molecule have been described that are mainly generated by the alternative splicing of exons 4, 5, and 6 (also named exons A, B, and C, respectively) of a transcript produced by a single gene. Additional antigenic variability of the CD45 molecules is achieved by glycosylation events. There are many potential sites for both O- and N-glycosylation located on the extracellular region of CD45 that are differentially used. This complex antigenic variability of the CD45 molecules is of great importance because of the existence of a tight regulation of the expression of the different CD45 isoforms during leukocyte development and lymphocyte activation.

CD45 functions as a lymphocyte membrane receptor capable of transducing signals via their phosphotyrosine phosphatase domains. In this regard, evidence suggesting that CD45RO is a ligand for CD22 has been reported. Nevertheless, although colateral associations between CD45 and a number of T-cell accessory molecules such as T-cell receptor (TCR), Thy-1, CD2, CD4, and CD8 have been described, no additional evidence of other putative ligands for this or other CD45 isoforms has been obtained. Recently, it has been shown that specific CD45 isoforms could differentially regulate TCR signaling. However, although evidence has been described indicating that tyrosine phosphorylation of CD45 by p50/IκB kinase is involved in the activation of CD45 phosphatase activity, the identity of the mechanism involved in the regulation of the phosphatase activity of CD45 remains elusive.

Several studies on the function of CD45 in T lymphocytes support a crucial role of CD45 in T-cell activation and development. Recent evidence also suggests that CD45 acts as a regulatory molecule that controls B-cell function. In this regard, CD45 has been involved in the regulation of B-cell proliferation triggered by anti-IgM or anti-CD40 monoclonal antibody (MoAb). Moreover, a recent report shows that CD45 phosphatase activity is elevated during mitosis, thus supporting the notion that CD45 could regulate cell division. CD45 appears also to be involved in the regulation of the B-cell antigen receptor function. Interestingly, using mice in which exon C' has been targeted by homologous recombination, Kishihara et al showed that, despite the apparently normal B-cell development in these mice, signaling through the B-cell antigen receptor was abrogated. Nevertheless, evidence indicating that CD45 is implicated in the regulation of isotype switching has been obtained. A recent report pointed out that in vivo administration of MoAb against a B-cell-restricted CD45 epitope abrogated the B-cell response to a T-dependent antigen, thus further supporting the important role of CD45 in B-cell function.

One of the key steps in the immune response is the regulation of leukocyte interactions. The correct establishment of cell-cell and cell-extracellular matrix interactions is essential for the proper development and function of leukocytes. We report herein that CD45 is implicated in the regulation of B-cell-cell interactions. We found that CD45-mediated B-cell homotypic adhesion was dependent on the leukocyte func-

From the Servicio de Inmunología and Servicio de Endocrinología, Hospital de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain.
Submitted November 15, 1995; accepted April 21, 1996. Supported by grants from INSALUD (Fondo de Investigaciones Sanitarias 94/1695) and Comunidad Autónoma de Madrid (CAM 028/92). J.M.Z. is the recipient of a postdoctoral fellowship from the Comunidad Autónoma de Madrid.
Address reprint requests to Manuel O. de Landázuri, MD, Servicio de Inmunología, Hospital de la Princesa, C/Diego de León 62, Madrid 28006, Spain.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.
© 1995 by The American Society of Hematology.

tion-associated antigen 1 (LFA-1)/intercellular adhesion molecule 1 (ICAM-1) and the LFA-1/ICAM-3 cell adhesion pathways. Furthermore, the engagement of CD45 by proaggregatory anti-CD45 MoAb induces membrane surface redistribution of both CD45 and LFA-1 molecules to cell-cell boundaries. Preliminary evidence is also reported, suggesting that the phosphotyrosine phosphatase activity of CD45 could be involved in this process.

**MATERIALS AND METHODS**

**Cells and tissues.** Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood from human volunteers or from freshly prepared buffy-coats by Ficoll-Hypaque (S.L. Rafer, Barcelona, Spain) centrifugation. Tonsils were obtained from children (age range, 4 to 12 years old), undergoing routine tonsillectomy. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation. B cells (CD19, CD20 >95%, CD3 <3%) were purified from PBMCs or tonsils using anti-CD19-coated magnetic beads (DynaL, Oslo, Norway) or by rosetting with 2-aminoethyl-isothiouronium bromide (Sigma Chemical Co, St Louis, MO) -treated sheep erythrocytes (Biomerieux, Marcy-L’Etoile, France). T cells (CD3 > 95%, CD19, CD20 < 3%) also were purified from tonsils or PBMCs by negative selection using anti-CD19 (tions) or both anti-CD19 and anti-CD56 (PBMC)-coated magnetic beads (DynaL) and depleted of adherent cells by adherence incubation in a plastic flask (Costar, Cambridge, MA). T-cell blasts were obtained by activation of PBMCs with 5 μg/mL phynoxaglutamin (Sigma) for 2 days and cultured in the presence of 50 U/mL of interleukin-2 (IL-2) for several days. Natural killer (NK) cells (CD16 and CD56 >95%, CD3 <3%) were purified from freshly prepared buffy coats as described 30 and activated with 50 U/mL of IL-2 during several days. Monocytes were also purified by buco centrifugation. Neutrophils were purified from buffy coats by dextran sedimentation as described.

Cells were grown in RPMI-1640 (Whitaker, Bioproducts Inc, Walkersville, MD) supplemented with 10% fetal calf serum (FCS; Biochrom, Seromed, Berlin, Germany), 2 mmol/L L-glutamine, 100 μg/mL of penicillin, and 50 pg/mL of streptomycin (all from Whittaker, Bioproducts Inc, Walkersville, MD), 95% air and 5% CO₂ atmosphere. Cell viability was determined by trypan blue exclusion, and cell death was determined by staining with trypan blue dye. All experiments were performed at least three times to ensure reproducibility.

**Flow cytometry analysis.** Flow cytometry analysis was performed on a FACScan cytometer (Becton Dickinson, San Jose, CA). For indirect immunofluorescence staining, cells were incubated at 4°C with hybridoma culture supernatant (100 μL for 10^6 cells) or 10 μg/mL of purified MoAb, washed, and then incubated with fluorescein isothiocyanate (FITC)-tagged goat-antimouse F(ab')₂ (Dakopatts, Copenhagen, Denmark). Immunoperoxidase staining. Tonsillar frozen sections were stained by using an indirect two-step immunoperoxidase method. Briefly, acetone-fixed 4-μm-thick sections were incubated with hybridoma culture supernatant for 30 minutes at room temperature, and for another 30 minutes with a peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts). Each incubation was followed by three washes with TBS (150 mmol/L NaCl, 20 mmol/L TRIS-HCI, pH 7.6). Then, sections were incubated with Graham-Karnovsky’s solution, containing 0.5 mg/mL 3,3-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide. The reaction was stopped by washing the sections with TBS. Sections were counterstained with Carazzi’s hematoxylin and then dehydrated and mounted by routine methods.

**Aggregation assay.** Cells were washed twice in RPMI 1640 medium and resuspended to a concentration of 2.5 X 10⁶ (cell lines) or 4 X 10⁶ (lymphocytes) cells/mL. Aliquots of 50 μL of cells suspensions were added to each well of flat-bottomed, 96-well microtiter plates (Costar). Then, 10 μL of MoAb culture supernatants and for another 30 minutes with a peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts). Each incubation was followed by three washes with TBS (150 mmol/L NaCl, 20 mmol/L TRIS-HCI, pH 7.6). Then, sections were incubated with Graham-Karnovsky’s solution, containing 0.5 mg/mL 3,3-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide. The reaction was stopped by washing the sections with TBS. Sections were counterstained with Carazzi’s hematoxylin and then dehydrated and mounted by routine methods.

**Flow cytometry analysis.** Flow cytometry analysis was performed on a FACScan cytometer (Becton Dickinson, San Jose, CA). For indirect immunofluorescence staining, cells were incubated at 4°C with hybridoma culture supernatant (100 μL for 10^6 cells) or 10 μg/mL of purified MoAb, washed, and then incubated with fluorescein isothiocyanate (FITC)-tagged goat-antimouse F(ab')₂ (Dakopatts, Copenhagen, Denmark). Immunoperoxidase staining. Tonsillar frozen sections were stained by using an indirect two-step immunoperoxidase method. Briefly, acetone-fixed 4-μm-thick sections were incubated with hybridoma culture supernatant for 30 minutes at room temperature, and for another 30 minutes with a peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts). Each incubation was followed by three washes with TBS (150 mmol/L NaCl, 20 mmol/L TRIS-HCI, pH 7.6). Then, sections were incubated with Graham-Karnovsky’s solution, containing 0.5 mg/mL 3,3-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide. The reaction was stopped by washing the sections with TBS. Sections were counterstained with Carazzi’s hematoxylin and then dehydrated and mounted by routine methods.

For quantitative measurement of cell aggregation, a modification of the method described by Keizer et al 40 was used. Cells were seeded in flat-bottomed microtiter plates as described above. The number of free cells was counted by using a special mask, consisting of squares (0.5 mm) under the plate. Within each well, at least five randomly chosen areas were counted, and the mean and the total number of free cells were calculated. Percent aggregation was determined by the following equation: Percent Aggregation = 100 X [No. of Free Cells/Total No. of Cells]. The experiments were performed in duplicate and the SD within each experiment was always less than 10%.

**Radiolabeling, immunoprecipitation, and electrophoresis.** Cells were washed twice in phosphate-buffered saline (PBS) and radioiodinated with chloroglycoluril (IODO-GEN; Pierce Chemical Co, Rockford, IL). 41 Cells were lysed in buffer containing 150 mmol/L
Fig 1. Homotypic cell adhesion induced by Lia1/11 MoAb. U-937 (A and B), Daudi (C and D), and Ramos (E and F) cells at a concentration of $1.2 \times 10^5$ cells/well were incubated for 6 hours at 37°C in the presence of 1.5 µg/mL of purified anti-HLA I W6/32 MoAb (A, C, and E) or 1.5 µg/mL of purified Lia1/11 MoAb (B, D, and F). Photomicrographs were taken after 6 hours from the beginning of the assay.

For biosynthetic labeling, cells were washed twice in Dulbecco’s modified Eagle’s medium (DMEM) without methionine and cysteine and resuspended at $4 \times 10^6$ cells/mL in this culture medium supplemented with 2 mmol/L L-glutamine and 5% dialyzed FCS. After 1 hour at 37°C, cells were pulsed with 0.5 mCi/mL of a mixture of [35S]methionine-cysteine (TRANS35S-LABEL, ICN Biomedicals Inc, Irvine, CA) for 20 minutes at 37°C and chased for different periods of time in the absence of the labeling mixture. When indicated, cells were incubated in the presence of 10 µg/mL tunicamycin (Sigma) (dissolved in Dimethyl sulfoxide) for 16 hours before the labeling and the drug was maintained during the rest of the assay.

Lysis of cells, immunoprecipitation and electrophoresis were as described above.

**Immunoblotting.** Cells were lysed and the polypeptides immunoprecipitated and processed as described above. Samples were subjected to SDS-PAGE, and resolved proteins were electrophoretically transferred to nitrocellulose (Trans blot transfer medium; Biorad, Hercules, CA) or Immobilon (Millipore, Bedford, MA) membranes. Blots were blocked with 3% bovine serum albumin (BSA) in TBS and then incubated with the respective MoAb culture supernatants diluted 1:3 in TBS containing 3% BSA, followed by incubation with antimouse κ chain 187.1 MoAb culture supernatant. Blots were finally incubated with iodine-125-labeled protein A ($5 \times 10^3$ cpm/mL), extensively washed in TBS containing 0.05% Tween-20, and subjected to autoradiography.

**Immunofluorescence microscopy.** Daudi cells ($2 \times 10^6$ cells/500 µL complete medium) were incubated in flat-bottomed, 24-well microtiter plates (Costar) in the presence of 1.5 µg/mL of the respective MoAb and were allowed to settle in a cell incubator at 37°C and 5% CO2 atmosphere. Aggregation was determined by direct visualization of the plate with an inverted microscope. Cells were fixed in PBS containing 2.5% formaldehyde and 5% sucrose during 15 minutes at room temperature and rinsed in TBS containing 0.1% sodium azide. To directly visualize the MoAbs that induce the cell aggregation, cells were stained with a 1:20 dilution of rhodamine-
Flow cytometry analyses of different leukocyte populations stained with Lia/1/11 MoAb. Peripheral blood lymphocytes (electronically gated) (A), purified peripheral blood B (B) and T (C) lymphocytes, activated peripheral blood T lymphocytes (D), freshly purified (E) or activated (F) NK cells, purified monocytes (G), purified granulocytes (H), purified platelets (I), unfractionated tonsillar lymphocytes (J), and purified tonsillar B (K) and T (L) lymphocytes were stained with Lia/ll MoAb, and only at low intensity (Fig 2G). In accordance with flow cytometry studies, immunoperoxidase staining of lymph nodes with Lia/11 MoAb showed that B cells located in the mantle were strongly positive. Interestingly, they were more brightly stained than B cells located in the germinal center, thus suggesting that epitopes that are recognized by Lia MoAbs could be regulated during B-cell maturation (Fig 3A). In contrast, the majority of T lymphocytes in the paracortical zone were almost negative (Fig 3A).

To identify the molecular structures recognized by the proaggregatory Lia MoAbs, biochemical studies were per-
formed. Figure 4A shows the pattern of immunoprecipitated polypeptides obtained with the three proaggregatory MoAbs Lia1/8 (lane 2), Lia1/11 (lane 3), and Lia1/15 (lane 4) from iodine-125-labeled Daudi cells. A single 220-kD polypeptide was specifically immunoprecipitated with these three MoAbs. This polypeptide displayed identical electrophoretic mobility to that precipitated with the conventional anti-CD45 D3/9 MoAb (Fig 4A, lane 5). Identical results were obtained by using U-937 cells (not shown).

None of the Lia MoAbs were able to recognize by flow cytometry analyses any of the CD45 molecules expressed on the mouse 300-19 cells, transfected with cDNAs coding for different CD45 isoforms (not shown). However, the polypeptides precipitated with these MoAb were identified as CD45 using different criteria. First, immunoprecipitation from iodine-125-labeled Daudi cell lysates of the polypeptide recognized by the Lia 1/11 MoAb (Fig 4B, lane 2) resulted in the depletion of the CD45 molecules (Fig 4B, lane 4). In reciprocal experiments, depletion of CD45 by precipitation with the anti-CD45 D3/9 MoAb (Fig 4C, lane 2) also prevented the immunoprecipitation of the polypeptide recognized by the Lia 1/11 MoAb (Fig 4C, lane 4). Second, western blot analysis of the polypeptides, precipitated either with the Lia1/15, Lia1/11, or D3/9 MoAb (Fig 4, D and E, lanes 1 through 3, respectively) and probed either with the Lia1/11 (Fig 4D) or with the anti-CD45 RP2/21 MoAb (Fig 4E), showed that CD45 and the polypeptides precipitated with Lia1/11 and Lia1/15 MoAb were antigenically related. Finally, partial digestion with the protease V8 of CD45 (precipitated with the D3/9 MoAb) and the polypeptides precipitated with Lia (1/8, 1/11, and 1/15) MoAbs showed an identical proteolytic degradation pattern (not shown). In addition, the precipitates obtained with the Lia MoAbs contained a phosphotyrosine phosphatase activity similar to that precipitated using the anti-CD45 D3/9 MoAb (not shown). Altogether, these results indicate that the MoAbs Lia1/11, Lia1/11, and Lia1/15 recognize a 220-kD isofrom of CD45.

Lia 1/11 recognizes an epitope encoded by the exon-A variable region of CD45 that can be masked by glycosylation. Antigenic complexity of CD45 is accomplished by both differential expression of alternatively spliced CD45 exons and glycosylation events. To determine the biochemical nature and topographic localization of Lia1/11 MoAb-recognized epitope, we first examined the contribution of carbohydrate structures to the formation of CD45 epitope recognized by Lia1/11 MoAb in Daudi B cells. Previously, we have described that unglycosylated intermediates of CD45 are not expressed on the cell surface. Therefore, we performed immunoprecipitation experiments with Lia MoAb from metabolically labeled Daudi cells (Fig 5). The Lia1/11 MoAb immunoprecipitated the different glycosylated intermediates of CD45 produced during the biosynthesis and maturation of this molecule (Fig 5A, lanes 5 through 7), as compared with the CD45 polypeptides immunoprecipitated with the conventional anti-CD45 D3/9 MoAb (Fig 5A, lanes 1 through 3). Furthermore, Lia1/11 MoAb was also able to immunoprecipitate the unglycosylated form of CD45 obtained by incubation of Daudi cells with the N-glycosylation inhibitor tunicamycin (Fig 5A, lane 8), a drug which inhibits the incorporation of both N- and O-linked carbohydrates on CD45 polypeptides. Interestingly enough, incubation of the Lia1/11 negative B-cell line Raji (Fig 5B, lanes 5 through 7) with tunicamycin enabled Lia1/11 MoAb to immunoprecipitate the unglycosylated form of CD45 (Fig 5B, lane 8).

On the other hand, we have previously described that the treatment of CD45 molecules with trypsin specifically causes the loss of the extracellular variable portion of CD45 glycoprotein. In an attempt to determine the topographic localization of the CD45 epitope recognized by the Lia1/11 MoAb, Daudi cells were incubated in the presence of trypsin. After cell treatment, the reactivity of different anti-CD45 MoAbs was analysed (Fig 6A). As expected, the reactivity of the conventional anti-CD45 D3/9 MoAb remained unaffected, whereas the reactivity of the restricted anti-CD45RA RP1/11 and anti-CD45RB MC5/2 MoAbs was abrogated. The reactivity of the Lia1/11 MoAb was also abolished, thus indicating that the epitope recognized by this MoAb is located on the variable region of CD45.

To determine more precisely the localization of the Lia1/11 epitope on the variable region of CD45, cells transfected with cDNAs encoding different CD45 isoforms were subjected to tunicamycin treatment followed by immunoprecipi-
tation with either Lial/11 MoAb (Fig 6C) or the conventional anti-CD45 D3/9 MoAb (Fig 6B). Finally, polypeptides bearing Lial/11 MoAb epitopes were detected by Western blot analysis. Lial/11 MoAb used as probe was only able to recognize the oligosaccharidated form of CD45 containing the region encoded by exon A (AB and ABC transfectants) in both D3/9 MoAb (Fig 6B, lanes 4 and 5, respectively) and Lial/11 MoAb (Fig 6C, lanes 4 and 5, respectively) precipitates. Interestingly, Lial/11 MoAb was able to recognize the glycosylated form of CD45ABC and CD45AB isoforms (Fig 6, B through D, lanes 4 and 5) in Western blot analysis, despite the fact that this cannot be detected by flow cytometry (not shown). However, Lial/11 MoAb showed a stronger reactivity with the tunicamycin-treated oligosaccharidated form of exon A containing CD45 polypeptides compared with the mature CD45 form (Fig 6, C and D, lanes 4 and 5). Altogether, these results indicate that the anti-CD45 Lial/11 MoAb recognizes an epitope located on the alternatively spliced A-encoded region of CD45 that can be masked by glycosylation on certain B-cell lines.

Homotypic cell adhesion triggered by CD45 Lial/11 MoAb involves LFA-1/ICAM-1 and LFA-1/ICAM-3 interactions and can be regulated by other CD45 MoAbs. The interaction of LFA-1 and VLA-4 integrins with their corresponding ligands from the Ig superfamily are major homotypic molecular adhesion pathways involved in cell adhesion of B and T lymphocytes.24,47 Thus, in an attempt to determine the involvement of these cell adhesion pathways in the aggregation triggered by CD45, cross-blocking cell adhesion experiments were performed by using MoAbs specific for the VLA-4 heterodimer complex (CD49d, CD29), the LFA-1 complex (CD11a, CD18), ICAM-1 (CD54), and ICAM-3 (CD50) as inhibitors. B lymphocytes purified from PBMCs were preincubated with different blocking MoAb during 20 minutes followed by incubation with the anti-CD45 Lial/11 MoAb (Fig 7A). Cell aggregation induced by Lial/11
remained unaffected by the anti-CD49d (VLA α-4) MoAb and by the anti-CD29 (VLA β-1) TS2/16 MoAb. In contrast, MoAb against both α and β subunits of the LFA-1 complex and also against ICAM-1 and ICAM-3 strongly diminished cell aggregation triggered by the anti-CD45 LiaI/11 MoAb. The LFA-1/ICAM-1 cell adhesion pathway was also involved in the aggregation triggered by the anti-CD45 LiaI/11 MoAb in the B-cell line Daudi (Fig 7B). In this case, anti-ICAM-3 MoAbs were not used because Daudi cells did not express this molecule (not shown). Taken together, these results indicate that the mechanism of homotypic cell adhesion triggered by CD45 in B cells is mediated through the LFA-1/ICAM-1 and LFA-1/ICAM-3 interactions.

We have previously described that some anti-CD45 MoAbs were able to downregulate cell aggregation triggered by the engagement of LFA-1 or ICAM-3 molecules. Similarly, cell aggregation induced by the LiaI/11 MoAb was also downregulated by some of these conventional anti-CD45 (D3/9 and TP1/41) MoAbs (Fig 7C). This effect was observed in both cases, when these MoAbs were added either before (Fig 7C) or after (not shown) the addition of LiaI/11 MoAb, thus indicating that this inhibitory effect was not caused by the fact that binding of these MoAbs was interfering with the interaction of LiaI/11 MoAb with CD45 molecule. Furthermore, cross-blocking cell-binding assays with radiolabeled and unlabeled anti-CD45 LiaI/11 and D3/9 MoAbs showed that both MoAbs did not cross-compete with each other for the binding to the molecule (not shown). On the other hand, incubation of Daudi cells with some other
conventional anti-CD45 MoAb, as RP1/10 and RP1/14, did not inhibit L1a/11 MoAb-induced cell aggregation (Fig 7C).

**L1a/11 MoAb induces coclustering of CD45 and LFA-1 polypeptides in cell aggregates.** Next, the cellular localization of CD45 as well as different cell adhesion molecules upon triggering of cell aggregation by anti-CD45 L1a/11 MoAb was studied by immunofluorescence. CD45 was mainly localized in regions of cell-cell contact (Fig 8A). This clustering of CD45 was specifically induced by L1a/11 MoAb because CD45 is localized all over cell membrane in cell aggregates induced by the anti-VLA α4 HPI/7 MoAb (not shown). Interestingly, LFA-1 also redistributed at cell-cell boundaries in L1a/11 MoAb-induced cell aggregates (Fig 8B), whereas ICAM-1 molecules appeared broadly distributed around the cell (Fig 8C), thus suggesting that the proaggregatory function of CD45 could regulate LFA-1 function. As expected, VLA β1 integrins, which were not involved in the adhesion triggered by L1a/11 MoAb, did not redistribute (Fig 8D).

**Effect of inhibitors of phosphotyrosine phosphatase and tyrosine kinase activities on CD45-mediated cell aggregation.** We next investigated the signal transduction pathways that could be involved in the CD45-mediated cell aggregation (Fig 9). First, we studied the involvement of the tyrosine phosphatase activity. By using the phosphotyrosine phosphatase inhibitor sodium orthovanadate, we observed a strong inhibition of the cell aggregation triggered by L1a/11 MoAb, whereas cell aggregation triggered by the proaggregatory anti-LFA-1α NK-L16 MoAb was not affected. These results suggest that L1a/11 MoAb could function by modulating the phosphotyrosine phosphatase activity of CD45. Furthermore, the tyrosine kinase inhibitor genistein, which inhibits the cell aggregation induced by NK-L16 MoAb (Fig 9), was also able to partially block cell aggregation triggered by L1a/11 MoAb, although other tyrosine kinase activity inhibitors, such as tyrphostin 25 (100 μmol/L) and herbimycin B (500 nmol/L), had no effect (not shown). In contrast, the protein kinase C (PKC)-specific inhibitor bisindolmaleimide did not affect the L1a/11 MoAb-mediated cell aggregation (Fig 9), whereas at same doses it abrogates PMA-induced cell-cell adhesion (not shown), thus suggesting that PKC does not play a crucial role in the regulation of this CD45-mediated cell aggregation.

**DISCUSSION**

In this report, we show that epitopes located on the alternatively spliced exon A region of CD45 are able to trigger intercellular adhesion in B lymphocytes. This activity requires the tyrosine phosphatase activity of CD45 and involves the LFA-1/ICAM-1 and LFA-1/ICAM-3 interactions. In addition, the engagement of CD45 induces clustering of CD45 and LFA-1 polypeptides at cell-cell boundaries. Interestingly, these CD45 epitopes are selectively expressed on B lymphocytes and NK cells, and we provide evidence suggesting that their expression could be regulated by glycosylation.

These results have been obtained by mean of the novel anti-CD45 L1a/8, L1a/11, and L1a/15 MoAbs. These...
MoAbs recognize a polypeptide of 220 kD that has been identified as CD45 by different biochemical criteria, such as preclearing experiments, cross-reactivity in Western blotting, partial proteolysis, immunoprecipitation of phosphotyrosine phosphatase activity, and Western blot analysis of CD45 transfectants. These MoAb appear to recognize epitopes on the exon A-encoded region of CD45 and are able to immunoprecipitate the different glycosylated intermediates produced during the biosynthesis and maturation of CD45 molecules. Furthermore, these MoAb immunoprecipitate the peptidic backbone of CD45 produced by incubation of the cell with the N- and O-glycosylation inhibitor tunicamycin, thus suggesting that these MoAbs recognize an epitope of peptidic nature. Interestingly enough, we report evidence indicating that the epitopes recognized by Lial/8, Lial/11, and Lial/15 MoAbs appear to be masked by glycosylation in several B-cell lines. This could explain the absence of reactivity of these MoAbs with the different CD45 transfectants in FACscan analyses. However, it is worthy to remark that Lial/11, although inefficiently, recognizes the glycosylated CD45 protein isoforms containing the exon A-encoded region in Western blot analyses, thus suggesting that glycosylation would mask the epitope that is recognized by Lial/11 MoAb on the cell-surface membrane.

The differential glycosylation of several surface polypeptides, including CD45, has been implicated in the regulation of leukocyte differentiation, development, and lymphocyte activation. In this regard, we have found that lymph node-resident B cells are differentially stained with the anti-CD45 Lial/11 MoAb. Thus, mantle B cells express a higher staining intensity of CD45 Lial/11 epitopes than more mature B cells localized in the germinal center, thus suggesting that the expression of this proaggregatory CD45 epitope could be regulated during B lymphocyte maturation. Nevertheless, we have been unable to detect any variation in the expression of Lial/11 epitope after activation of purified peripheral or tonsillar B lymphocytes with PMA and anti-μ chain of IgM for 1 to 3 days (J.M. Zapata, unpublished observations). In contrast, CD45 Lial/11 epitope is present on peripheral blood NK cells, and its expression is downregulated upon in vitro activation of NK cells. This could be related to the downregulation of the expression of the different CD45 isoforms carrying variable exons in activated NK cells.

Our results clearly pointed out a role for CD45 in the regulation of cell adhesion in B cells. We have shown that purified B lymphocytes and the cell lines tested that express the CD45 epitope recognized by Lial/11 MoAb can be aggregated upon incubation with this MoAb. An exception was the pre-B-cell line NALM-6, which did not express LFA-1, thus further supporting the notion that the cell adhesion pathway triggered by CD45 is LFA-1 dependent. Several other molecules expressed on the surface of leukocytes have also been involved in the regulation of cell-cell adhesion. The antibody engagement of some of these molecules, including CD19, CD20, CD39, CD40, and major histocompatibility complex (MHC) class II trigger homotypic cell adhe-
sion in B cells involving both LFA-1-dependent and -independent interactions. Interestingly, it has been shown that the engagement of CD45 using a conventional anti-CD45 MoAb was able to inhibit the cell aggregation induced through all these molecules. Furthermore, we have recently shown that several anti-CD45 MoAb could also downregulate both the LFA-1-dependent and the ICAM-3-dependent homotypic cell adhesion triggered by direct engagement of these adhesion molecules. Interestingly, we show here that these anti-CD45 MoAb are also able to inhibit cell aggregation triggered by the anti-CD45 Lia1/11 MoAb. Altogether, these results indicate that CD45 is able to both activate or inhibit the LFA-1-dependent and ICAM-3-dependent cell-cell adhesion pathways, and that these functions can be modulated by engagement of different epitopes on the CD45 molecule. This is in agreement with what has recently been described; some anti-CD45 MoAb could trigger LFA-1-dependent intercellular cell adhesion of T lymphocytes at different maturation and activation stages. Taken together, these results suggest that CD45 could function as a modulator of the LFA-1 cell adhesion pathway in leukocytes. Accordingly, it has been shown that anti-CD45 MoAb inhibit the calcium mobilization induced via LFA-1 molecules in NK cells.

It is interesting to note that, in purified B lymphocytes, the LFA-1-dependent cell aggregation triggered by anti-CD45 Lia1/11 MoAb involved LFA-1 interactions with ICAM-1 and ICAM-3. This result is in agreement with previous data in T lymphocytes showing that the mechanism of LFA-1/ICAM interactions involves the initial binding of ICAM-3 to LFA-1, and that this interaction appears to increase the avidity of LFA-1 for ICAM-1, thus allowing the establishment of a more stable cell-cell interaction.

On the other hand, we have shown that cell aggregation triggered by CD45 induces clustering of CD45 and LFA-1 molecules. Evidence has been described suggesting that CD45 interacts with the cytoskeletal protein fodrin, and that LFA-1 interacts with the actin-binding protein α-actinin. These cytoskeletal interactions could be involved in driving these molecules to the cell-cell boundaries, as well as to participate in the regulation of the avidity of LFA-1 for their ligands. In this regard, the crucial role of the LFA-1/cytoskeletal interactions in the regulation of LFA-1 function has recently been described.

Our studies on the signaling pathways involved in the regulation of CD45-mediated cell aggregation by using inhibitors of both phosphotyrosine phosphatase and tyrosine kinase activities suggest that Lia MoAb engagement of CD45 may indeed be modulating its phosphotyrosine phosphatase activity, which appears to be involved in the formation of cell aggregates. On the other hand, the PKC inhibitor bisindolmaleimide had no effect on the cell-aggregation triggered by the anti-CD45RA Lia 1/11 MoAb, thus suggesting PKC does not appear to play a crucial role in this process. Interestingly, the cyclic adenosine monophosphate/cyclic guanosine monophosphate-dependent kinase inhibitor H8, was able to block the cell-cell adhesion triggered by Lia1/11 MoAb. This inhibitory effect was observed at concentrations that did not inhibit PMA-induced cell clustering, although the inhibitory effect was highest at concentrations that also block the effect of PMA (Zapata et al, unpublished observations). These results are in agreement with those recently reported by Lorentz et al, using a heterotypic cell aggregation system between T-cells and monocytes. Altogether, these results suggest that CD45 could be regulating LFA-1 function in a complex fashion that deserves further investigation.

ACKNOWLEDGMENT

We thank Drs G.D. Johnson (BU12), Neil R. Cooper (HB5), Bice Perussia (B73.1), Jean de Vries (SPV-3T3), Tim Springer (RR1), C. Figdor (NK1-L16), and Robert Rothlein (RG5) for kindly providing the MoAbs indicated; Dr Michel Streuli for kindly providing murine cell line transfected with different human CD45 cDNAs; Dr Paloma Sanchez-Mateos for her invaluable help with the immunofluorescence; David Hernandez and Mariano Viton for expert technical assistance in flow cytometry analysis; Angeles Ursa and Milagros Sanchez de Matias for the Lia MoAb; and Nigel Barnstable for helpful comments.

REFERENCES

CD45 REGULATED INTERCELLULAR ADHESION OF LEUKOCYTES

45. Fraker PI, Speck JC: Protein and cell membrane iodinations with a sparingly soluble chloroamide-1,3,4,6-tetrachloro-3,6-diphenyl glycoluril. Biochem Biophys Res Comm 80:849, 1978
49. Smith SH, Rigley KP, Callard RE: Activation of human B cells through the CD19 surface antigen results in homotypic adhesion by LFA-1 dependent and independent mechanism. Immunology 73:293, 1991
59. Lockehwar VB, Bourguignon LYW: Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytokine. J Biol Chem 267:21551, 1992
B-cell homotypic adhesion through exon-A restricted epitopes of CD45 involves LFA-1/ICAM-1, ICAM-3 interactions, and induces coclustering of CD45 and LFA-1

JM Zapata, MR Campanero, M Marazuela, F Sanchez-Madrid and MO de Landazuri