Brief Definitive Report

Identification and Characterization of a 100-kD Ligand for CD6 on Human Thymic Epithelial Cells

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Summary

CD6 is a 130-kD glycoprotein expressed on the surface of thymocytes and peripheral blood T cells that is involved in TCR-mediated T cell activation. In thymus, CD6 mediates interactions between thymocytes and thymic epithelial (TE) cells. In indirect immunofluorescence assays, a recombinant CD6-immunoglobulin fusion protein (CD6-Rg) bound to cultured human TE cells and to thymic fibroblasts. CD6-Rg binding to TF and TE cells was trypsin sensitive, and 54 ± 4% of binding was divalent cation dependent. By screening the blind panel of 479 monoclonal antibodies (mAbs) from the 5th International Workshop on Human Leukocyte Differentiation Antigens for expression on human TE cells and for the ability to block CD6-Rg binding to TE cells, we found one mAb (J4-81) that significantly inhibited the binding of CD6-Rg to TE cells (60 ± 7% inhibition). A second mAb to the surface antigen identified by mAb J4-81, J3-119, enhanced the binding of CD6-Rg to TE cells by 48 ± 5%. Using covalent cross-linking and trypsin digestion, we found that mAb J4-81 and CD6-Rg both bound to the same 100-kD glycoprotein (CD6L-100) on the surface of TE cells. These data demonstrate that a 100-kD glycoprotein on TE cells detected by mAb J4-81 is a ligand for CD6.

Intrathymic development of human T cells is regulated by the interactions between thymocytes and stromal cells of the thymic microenvironment (for a review see reference 1). Both epithelial and fibroblast components of thymic stroma are important for thymocyte maturation (2). mAb-mediated inhibition of thymocyte-stromal cell binding has shown that CD2, CD54, CD58, LFA-1 (CD11a/CD18), very late antigen 3 (VLA-3) (CD49c/CD29), VLA-4 (CD49d/CD29), VLA-6 (CD49f/CD29), and fibronectin serve as adhesion receptors for thymocyte–stromal cell interactions (3–7). Vollger et al. (3) screened for the ability of mAbs to inhibit thymic epithelial (TE) cell–thymocyte binding and found that CD6 mAbs partially inhibited TE-thymocyte binding, suggesting that CD6 may also be involved in thymocyte–stromal interactions.

CD6 is a 130-kD glycoprotein that is differentially expressed on the surface of mature human thymocytes, the majority of peripheral blood T cells, a subset of B cells, and neurons of the cerebral cortex (8–10). CD6 is a member of the macrophage scavenger receptor protein family and is homologous to the CD5 molecule (11). The role of CD6 in T cell development has not been identified. On peripheral blood T cells, CD6 is involved in T cell activation as CD6 mAb T12 can augment T cell proliferation in autologous mixed lymphocyte reactions (MLR) (12). mAbs to CD6 also augment TCR-mediated T cell activation (13–16), and CD6 is tyrosine phosphorylated after TCR-mediated T cell triggering (17). Clinically, anti-CD6 mAbs (T12) have been used to deplete T cells from bone marrow transplants and prevent GVHD (18). CD6 may be involved in autoimmunity as $CD3^+$, $CD6^-$ T cells that survive T12-ricin treatment show decreased alloreactivity compared with $CD6^+$ T cells (19), and the anti-CD6 mAb UMCD6 inhibited the autoreactive response of cloned T cells in autologous MLRs (20).

In this study, we have used CD6-Ig fusion proteins and a panel of 479 mAbs to demonstrate that TE cells express a 100-kD divalent cation–independent ligand for CD6 that is recognized by mAbs J4-81 and J3-119.

Materials and Methods

Cells and Culture Conditions. TE cells and thymic fibroblasts (TF) were cultured by an explant technique as described (21, 22). Human thymus tissue was obtained from the Department of Pathology, Duke University Medical Center, as discarded tissue from children undergoing corrective cardiovascular surgery, and thymocytes prepared as described (23). COS-M6 cells and HBL-100 cells (American Type Culture Collection [ATCC], Rockville, MD) were grown in DMEM containing 10% FCS, 1 mM sodium pyruvate, 0.025

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/04/1563/06 $2.00
Volume 181 April 1995 1563–1568

μg/ml amphotericin B, 100 U/ml penicillin, and 100 μg/ml streptomycin.

mAbs. Antibodies used in this study were: P3X63/Ag8 (control mAb; ATCC), A3D8 (anti-CD44; 24), J4-81, and J3-119 (25), and the blind panel of 479 mAbs from the 5th International Workshop on Human Leukocyte Differentiation Antigens (HLDA-V) (26).

Detection of Cell Surface Antigens. Cells, suspended in either PBS or DMEM (with or without 10 mM EDTA) containing 2% BSA and 0.1% NaN_3, were incubated with a recombinant CD6-Ig fusion protein (CD6-Rg) (27), CD5-Rg (28), or human IgG (Sigma Chemical Co., St. Louis, MO) (100 μg/ml) for 30 min at 4°C and washed with PBS containing 2% BSA and 0.1% NaN_3. Fluorescein-conjugated goat anti-human IgG1 (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used as a secondary reagent. Cells were analyzed on a FACStar$^{Plus®}$ flow cytometer (Becton Dickinson, & Co., Mountain View, CA). To determine trypsin sensitivity of fusion protein interactions, cells were incubated with 0.2% trypsin in PBS containing 1 mM EDTA for 30 min at 37°C and washed before reactivity with fusion proteins.

Screening of mAbs for Inhibition of CD6-Rg Binding to TE Cells. mAbs from the blind panel of the HLDA-V and a panel of anti-integrin mAbs were screened for reactivity to the surface of TE cells (29). Of the 154 mAbs that reacted with TE cells, 126 were used in this assay. TE cells (10^5) were incubated with ascites or purified mAb (1:100) for 15 min at 4°C. Either CD5-Rg or CD6-Rg (5 μg) were added and allowed to react for 2 h at 4°C, washed, and, labeled with a fluorescein-conjugated antiserum specific for the Fc portion of human IgG (Sigma Chemical Co.). To account for any cross-reactivity of fluorescein-conjugated anti-human IgG with murine Ig, binding was determined as the difference in fluorescence (ΔFL) between samples containing CD6-Rg and CD5-Rg.

Immunoprecipitation and Protein Cross-linking Conditions. TE cells, surface labeled with ^{125}I as described (30), were incubated with mAbs (1:100 ascites), purified Ig (200 μg/ml), or Ig fusion proteins (200 μg/ml) for 2 h in DME/5% FBS. After washing with cold PBS, bound Igs were cross-linked to cell surface proteins with 1 mM 3,3′ dithiobis(sulfo-succinimidyl propionate) (DTSSP) (Pierce Chemical Co., Rockford, IL) in PBS for 60 min at 4°C. After addition of 20 mM Tris-HCl (pH 8.0), the cells were washed with cold PBS. Cells were lysed in PBS containing 1% NP-40, 1 mM PMSF, 0.1 mM Nα-p-Tosyl-L-lysine chloromethyl ketone (TLCK) and 0.1% NaN_3. Ig complexes were purified with protein A–Sepharose beads (Sigma Chemical Co.). Before SDS-PAGE, protein complexes were solubilized with SDS loading buffer (2% SDS, 10 mM Tris-HCl, pH 7.4, 20% glycerol, bromphenol blue) containing 2% 2-ME. To confirm identity of proteins, purified protein complexes were treated with 0.2% trypsin in 1 mM EDTA for 30 min at 25°C (31) before solubilization and cross-linker cleavage. After SDS-PAGE, the gels were fixed, dried, and exposed to autoradiography film.

Immunohistology. Normal human tissues were obtained as discarded tissue from the Department of Pathology, Duke University Medical Center and frozen in liquid nitrogen. Indirect immunofluorescence (IF) assays of mAb reactivity on acetone-fixed tissue sections were performed as described (32).

Results

CD6-Rg Binding to Cells of the Thymic Microenvironment. The ability of CD6-Rg fusion protein to bind to TE cells, thymic fibroblasts, and thymocytes was determined by indirect IF and flow cytometry. CD6-Rg bound to the surface of TE cells as well as to thymic fibroblasts, but not to thymocytes (Fig. 1 *A*). Binding of CD6-Rg to TE cells was trypsin sensitive and partially dependent upon divalent cations (Fig. 1 *B*). CD6-Rg bound well to TE cells in a buffer containing DME and 5% fetal bovine serum, but in the presence of 10 mM EDTA, binding of CD6-Rg to TE cells was inhibited by 54 ± 4% (n = 5, p <0.001, Fig. 1 *B*).

Antibody-mediated Inhibition of CD6-Rg Binding to TE Cells. To begin to identify the CD6 ligand(s), a panel of 479 mAbs from HLDA-V was screened for reactivity to the surface of TE cells by indirect IF and flow cytometry. Of the 154 mAbs that reacted with the surface of TE cells (29), 126 mAbs were used in assays to inhibit the binding of CD6-Rg to TE cells. Of the 122 mAbs that did not react with the secondary antiserum, only one (J4-81) inhibited the binding of CD6-Rg to TE cells. mAb J4-81 inhibited the binding of CD6-Rg to TE cells by 60 ± 7% (n = 10, p <0.001) and to the breast cell line HBL-100 by 40 ± 3% (n = 3, p <0.02) (Table 1), which has also been shown to bind CD6-

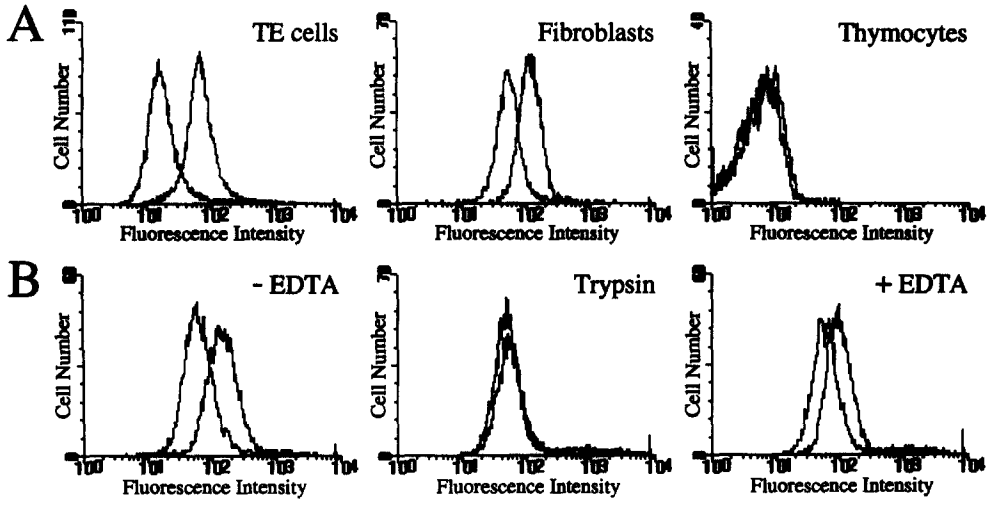

Figure 1. Thymic epithelial cells and thymic fibroblasts express a trypsin-sensitive surface ligand for CD6. (*A*) Representative histograms depicting the reactivities with CD6-Rg of thymic epithelial cells, thymic fibroblasts, and thymocytes. Also shown in each panel are background levels of fluorescence with control fusion protein CD5-Rg. (*B*) The binding of CD6-Rg to TE cells in either divalent cation–containing media (– EDTA), trypsin-treated TE cells in media (*trypsin*), or TE cells in PBS + 10 mM EDTA (*EDTA*) was tested. Shown are histograms of a representative experiment (from >10 performed) depicting CD6-Rg binding to TE cells as determined by indirect IF and flow cytometry.

Table 1. *mAb Inhibition of CD6-Rg Binding to TE and HBL-100 Cells*

Cell Type	mAb	Δ FL*	Percent binding‡	Percent inhibition§
TE cells	P3	52	100	0
	CD9	60	115	−15
	CD24	48	92	8
	CD40	48	92	8
	CD46	57	110	−10
	CD51	46	88	12
	CD54	44	85	15
	CD58	45	87	13
	CD59	52	100	0
	CD63	53	102	−2
	CD66	56	108	−8
	J4-81	21	40	60
HBL-100	P3	58	100	0
	J4-81	34	59	41

* ΔFL = Fluorescence (CD6-Rg) − fluorescence (CD5-Rg). Shown is the binding of CD6-Rg compared with control (CD5-Rg) in the presence of selected mAbs that bind well to the surface of TE cells.
‡ = 100 × [ΔFL(experimental mAb) − ΔFL(P3)]/ΔFL(P3).
§ = 100 × [ΔFL(P3) − ΔFL(experimental mAb)]/ΔFL(P3).

Rg (27). In flow cytometry assays, both TE and HBL-100 cells reacted strongly with mAb J4-81 (data not shown).

mAb J3-119, reported to react with a second epitope on the molecule detected by J4-81 (25), enhanced the binding of CD6-Rg to TE cells (Fig. 2) by 48 ± 5% ($n = 6, p < 0.005$), and to HBL-100 cells by 45 ± 11% ($n = 3$, $p < 0.1$). To confirm that mAb J3-119 recognized the same protein as mAb J4-81, the ability of mAb J3-119 to block the binding of biotinylated J4-81 to TE cells was tested. Whereas mAb A3D8 to CD44 (which binds well to the surface of TE cells; 29) had no effect on J4-81 binding, both J4-81 and J3-119 mAbs inhibited the binding of biotinylated J4-81 (99 and 82%, respectively; data not shown).

mAb J4-81 and CD6-Rg Bind 100-kD TE Cell Proteins that Are Identical. To identify the TE cell surface protein(s) that bound to CD6-Rg, we devised a strategy whereby CD6-Rg interactions with CD6 ligand(s) on surface ^{125}I-labeled TE cells were stabilized with DTSSP, and CD6-Rg–containing complexes were purified using protein A–Sepharose beads. Using this strategy, CD6-Rg specifically reacted with a 100-kD TE cell surface protein (Fig. 3) termed CD6L-100. mAb J4-81 cross-linked to ^{125}I-labeled TE cells also yielded a 100-kD protein that migrated with the 100-kD protein recognized by CD6-Rg (Fig. 3). Trypsin digestion studies showed that the 100-kD protein identified by J4-81 had an identical trypsin digestion pattern to the protein identified by CD6-Rg (Fig. 3), indicating that both proteins were identical. CD6L-100 was determined to be a glycoprotein as mAb J4-81 immunoprecipitated CD6L-100 from extracts of TE cells metabolically labeled with [^{3}H]glucosamine (data not shown).

The 100-kD CD6 Ligand is a Divalent Cation–independent Ligand for CD6. CD6-Rg was able to immunoprecipitate CD6L-100 in the presence and absence of divalent cations, suggesting that CD6L-100 may be a divalent cation–independent CD6 ligand. Further, J4-81 only partially inhibited the binding of CD6-Rg to TE cells in the presence of divalent cations. Thus, there may be more than one ligand for CD6. mAb J4-81 almost completely inhibited (80 ± 10% inhibition, $p < 0.1$) CD6-Rg binding to TE cells in the presence of EDTA (Fig. 2), confirming that it is primarily involved in divalent cation-independent CD6–CD6 ligand interactions. In contrast, mAb J3-119 enhanced CD6-Rg binding by 47 ± 7% ($p < 0.1$) in the absence of divalent cations (Fig. 2).

Tissue Distribution of mAb J4-81 Reactivity. The tissue distribution of CD6L-100 was examined on frozen sections of a variety of human tissues by indirect IF (Table 2). The reactivity of mAb J4-81 with human tissues was broad. Whereas CD6 was expressed on thymocytes in postnatal human thymus,

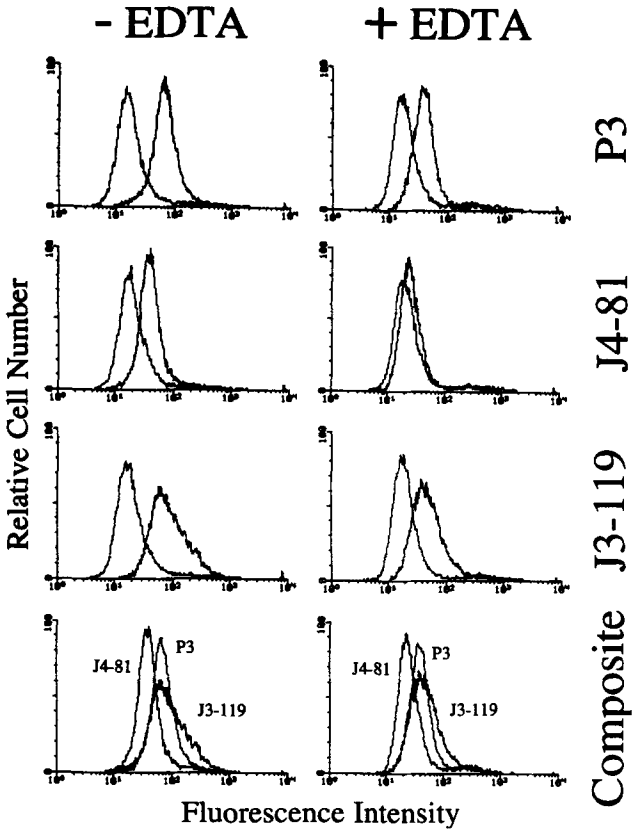

Figure 2. Effect of preincubation of J4-81 and J3-119 on CD6-Rg binding to TE cells. Shown are histograms of CD6-Rg binding to the surface of human TE cells, as detected by indirect IF and flow cytometry, in the presence of mAbs P3 (control), J4-81, or J3-119 in media containing divalent cations (− EDTA) or no divalent cations (+ EDTA). The binding of control human IgG is shown in each panel. The bottom panels show composite summary histograms of CD6-Rg binding in the presence of either P3, J4-81, or J3-119. Data are representative of 10 experiments in the presence of divalent cations and 2 experiments with no divalent cations.

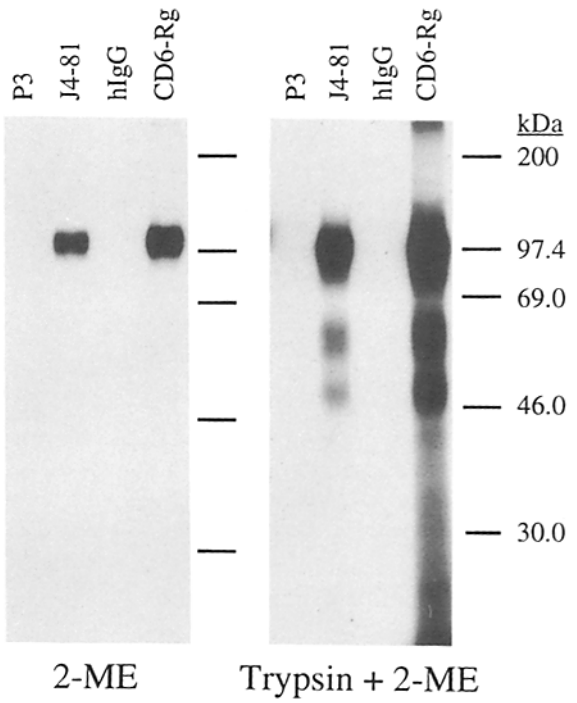

Figure 3. mAb J4-81 and CD6-Rg bind to the same 100-kD glycoprotein on the surface of human TE cells. Shown are autoradiographs of ^{125}I-labeled TE cell surface proteins cross-linked to either mAb P3 (control), mAb J4-81, hIgG1 (human Fc control), or CD6-Rg. The cross-linked proteins were treated with either 2-ME alone (to cleave the cross-linker) or trypsin and 2-ME before electrophoresis. Both CD6-Rg and mAb J4-81 bound to 100-kD proteins with identical trypsin digestion patterns. Data are representative of two separate experiments.

Table 2. *Reactivity of mAb J4-81 in Sections of Normal Human Tissues*

Tissue (number tested)	J4-81 Reactivity
Thymus (5)	Hassall's bodies, epithelium, fibroblasts
Spleen (2)	Scattered mononuclear cells
Lymph node (1)	Scattered mononuclear cells
Tonsil (2)	Pharyngeal epithelium, lymphocytes
Appendix (1)	Fibroblasts, lymphoid cells, epithelium
Colon (2)	Fibroblasts, epithelium
Esophagus (2)	Basal epithelium
Breast (2)	Epithelium, fibroblasts
Liver (2)	Hepatocytes, Kupfer cells, fibroblasts
Pancreas (2)	Acinar and islet cells
Kidney (2)	Fibroblasts, subset of tubules, Bowman's capsule
Skin (2)	Perivascular fibroblasts, epithelium
Brain (2)	Neurons

The cell types reacting with mAb J4-81 in each of the tissue types are listed.

CD6L-100 was expressed on cortical and medullary TE cells and Hassall's bodies (Fig. 4). In tissues other than thymus, J4-81 reacted with epidermal keratinocytes, gut epithelium, breast epithelium, pancreatic acinar cells, pancreatic islet cells, hepatocytes, renal tubular epithelium, neurons of the cerebral cortex, and fibroblasts.

Discussion

In this study, we have shown that human thymic epithelial cells and fibroblasts express a ligand for CD6, termed CD6L-100. We have found two mAbs, J4-81 and J3-119, that bind to CD6L-100 and alter the binding of CD6-Rg to TE cells. Cross-linked immunoprecipitation experiments and trypsin digestion revealed that the 100-kD molecule bound by CD6-Rg and by J4-81 were identical.

Whereas the role of CD6 as an accessory molecule in T cell activation is well established (12–15), its role in T cell development is not clear. Vollger et al. (3) initially suggested that CD6 may be an adhesion molecule involved in TE-thymocyte binding as the anti-CD6 mAbs T12 and 12.1 were able to partially block the binding of human thymocytes to cultured human TE cells. Identification of the ligand(s) for CD6 may provide a better understanding of the role of CD6 in T cell differentiation and activation (16, 20, 27).

Partial inhibition of CD6-Rg binding to TE cells and thymic fibroblasts by EDTA suggested that there may be more than one ligand for CD6, or that the CD6 ligand may have different states of activation depending on divalent cations. Interestingly, a 31-kD surface molecule was frequently immunoprecipitated along with CD6L-100 from B cells by mAbs J4-81 and J3-119 (25). Whereas we have clearly established that CD6L-100 is a divalent cation-independent ligand for CD6, other ligands for CD6 may exist and CD6L-100 may complex with other proteins that regulate CD6 binding (Wee, S. F., D. D. Patel, B. F. Haynes, and A. Aruffo, unpublished observations).

CD6L-100 has a broad tissue distribution, with expression on a variety of cell types in normal tissues as defined by mAb J4-81 (Fig. 4, Table 2). mAb J4-81 was also tested in the blind panel of HLDA-V, and was shown to react with the surface of the following human cell types: $CD34^{mid}$ and $CD34^{hi}$ bone marrow cells, resting and TNF-activated human umbilical vein endothelial cells, dermal endothelial cells, activated peripheral blood B and T cells, a variety of B cell and T cell lymphomas, monocytes, myeloid leukemias, thymic epithelial cells, epidermal keratinocytes, skin and lung fibroblasts, and a variety of stromal cell carcinomas (26). The broad distribution of CD6L-100 is consistent with the previous report that CD6-Rg binds to a number of murine lymphoid tissues and transformed human cell lines (27). The expression of CD6L-100 in normal noninflamed nonlymphoid tissues combined with the accessory role of CD6 in T cell activation implies that CD6–CD6L interactions may be functional in the earliest stages of T cell activation, before upregulation of MHC and adhesion molecules by T cell–liberated cytokines. CD6L-100 expression did not change with IFN-γ activation of TE cells (29) or TNF-α activation of endothelial

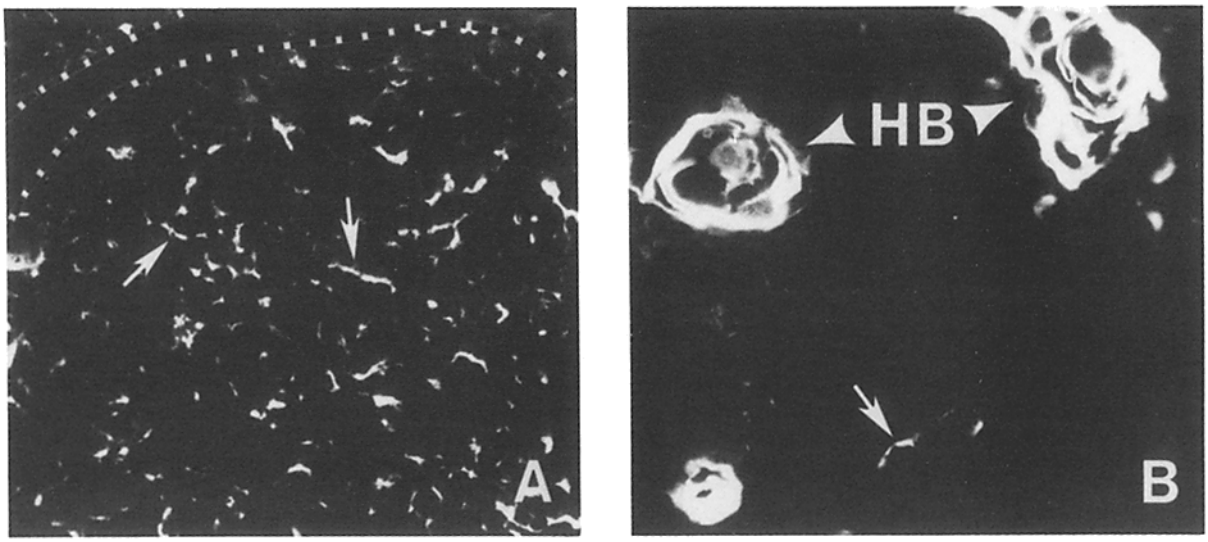

Figure 4. Expression of CD6L-100 in postnatal human thymus. Shown are photomicrographs (representative of experiments on five thymuses) of frozen sections of 2-mo-old human thymus stained by indirect IF with mAb J4-81. (*A*) A section of thymus cortex and *B* shows a section of thymus medulla with Hassall's bodies (*HB*). The thymic capsule is indicated by dashed lines in *A*. Thymic epithelial cells (*arrows*), in both the cortex and in and around HB in medulla, reacted with mAb J4-81. Thymocytes did not react with mAb J4-81. An identical pattern was seen using mAb J3-119 (not shown). ×400.

cells (26). CD6–CD6L interactions may also be important in the nervous system as neurons of the cerebral cortex express both CD6 (10) and CD6L-100.

Osorio et al. (16) have suggested that the ability of CD6 to enhance antigen-TCR signaling could be physiologically significant especially in cases of low levels of antigen or in antigen interaction with TCRs of low avidity for the antigen. This theory fits well with the recent findings that whereas $CD6^-$ peripheral T cells have normal proliferative responses to T cell mitogens (PHA, anti-CD2 mAb, and immobilized anti-CD3 mAb) and soluble antigen (tetanus toxin) plus APC, $CD6^-$ T cells are less alloreactive than unfractionated PB T cells (19). Further, CD6 mAb UMCD6 inhibited the response of cloned T cells in autologous MLRs (20). Thus, inhibition of CD6–CD6 ligand interactions by depletion of $CD6^+$ peripheral blood T cells or therapeutic use of anti-CD6 mAbs, soluble CD6, anti-CD6 ligand mAbs, or soluble CD6 ligands may decrease autoreactivity in specific autoimmune disease states.

Finally, we have defined two different epitopes of the CD6L-100 molecule using mAbs J4-81 and J3-119. mAb J4-81 inhibited CD6-Rg binding to TE cells whereas J3-119 enhanced CD6-Rg binding. These two epitopes may overlap as J3-119 inhibited the binding of J4-81 to TE cells. Alternatively, mAb J3-119 inhibition of J4-81 binding could also have been due to a change in the conformation of CD6L-100 induced by J3-119 binding. Thus, the enhanced binding of CD6-Rg in the presence of mAb J3-119 suggested that mAb J3-119 may either induce or stabilize the active conformation of CD6L-100. Similarly, mAb J4-81 may either be directed to the CD6 binding site or stabilize the inactive conformation of CD6L-100. Nucleotide sequence analysis of the gene encoding CD6L-100 and functional studies using mAbs J4-81 and J3-119 in assays of cell interactions, T cell autoreactivity, and T cell responses to specific antigen should help further define the roles of CD6–CD6 ligand interactions in T cell development and T cell effector function.

We thank Brett Modrell for CD6-Rg preparations, and Kim McClammy for secretarial assistance.

This work was supported by National Institutes of Health grant CA28936 to B. F. Haynes, Department of Energy grant DE-F006-86-ER60409 to A. Aruffo, and the Bristol-Myers Squibb Pharmaceutical Research Institute.

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Received for publication 6 October 1994 and in revised form 16 December 1994.

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