

The pattern of expression of CD147/neurothelin during human T-cell ontogeny as defined by the monoclonal antibody 8D6

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A novel monoclonal antibody termed 8D6 was generated against the human leukemic T-cell line Molt 13 and was found to recognize the neurothelin structure, recently termed CD147. UM8D6 precipitated a single band from HSB2 cells of molecular weight 35 kDa nonreduced and 40 kDa reduced. CD147 is broadly expressed on human hematopoietic cells and is expressed more intensely on thymocytes than on mature peripheral blood T cells. A clear distinction was found by three-color flow cytometry between antigen density on single positive mature thymocytes subsets compared with the corresponding subset from peripheral blood. The 8D6 antibody did not have direct effects on T-cell activation or apoptosis, and the function of CD147 in development and activation of T cells is not yet clear. Nevertheless, its highly regulated expression during T-cell differentiation and the structural characteristics of this antigen suggest that it has an interesting and important role in T-cell biology.

**A. H. Kirsch, L. A. Diaz, Jr.,
B. Bonish, P. A. Antony and D. A. Fox**

¹Division of Rheumatology, Multipurpose Arthritis and Musculoskeletal Diseases Center, and Specialized Center of Research in Rheumatoid Arthritis, University of Michigan, Ann Arbor, USA

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Cells of lymphoid origin and the central nervous system share a variety of surface markers, such as CD6, CD90 (Thy-1) and various adhesion molecules. Neurothelin, a glycoprotein which is expressed on endothelial cells, is thought to play an important role in the blood brain barrier (1). Other names for this molecule include HT7 (chick and mouse), OX-47 (rat), basigin (mouse), M6 (human) and EMMPRIN (1–4).

Neurothelin-related determinants are also expressed on cells of lymphoid origin. The OX-47 antigen is found at low levels on rat lymphocytes and is upregulated upon activation in both T and B cells (2). The M6 antigen, present on peripheral granulocytes of patients with rheumatoid and reactive arthritis, is also expressed on a variety of T-cell, B-cell, erythroid, and myeloid cell lines (3). This structure has recently been designated CD147 by the 6th International Workshop and Conference on Human Leukocyte Differentiation Antigens (4). However, information is not yet available on the developmental expression of neurothelin by human lymphoid cells.

This article describes the pattern of expression

of human CD147/neurothelin as recognized by a new monoclonal antibody termed 8D6. CD147/neurothelin is widely expressed on cells of hematopoietic origin and has a specific pattern of expression during the development of T-lineage cells.

Material and methods

Antibodies

Production of the 8D6 monoclonal antibody. A subtractive immunization technique was used to develop monoclonal antibodies to less immunogenic antigens or epitopes on the thymocyte cell surface. A 6-week-old female BALB/c mouse was serially immunized (3 immunizations at 2-week intervals) with Peer cells, a human T-cell leukemic line. Each immunization was followed by treatment with cyclophosphamide, as previously described (5). The same mouse was then immunized and boosted twice with cells from a second leukemic T-cell line, Molt 13. Hybridoma 8D6 (IgG1) was subcloned and propagated in ascites form in pristane-primed mice.

Other antibodies: 7F8 (anti-CD98) was produced using the method described above (5). Anti-CD3-tricolor, anti-CD4-phycoerythrin (PE), anti-CD8-PE were obtained from Caltag. Fluorescein isothiocyanate-conjugated goat anti-mouse antibody was obtained from Coulter. OKT3 (anti-CD3) was produced from hybridoma cells obtained from ATCC, and UMCD2 (anti-CD2) was produced as previously described (6). Anti-AD2 (7) was a gift from B. Haynes and D. Patel of Duke University.

Cells

Human peripheral blood mononuclear cells were obtained from venous blood of healthy volunteers by centrifugation over Ficoll-Hypaque. Thymic tissue was obtained as excess surgical material from children who underwent corrective cardiac surgery. It was finely minced and pressed through a stainless steel mesh to prepare a single-cell suspension. Thymocytes and peripheral blood mononuclear cells were separated from red blood cells and debris by centrifugation over Ficoll-Hypaque and washed three times.

The human T-cell leukemic lines HSB-2, Jurkat, Peer, Hut-78 and Molt 13 were maintained in continuous cell culture in our laboratory.

Immunoprecipitation

Cells of the HSB-2 T cell line were metabolically labeled with [35]-S-methionine for 4.5 h and then lysed and precleared as previously described (5), immunoprecipitated by incubating the lysate first with 8D6 antibody, followed by addition of protein-G Sepharose beads. Immunoprecipitates were analyzed by SDS-PAGE under reducing and non-reducing conditions.

Flow cytometry analysis

Single-color flow cytometry. Monoclonal antibodies were used at saturating concentrations. Surface antigen expression was determined using indirect or direct immunofluorescence using an Epics 3 or Elite Cell Sorter (Coulter Electronics, Hialeah, FL, USA). Cell populations of interest were selected using forward and right angle light scatter gating.

Three-color flow cytometry. Thymocytes and peripheral blood mononuclear cells obtained on the same day were placed into tubes (5.0×10^5 cells per tube) in 50 μ l of phosphate-buffered saline (PBS). Fifty μ l of antibody (8D6, 7F8 or controls) was added to appropriate tubes and incubated for 30 min at 4°C. After washing twice with 2 μ l of PBS,

5 μ l of CD4-PE or CD8-PE and 50 μ l of CD3-TC (or appropriate controls) were added and incubated for 30 min at 4°C. Cells were washed twice with 2 ml of PBS and resuspended in fixative (formaldehyde 1%). Cells were analyzed by a Coulter Elite cell sorter.

T-cell activation

Proliferation assays. Mononuclear cells and thymocytes (1×10^5 cells/well) were cultured in triplicate in round-bottom 96-well plates in RPMI 1640 (Bio Whittaker) supplemented with 10% FCS (Gibco), 2 mM glutamine and penicillin (100 U/ml)/streptomycin (100 U/ml) at a total volume of 0.2 ml/well. Cells were stimulated with PHA, PMA, IL-2, anti-CD2, anti-CD3, and anti-CD98. At day 4 of culture, wells were pulsed overnight with tritiated thymidine (0.8 μ Ci/well) and harvested, and thymidine incorporation was measured.

Kinetics of 8D6 expression. Peripheral blood mononuclear cells were cultured in RPMI supplemented with 10% FCS, glutamine, and penicillin/streptomycin at 1×10^6 cells/ml alone or with various mitogens including PMA (1 ng/ml), PHA (0.5 μ g/ml), ionomycin (0.5 μ g/ml), or a combination of PMA and ionomycin for 4 days at 37°C. On days 2 and 4, a subset of cells under each condition were stained with 8D6 and several other antibodies including OKT3, anti-Tac (anti-CD25/IL-2R), 7F8 (anti-CD98).

Apoptosis experiments

Peripheral blood mononuclear cells were stimulated with PHA and then maintained in IL-2-containing medium as described above. Every 3–4 days, a subset of the blasts were subcultured in medium alone, or supplemented with 8D6 antibody (1:500) (or other agents with potential effects on apoptosis) for 24 h. Cells were fixed in 1% formaldehyde for 15 min at 0–4°C and then rinsed in PBS. One million cells were resuspended in 50 μ l of cacodylate buffer [0.2 M potassium cacodylate, 25 mM Tris-HCl (pH 6.6), 2.5 mM cobalt chloride, 0.25 mg/ml bovine serum albumin, 100 μ l/ml terminal deoxynucleotidyl transferase, 0.5 mM biotin-16 dUTP] and incubated for 30 min at 37°C. Cells were rinsed in PBS and resuspended in 100 μ l of saline-citrate buffer [2.5 μ g/ml fluoresceinated avidin, 0.1% Triton X-100, 5% (w/v) non-fat dry milk, 0.6 M NaCl and 0.06 M sodium citrate] and incubated for 30 min at room temperature in the dark. Cells were rinsed in PBS containing 0.1% Triton X-100 and resuspended in 1 ml of PI buffer

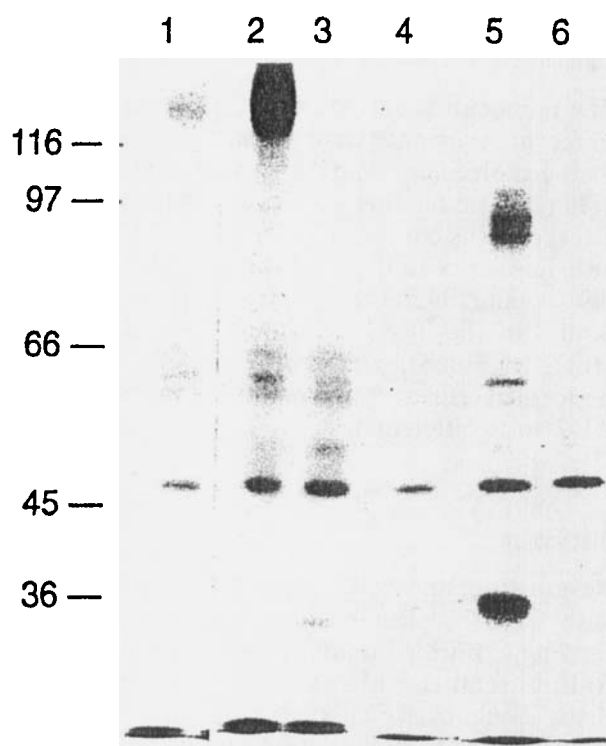


Figure 1. Structures recognized by the novel monoclonal antibody 8D6. Cells of the HSB-2 T cell line were metabolically labeled with [³⁵S]-methionine, lysed, precleared, immunoprecipitated, and analyzed by SDS-PAGE. Lanes 1–3, non-reduced; lanes 4–6 reduced. Lanes 1 and 4, control murine IgG1 monoclonal antibody. Lanes 2 and 5, monoclonal antibody 7F8, which recognizes the CD98 heterodimer. Lanes 3 and 6, monoclonal antibody 8D6, which recognizes a structure at ~35 kDa nonreduced and ~40 kDa reduced.

(PBS, 5 µg/ml PI, 0.1% RNase A). Staining was analyzed by two-color flow cytometry.

Results

Characterization of structures recognized by the novel monoclonal antibody 8D6

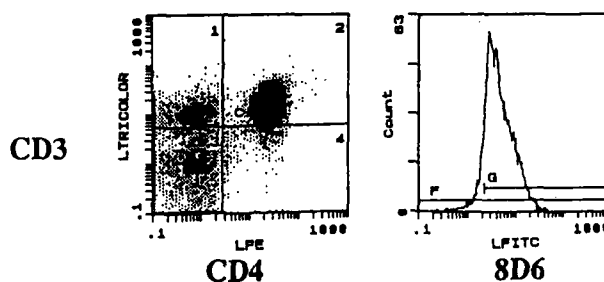
The 8D6 antibody recognizes an antigen on HSB-2 cells of approximately 35 kDa under non-reducing conditions and approximately 40 kDa under reducing conditions, suggesting the presence of an intrachain disulfide bond (Figure 1). By comparing this biochemical data and the pattern of expression of the 8D6 antigen with known antigens from the Fifth International Leukocyte Workshop, the 8D6 antigen appeared to be a novel cell surface structure, not defined by CD classifications of the Fifth Workshop. Subsequently, in studies performed by the Sixth Workshop, 8D6 was found to identify CD147/neurothelin in binding assays both to transfectants and to beads coated with a CD147-Rg fusion protein (4).

8D6/neurothelin is expressed on a variety of cell types and is expressed more strongly on differentiated thymocytes than on mature T cells

The 8D6 antibody binds to resting and activated peripheral blood mononuclear cells, thymocytes and transformed cell lines of both T-cell and non-T-cell origin. T-cell-derived populations that express 8D6 include Jurkat, HSB2, Peer, Molt 4 and Molt 13. Other cells that bind 8D6 include UM-Mel-1, a melanoma line, and HepG2, a hepatoma line. This antigen is also expressed on synovial T cells and synoviocytes.

Three-color flow cytometry analysis was performed to more precisely compare expression of CD147/neurothelin on thymocytes and peripheral blood T cells (Figure 2). The use of monoclonal antibodies to CD3 and CD4 or CD3 and CD8 permitted selection of high CD3 (CD4⁺CD8⁻ or CD4⁻CD8⁺), mature thymocytes, with CD3 density comparable to peripheral blood T lymphocytes. This approach also permitted comparison of expression of 8D6/neurothelin on the CD4 and CD8 subsets. The results shown in Table 1 indicate that mature CD4⁺ and CD8⁺ thymocytes have a

PBMC's



Thymocytes

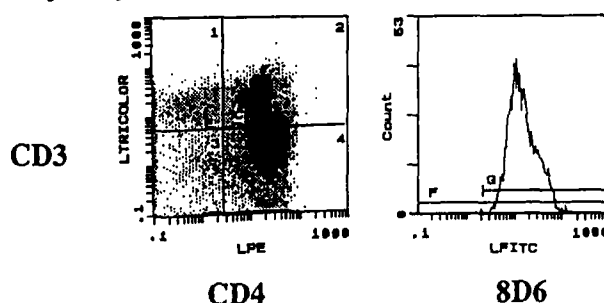


Figure 2. Three-color analysis of monoclonal antibody 8D6 reactivity with CD4⁺ thymocytes and T cells. Histograms of thymocytes and peripheral blood mononuclear cells stained directly with tricolor-conjugated anti-CD3 and phycoerythrin-conjugated anti-CD4, and indirectly with monoclonal antibody 8D6. **Left.** Two-color histograms that identify the CD3⁺CD4⁺ subset in quadrant 2. The tricolor (CD3) background cursor is set to exclude low CD3⁺ (stage II) thymocytes. **Right.** Single-color histograms of 8D6 fluorescence gated on cells in quadrants 2 of adjacent panels.

Table 1.
Relative binding of antibodies to thymocyte and peripheral blood T-cell subsets

Antibody	Cell type	Percentage positive	Net mean fluorescence (background subtracted)
8D6	CD3hi,CD4 ⁺ thymocyte	99.4	15.515
8D6	CD3 ⁺ ,CD4 ⁺ PBMC	89.7	4.214
8D6	CD3hi,CD8 ⁺ thymocyte	99.6	13.176
8D6	CD3 ⁺ ,CD8 ⁺ PBMC	95.1	4.46
CD98	CD3hi,CD4 ⁺ thymocyte	53.4	2.355
CD98	CD3 ⁺ ,CD4 ⁺ PBMC	16.4	0.884
CD98	CD3hi,CD8 ⁺ thymocyte	63.0	2.816
CD98	CD3 ⁺ ,CD8 ⁺ PBMC	50.6	1.52

Net mean fluorescence intensity (in arbitrary units) was calculated from single-color fluorescence histograms of CD147 (8D6) and CD98 staining, gated on thymocyte and peripheral blood T-cell subsets, as in Figure 2.

higher expression of both CD147/neurothelin antigen and the CD98 antigen (formerly termed the 4F2 antigen) than peripheral blood T lymphocytes. This is most apparent when comparing the net mean fluorescence (Table 1). This suggests that the expression of this antigen is developmentally regulated, decreasing as thymocytes mature to become peripheral blood T cells.

Expression of 8D6/neurothelin during short-term activation

Peripheral blood mononuclear cells in short-term culture augment IL-2 receptor expression in the presence of various mitogens, including PHA, PMA ionomycin or a combination of ionomycin and PMA. In contrast, expression of 8D6/neurothelin decreases slightly by day 4, as judged by net mean fluorescence (Figure 3). Similar results were obtained in the "blind panel" analysis conducted by the Sixth Leukocyte Differentiation Antigen Workshop (7). In studies using T-cell lines maintained in medium containing IL-2, expression of CD147 was found to gradually increase after day 5 of culture, and high expression is found on long-term activated cells (data not shown).

Functional assays

The effects of 8D6 on thymocytes and peripheral blood mononuclear cells were examined by coculturing cells with PHA, PMA, IL-2, anti-CD2, anti-CD3 and anti-CD28. No consistent inhibitory or costimulatory effects of the 8D6 antibody on T-cell or thymocyte proliferation under these conditions were found (data not shown). 8D6 did not appear to affect apoptosis in cultured thymocytes (data not shown).

Comparison with AD2

The monoclonal antibody AD2 has been reported to recognize an antigen of similar molecular weight to the molecule immunoprecipitated by antibody 8D6 (7). The binding of 8D6 and AD2 to various cell types was compared. 8D6 and AD2 both bind at high density to the T-cell lymphoma line, Jurkat (99.7% and 99.5%, respectively). However, AD2 bound to the T-cell lymphoma lines HSB-2 and Hut78 at much lower density than 8D6. Studies performed during the Sixth Workshop clustered AD2 to a different novel antigen termed CD165 (7).

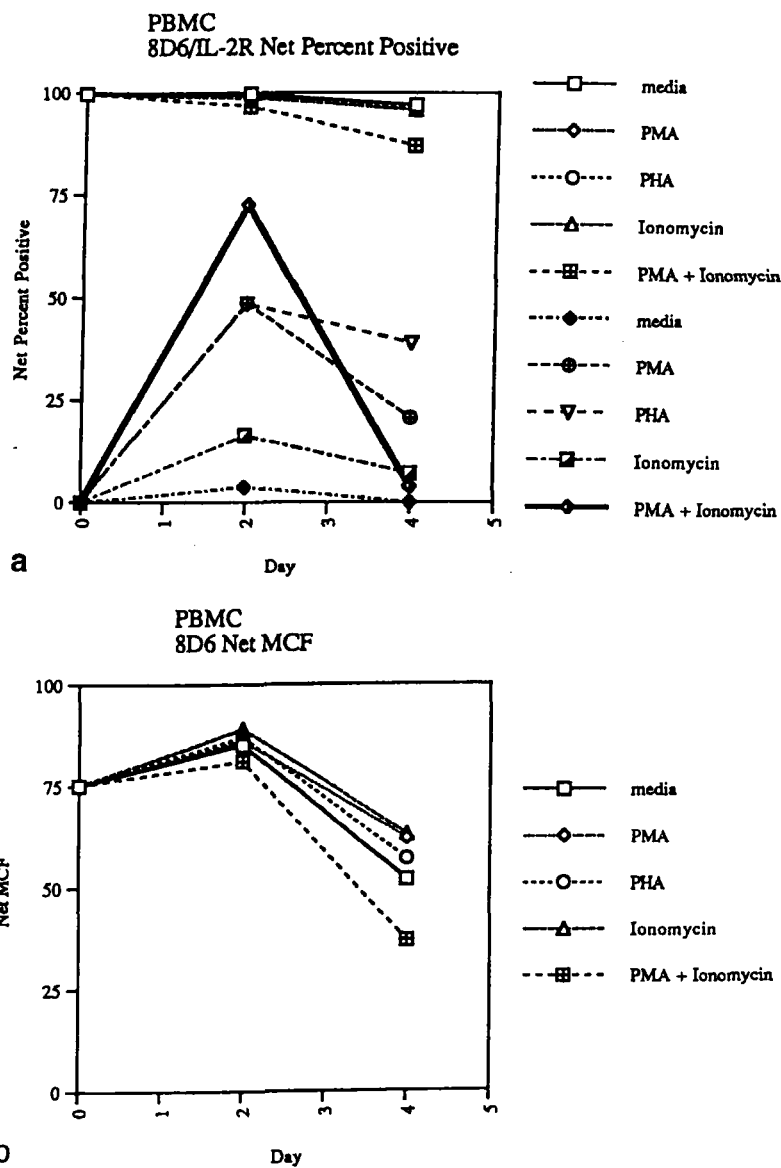
Discussion

Reactivity of the novel monoclonal antibody 8D6 with CD147/neurothelin has been established by binding to purified soluble protein, specific binding to transfectants, and dendrogram clustering in the Sixth Leukocyte Differentiation Antigen Workshop analysis. Immunoprecipitation of radio-labeled HSB-2 cells reveals a single band, which increases slightly in apparent molecular weight upon reduction, consistent with the intrachain disulfide bonds in the immunoglobulin-like regions of CD147. CD147/neurothelin is a highly glycosylated protein with a polypeptide core of 27.4 kDa. The apparent molecular weight of immunoprecipitated material from different cell sources varies, with sizes ranging from 35 to 60 kDa. It is likely that the antigen expressed on HSB-2 cells is somewhat less glycosylated than on other cell populations, given the relatively lower molecular weight and the relatively narrow band resolved by SDS-PAGE. In contrast, immunoprecipitation of biotin labeled protein from Jurkat cells yielded a broad band in the range of 50–60 kDa with a slight apparent shift to a higher molecular mass upon reduction (data not shown).

During the course of these studies, a monoclonal antibody termed AD2 was described with similar biochemical characteristics (8). However, cross-blocking studies and comparison of flow cytometric patterns on various cell lines indicated that AD2 did not recognize the same antigen as 8D6. Subsequently, the workshop analysis has clustered AD2 to a distinct novel CD designation, CD165 (7).

CD147/neurothelin, although described initially as a tumor cell antigen and as a component of the blood-brain barrier, is expressed widely on hematopoietic cells, with high expression on all cell lines examined. The data in this report demonstrate a particularly interesting pattern of expression on human T-lineage cells, with high-inten-

Figure 3. Expression of 8D6/neurothelin during lymphocyte cultures, as analyzed by flow cytometry. A. Net percentage of cells positive for 8D6 (upper 5 lines and symbols) or CD25/IL-2R (lower five lines and symbols). B. Mean channel fluorescence (MCF) for 8D6/neurothelin, expressed in arbitrary units.



sity expression on thymocytes and somewhat lower levels of expression on mature peripheral blood T cells. When mature single-positive CD4⁺ or CD8⁺ thymocytes were compared with the corresponding peripheral blood T-cell subset, three-fold or greater differences in antigen density were noted. A similar, although less striking, difference in antigen density was noted for CD98. Other T-cell differentiation antigens, such as CD3 and CD6, are expressed less strongly on thymocytes than on mature T cells. It is not known yet whether cord blood T cells resemble thymocytes or resemble T lymphocytes from adult peripheral blood in terms of expression of CD147 and CD98. Although expression of CD147 is strong on long-term activated T cells and T-cell lines, it does not appear to be induced early in the course of T-cell activation in a manner comparable to the CD25 subunit of the IL-2 receptor or the CD98 antigen.

The regulated expression of CD147 during T-cell differentiation strongly suggests a function or functions for this molecule that may be specific to particular points in T-cell development. Studies thus far using the 8D6 monoclonal antibody have not yielded information as to the precise nature of the role of this structure in T-cell biology. Thus, 8D6 was neither mitogenic nor comitogenic for T cells and failed to inhibit T-cell proliferative responses. Furthermore, it did not induce or prevent apoptosis of cultured human thymocytes. It has been speculated that CD147 may mediate adhesive function in certain cells, although no data to support this are yet available for lymphocytes. Designation of neurothelin to a new specific CD cluster and definition of a variety of monoclonal antibodies reactive with this molecule should now lead to progress in understanding its role during lymphocyte development and activation.

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Address:
David A. Fox
Division of Rheumatology
University of Michigan
Room 3918
Taubman Center
P.O. Box 0358
Ann Arbor, MI 48109-0358
USA
Fax +1 313-763-2025