SIALOPHORIN, A SURFACE SIALOGLYCOPROTEIN
DEFECTIVE IN THE WISKOTT-ALDRICH SYNDROME, IS
INVOLVED IN HUMAN T LYMPHOCYTE PROLIFERATION

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It has been well established that T lymphocytes can be triggered by antigen interacting with the T cell antigen receptor (TcR)1 or by antibodies binding to the associated structure CD3. Recent findings (1) suggest the existence of alternative pathways of T lymphocyte activation. Certain mAbs to the cell surface molecule CD2 (T11, LFA-2) activate T lymphocytes. T lymphocyte activation is dependent on the epitope recognized by the anti-CD2 mAb. mAbs to a number of other surface antigens provide a costimulatory signal. mAbs to CD5 (Tp67) (2) and CDw28 (Tp44) (3) augment T lymphocyte proliferation and IL-2 production. In the presence of PMA, mAbs to Tp44 stimulate purified T lymphocytes directly (3). We report here that an mAb to the surface protein sialophorin stimulates T lymphocyte proliferation in a manner similar to mAbs to CD3 and CD2.

Sialophorin, previously called gpL115, was found to be deficient in quantity and/or defective in lymphocytes of patients with the X-linked immunodeficiency Wiskott-Aldrich syndrome (4–6). A molecule indistinguishable from sialophorin of normal lymphocytes was purified to homogeneity from cells of the lymphoblastoid cell line CEM (6, 7). Sialophorin of CEM cells consists of >50% carbohydrate, primarily ~100 O-linked units of sialic acid, galactose, and galactosamine, and a single ~520-amino-acid polypeptide chain rich in serine, threonine, and proline (7, 8).

In this report, the mAb L10 which recognizes a surface epitope of sialophorin (6) was used to investigate the expression and the function of the molecule. Fluorescent staining with mAb indicated that sialophorin is expressed on all cells in the thymus and on a fraction of bone marrow and peripheral blood cells. Functional studies demonstrate that L10 mAb triggers the proliferation of

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1 Abbreviations used in this paper: FITC-GAM, fluorescein-conjugated goat F(ab')2 anti-mouse antibody; TcR, T cell antigen receptor.

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peripheral blood T cells. T lymphocyte stimulation by L10 mAb is dependent on the presence of monocytes. The possibility that sialophorin provides another pathway of T lymphocyte activation is discussed.

Materials and Methods

Culture Medium. Cells were grown in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco Laboratories, Grand Island, NY), 10 mM Hapes (Sigma Chemical Co., St. Louis, MO), and 25 μM 2-ME (Eastman Organic Chemicals, Rochester, NY). The long-term cytolytic T lymphocyte (CTL) line was maintained in 10% human conditioned medium.

IL-2-containing Human Conditioned Medium. IL-2-containing conditioned medium was prepared as described (9) by culturing washed mononuclear cells isolated from plateletpheresis residue at 3 × 10^6/ml in RPMI 1640 with 3% FCS, 0.15% PHA (Bacto PHA-P; Difco Laboratories, Inc., Detroit, MI), 2.8 μM indomethacin (Sigma Chemical Co.), 3 mM lithium chloride, and 50 μM hydroxyurea (Sigma Chemical Co.). The conditioned medium was collected after 72 h and subjected to fractional precipitation with ammonium sulfate. The 50–75% saturated ammonium sulfate fraction was dialyzed, filter-sterilized, and stored at −20°C.

Cell-Surface Immunofluorescence. Cells were washed twice with PBS containing 2.5% FCS and 0.02% sodium azide. In dual parameter analysis, the cells were incubated on ice for 30 min with excess fluorescein- and phycoerythrin-conjugated mAb. The cells were washed three times, fixed in 1% paraformaldehyde and examined on a FACS I analyzer (Becton Dickinson & Co., Mountain View, CA). In single parameter analysis, the cells were incubated with mAbs, washed and incubated with a 1:10 dilution of fluorescein-conjugated goat F(ab')2 anti-mouse IgG antibody (FITC-GAM, Tago, Burlingame, CA) on ice for 30 min and were washed and fixed.

Proliferation Assay. Peripheral blood mononuclear cells were isolated by Ficoll/Hypaque centrifugation (Lymphocyte Separation Medium; Bionetics Laboratory Products, Charleston, SC). Threefold dilutions of cells were cultured in triplicate in round-bottom microtiter wells (Linbro Chemical Co., Hamden, CT). mAbs were added at the start of the cell culture. The cells were pulsed with 1 μCi [3H]thymidine per well for 24 h before harvest at 72, 96, or 120 h.

Treatment with mAbs and Complement. Peripheral blood or bone marrow mononuclear cells isolated by Ficoll/Hypaque centrifugation were incubated at 2 × 10^7/ml with 10 μg/ml mAb on ice for 30 min. The cells were pelleted and incubated with rabbit complement (Pel-Freeze Biologicals, Rogers, AR) at a 1:3 dilution for 1 h at 37°C and then washed. The efficiency of complement lysis was assessed by trypan blue exclusion and immunofluorescence analysis.

Monoclonal Antibodies. Hybridoma cells producing mAbs were grown as ascites in BALB/c mice. The antisialophorin mAb L10 is an IgG1 which recognizes native and sialidase-treated sialophorin (6). MMA mAb recognizes the surface molecule MMA on phagocytes (10). The mAbs defining cell surface molecules associated with T cell activation, 4F2 (11) and TS2/7 (anti-VLA-1; reference 12), were used at a 1:200 dilution of hybridoma ascites. Other mAbs were obtained as follows: OKT3, OKT11 (Ortho Pharmaceutical, Raritan, NJ) and Leu-1, Leu-3a, Leu-8, Leu-11, Leu-12, Leu-15, Leu-M5, anti-II-2-R, and anti-HLA-DR (Becton Dickinson & Co.). Where indicated, IgG was purified from ascites by protein A–Sepharose chromatography (15).

Preparation of F(ab')2 Antibodies. F(ab')2 fragments were prepared from purified IgG as described (13). Briefly, 200 μl of papain-Sepharose (gift of Dr. S. Herrmann, Dana-Farber Cancer Institute, Boston, MA) was activated with 50 mM cysteine hydrochloride (Sigma Chemical Co.) for 30 min at 37°C. Papain-Sepharose was washed and incubated with purified mAb IgG for 6 h at 37°C. The resin was removed by centrifugation and Fc fragments and intact IgG were separated from F(ab')2 antibodies by Protein A–Sepharose chromatography.
FIGURE 1. Surface expression of sialophorin on peripheral blood mononuclear cells assessed by dual parameter immunofluorescence. Peripheral blood mononuclear cells were stained with FITC-conjugated L10 mAb (5 μg/ml). Phycoerythrin-conjugated mAb added at 5 μg/ml for the second parameter were: anti-CD4 (Leu-3a), anti-CD8 (Leu-2), anti-CD19 (Leu-12), anti-CD16 (Leu-11), anti-CD11 (Leu-15), and Leu-M3.

Monocyte Purification. Leukocytes from plateletpheresis residues were washed in culture medium and fractionated by Ficoll/Hypaque centrifugation. The mononuclear cells at 5 x 10⁷/ml in culture medium were placed on a rocker for 30 min at 4°C. Aggregated cells were isolated using unit gravity sedimentation (14). These cells were underlayered with 3 ml cold FCS and harvested after 15 min of unit gravity sedimentation. The pelleted cells were incubated at 10⁷/ml in 75-cm² culture flasks at 37°C for 30 min. The adherent cells were vigorously washed with culture medium. Culture medium containing 20% FCS was added to the remaining adherent cells, which were harvested after culture for 12 h. These purified monocytes were used to reconstitute autologous monocyte-depleted PBMC in proliferation assays.

Results

Distribution of Sialophorin. Previous studies (6) used surface radioiodination to demonstrate sialophorin expression on the surface of lymphocytes and CEM lymphoblastoid cells. To further investigate the distribution of sialophorin, we performed dual-parameter immunofluorescence analysis of peripheral blood cells. Staining of freshly isolated blood mononuclear cells with fluorescein conjugates of the antisialophorin mAb L10 demonstrated that sialophorin is expressed on both CD4⁺ (T4, Leu-3a) and CD8⁺ (T8, Leu-2) cells (Fig. 1) as well as all CD2⁺ (T11, Leu-5) and CD5⁺ (T1, Leu-1) T lymphocytes (data not shown). L10 staining was also observed on some peripheral B lymphocytes defined by the B cell mAb Leu-12 (anti-CD19) (Fig. 1). Thus, staining with L10 indicates that sialophorin is expressed on all T lymphocytes and on a subset of B lymphocytes.

In addition, L10 mAb stained 50% of CD11⁺ (Leu-15) peripheral blood mononuclear cells (Fig. 1). The CD11 antigen, defined by Leu-15/OKM1/Mo1 mAb, is expressed on monocytes, NK cells, and some T lymphocytes (15, 16). Sialophorin expression on NK cells is indicated by the detection of a population of L10⁺ cells that stained with an mAb to the NK marker CD16 (Leu-11 mAb, anti-Fc receptor) (Fig. 1). Peripheral blood mononuclear cells that stained with the monocyte marker mAb LeuM3 did not stain with L10 (Fig. 1).

L10 mAb staining indicated that sialophorin is expressed on virtually all
ANTISIALOPHORIN ANTIBODY ACTIVATES T LYMPHOCYTES

TABLE I

Surface Expression of Sialophorin

| Cells          | Positive | MFI |
|----------------|----------|-----|
| Thymocytes     | 92       | 29.9|
| Bone marrow    | 29       | 22.5|
| PBMCs          | 62       | 33.0|

Cells were incubated with L10 mAb followed by FITC-labeled second antibody. The extent of binding of mAb is expressed as mean fluorescence intensity (MFI) after subtraction of blank fluorescence (cells without mAb; 3.7–6.4 MFI). Thymocytes were obtained from donors <1 yr old undergoing cardiac surgery. Bone marrow cells obtained from normal adult volunteers were depleted of T cells by treatment with Leu-1 mAb (anti-CD5) and complement.

TABLE II

Effect of L10 mAb on Cytolytic Activity

| L10 mAb (μg/ml) | Cytolytic activity |
|-----------------|-------------------|
|                 | NK effector cells | CTL effector cells |
| 0               | 52                | 38               |
| 5               | 60                | 47               |
| 10              | 61                | 55               |
| 30              | 62                | 56               |

Cytolytic activity is expressed as percent specific ⁵¹Cr release in 4 h. The NK effector cells, freshly isolated peripheral blood mononuclear cells, were tested at 100:1 against K562 target cells and the CTL line at 12:1 against JY cells (HLA-A2; B7; DR4,6). The CTL effector line was a long-term line maintained by weekly stimulation with irradiated JY cells and conditioned medium containing IL-2.

thyrmocytes (Table I). To investigate sialophorin expression on bone marrow cells, the total population was depleted of T cells by treatment with Leu-1 mAb (anti-CD5) and complement. The residual bone marrow cells were CD2⁻ and CD3⁻. However, 29% of the residual bone marrow cells were L10⁺, indicating expression of sialophorin (Table I).

L10 mAb did not stain erythrocytes or other mesenchymal cells, such as primary fibroblasts and a rhabdomyosarcoma cell line RD (data not shown).

L10 mAb Stimulates the Proliferation of T Lymphocytes. The function of several surface molecules, including LFA-1, CD4, and CD8, was demonstrated by the ability of appropriate mAbs to inhibit CTL and NK function. In the initial functional evaluation of sialophorin, it was observed that, rather than inhibiting CTL and NK function, L10 mAb augmented cytolytic T cell activity (Table II). The marginal stimulation of T cells and NK cells in cytotoxicity assays suggested that L10 mAb might be stimulatory for T lymphocytes.

Purified L10 mAb was compared with mAbs recognizing other T cell surface antigens for their ability to stimulate T cell proliferation. In multiple experiments, mAbs recognizing the T cell antigens sialophorin and CD3 stimulated >10-fold increase in [³H]thymidine incorporation. In contrast, mAbs recognizing CD2, CD4, CD5, CD8, and Leu-8 antigens did not stimulate [³H]thymidine
incorporation (Fig. 2). L10 mAb was comparable to anti-CD3 mAb in its ability to stimulate proliferation. Both L10 mAb and anti-CD3 mAb stimulated maximal [³H]thymidine incorporation at 72 h (Fig. 3). The concentration of L10 mAb required for optimal proliferation was 100 ng/ml. Anti-CD3 mAb was more efficient than L10 mAb at stimulating proliferation in the range of 10–100 ng/ml (Fig. 4).

The distribution of sialophorin molecules indicated that L10 could be stimulating NK cells or B cells, in addition to T cells. To identify the peripheral blood mononuclear cells stimulated by L10, we used mAbs and complement to deplete various populations. Treatment of peripheral blood mononuclear cells with the
Table III

Monocyte Dependence of L10 mAb-Stimulated T Cell Proliferation

| Exp. | Responding cells                          | [³H]Thymidine incorporation (cpm x 10⁴) |
|------|------------------------------------------|---------------------------------------|
|      |                                          | No mitogen  | OKT3 mAb | L10 mAb |
| A    | PBMC                                     | 0.9        | 34.2     | 39.3    |
|      | PBMC, T-depleted                         | 0.5        | 0.9      | 0.8     |
| B    | PBMC                                     | 1.4        | 47.1     | 41.7    |
|      | PBMC, monocytes depleted                 | 3.7        | 7.5      | 12.8    |
|      | PBMC, monocytes depleted, monocytes added| 2.2        | 58.4     | 66.1    |

[³H]Thymidine incorporation (24-h pulse) is presented as cpm x 10⁴ assessed after 72 h of culture. The mean of triplicate values is shown; variance was usually ±10%. Depletion of T cells by treatment with Leu-1 mAb and complement was ≥98% complete, as demonstrated by Leu-1 mAb fluorescent staining. Monocytes were depleted using MMA mAb and complement. Where indicated, 10% purified monocytes were added to the monocyte-depleted cultures. Intact OKT3 and L10 mAb were at 100 ng/ml.

Pan-T cell mAb Leu-1 (anti-CD5) and complement completely abrogated the proliferative response to L10 mAb, indicating that peripheral blood T lymphocytes were responsible for [³H]thymidine incorporation (Table III, Exp. A). Similar results were obtained when a combination of anti-CD3 and anti-CD2 mAb and complement were used for cell depletion (data not shown).

Monocyte Dependence of L10-stimulated Proliferation. When monocytes were depleted from peripheral blood mononuclear cells by treatment with MMA mAb and complement, L10-stimulated proliferation was not observed (Table III, Exp. B), indicating that monocytes are required for stimulation of T lymphocytes by L10 mAb. This finding suggested that monocytes are needed for monokine production and/or Fc receptor binding of L10 mAb. F(ab')2 fragments of L10 mAb were found to be 50-fold less effective than intact L10 IgG in stimulating T cell proliferation (Fig. 4), suggesting that an important contribution of monocytes is binding of intact L10 mAb via the Fc receptor. The addition of supernatants from PHA-activated mononuclear cells did not restore L10-dependent proliferation to monocyte-depleted T cells (data not shown), suggesting that the monocyte dependence is not due solely to a requisite monokine.

Expression of Activation Antigens after L10 Stimulation. To further investigate the activation of T lymphocytes by L10, the surface expression of T cell activation antigens was evaluated 5 d after stimulation. The activation antigens HLA-DR, IL-2-R, and 4F2 were induced by L10 mAb, by the anti-CD3 mAb OKT3, and by PHA (Table IV). None of these agents induced the expression of the late activation antigen VLA-1. Thus, the induction of early activation antigens by L10 mAb is similar to the induction by anti-CD3 mAb. The anti-CD3 mAb OKT3 effectively modulated the CD3 antigen. In contrast, no modulation of the sialophorin antigen was observed at 5 d.

Discussion

Fluorescent staining with the mAb L10 demonstrated that the heavily glycosylated surface protein sialophorin is expressed on all T lymphocytes in peripheral
Peripheral blood mononuclear cells were incubated with PHA-P (3 μg/ml), OKT3 mAb (100 ng/ml), and L10 mAb (100 ng/ml) for 5 d. Expression of surface antigens is presented as the MFI of cells stained with the detecting mAb and FITC-GAM with blank fluorescence (cells without mAb) substracted. Parallel cell cultures that were washed twice 48 h before harvest to remove surface-bound antibody gave similar results (data not shown).

The time course and magnitude of mAb-induced T lymphocyte proliferation are similar for sialophorin and CD3. In addition, both molecules require the Fc portion of the antibody for stimulation. Either substitution of F(ab')2 mAb or depletion of monocytes significantly diminished the proliferative response of T lymphocytes to L10 mAb. The Fc region may be required for binding the mAb to monocytes and presenting it to T cells. As suggested by others (17, 18), mAb binding by the monocyte Fc receptor may be required to facilitate clustering or aggregation of the corresponding surface antigen.

Surface expression of the activation antigens HLA-DR, IL-2-R, and 4F2 provided an additional measure of T cell activation. The magnitude and time course of expression of activation antigens was similar for cells stimulated by PHA, anti-CD3 mAb, and L10 mAb. The induction of IL-2-R by all three agents suggests a common pathway dependent on IL-2 growth factor.

The failure of the dual-parameter mAb staining experiments to detect sialophorin on peripheral blood Leu-M3+ cells is not readily explainable since another study detected sialophorin expression on isolated monocytes using the technique of immunoblotting with L10 mAb (19). Immunoblotting analysis with L10 mAb also demonstrated sialophorin expression on blood neutrophils, lymphocytes, and platelets (19).

The chemical characteristics of the sialophorin molecule are similar to those of the rat surface molecule defined by W3/13 mAb (20, 21). Like sialophorin, W3/13 antigen (also called leukocyte sialoglycoprotein) is a heavily glycosylated single-chain polypeptide rich in serine, threonine, and proline with multiple O-linked carbohydrate units of sialic acid, galactose, and galactosamine (21). W3/13 mAb was found to stain all thymocytes, T (but not B) lymphocytes, bone marrow neutrophils, and brain cells (20).

Highly glycosylated surface proteins resembling sialophorin have been de-
tected by others on human lymphoid cells (reviewed in references 7 and 8). Expression of the human sialophorin-like molecule L-LSGP was detected by fluorescent mAb staining on most thymocytes, virtually all T4+ and T8+ peripheral blood lymphocytes, and 25% of circulating B lymphocytes, but not on peripheral blood monocytes, neutrophils, and platelets (22).

The association of sialophorin with stimulation of T lymphocytes, on the one hand, and, on the other hand, the defect of sialophorin in lymphocytes of Wiskott-Aldrich syndrome patients suggest intriguing possible relationships. The Wiskott-Aldrich syndrome is an X-linked trait characterized clinically by both lymphocyte and platelet dysfunction. Successful bone marrow transplantation indicates that the defect resides in lymphohematopoietic cells (23–26). Structural correlates of the T cell functional defect have been identified in Wiskott-Aldrich syndrome. At the cellular level, gross abnormalities of lymphocyte surface architecture were identified (27) and, at the molecular level, lymphocytes from Wiskott-Aldrich patients were found to be deficient in sialophorin and/or to express defective molecules (5, 6). The deficiency/defect of the sialophorin molecule in Wiskott-Aldrich syndrome cells and the capacity of sialophorin to function in normal T cell proliferation as reported here suggest the involvement of sialophorin in the pathogenesis of the Wiskott-Aldrich syndrome.

In conclusion, sialophorin has the capacity to function in T cell activation. The antisialophorin mAb L10 stimulates T cell proliferation in a manner similar to anti-CD3 mAb. Remaining questions concern the identity of the putative physiological ligand for sialophorin, the mode of transmembrane signaling by sialophorin, and the role of sialophorin defects in the Wiskott-Aldrich syndrome.

Summary

The mAb L10 was used to determine the distribution and the function of sialophorin, the heavily glycosylated surface molecule that is deficient/defective in lymphocytes of patients with the X-linked immunodeficiency Wiskott-Aldrich syndrome. Dual-parameter FACS analysis indicated that sialophorin is expressed on CD4+ and CD8+ lymphocytes, on a subpopulation of peripheral blood B lymphocytes, on all thymocytes, and on a subpopulation of bone marrow cells. Functional studies demonstrated that L10 mAb stimulates the proliferation of peripheral blood T lymphocytes as measured by stimulation of [3H]thymidine incorporation. The time course and magnitude of increased [3H]thymidine incorporation by T lymphocytes in response to L10 mAb paralleled that induced by anti-CD3 mAb. Effective stimulation was dependent on the presence of monocytes and the Fc portion of L10 mAb. Stimulation of lymphocytes by L10, like stimulation by anti-CD3 mAb, involves increased expression of 4F2, HLA-DR, and IL-2-R. These observations suggest that sialophorin functions in T cell activation.

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