Altered O-Glycan Synthesis in Lymphocytes from Patients with Wiskott-Aldrich Syndrome

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Summary
The only molecular defect reported for the X-linked immunodeficiency Wiskott-Aldrich syndrome (WAS) is the abnormal electrophoretic behavior of the major T lymphocyte sialoglycoprotein CD43. Since the 70 to 80 O-linked carbohydrate chains of CD43 are known to influence markedly its electrophoretic mobility, we analyzed the structure and the biosynthesis of O-glycans of CD43 in lymphocytes from patients with WAS. Immunofluorescence analysis with the carbohydrate dependent anti-CD43 antibody T305 revealed that in 10 out of the 12 WAS patients tested increased numbers of T lymphocytes carry on CD43 an epitope which on normal lymphocytes is expressed only after activation. Other activation antigens were absent from WAS lymphocytes. Western blots of WAS cell lysates displayed a high molecular mass form of CD43 which reacted with the T305 antibody and which could be found on in vivo activated lymphocytes but was absent from normal unstimulated lymphocytes. To examine the O-glycan structures, carbohydrate labeled CD43 was immunoprecipitated and the released oligosaccharides identified. WAS lymphocyte CD43 was found to carry predominantly the branched structure NeuNAcα2→3Galβ1→3 (NeuNAcα2→3Galβ1→4GlcNAcβ1→6) GalNAcOH whereas normal lymphocytes carry the structure NeuNAcα2→3Galβ1→3 (NeuNAcα2→6) GalNAcOH. Only after activation NeuNAcα2→3Galβ1→3 (NeuNAcα2→3Galβ1→4GlcNAcβ1→6) GalNAcOH becomes the principal oligosaccharide on CD43 from normal lymphocytes. Analyzing the six glycosyltransferases involved in the biosynthesis of these O-glycan structures it was found that in WAS lymphocytes high levels of β1→6 N-acetyl-glucosaminyl transferase are responsible for the expression of NeuNAcα2→3Galβ1→3 (NeuNAcα2→3Galβ1→4GlcNAcβ1→6) GalNAcOH on CD43. The gene responsible for WAS has not yet been identified but the results presented in this study suggest that the primary defect in WAS may affect a gene which is involved in the regulation of O-glycosylation.

The X-linked, recessive immunodeficiency, Wiskott-Aldrich syndrome (WAS), is characterized by thrombocytopenia with reduced platelet volume and impaired aggregation, immunological defects affecting both humoral and cellular responses, and eczema (1). The T lymphocytes in WAS show an abnormal morphology (2), a reduced response in the mixed lymphocyte reaction (3), and a progressive depletion of the T lymphocyte dependent areas of spleen and lymph nodes leading to lymphopenia (4). The only molecular defect described so far in WAS is the lack or decreased expression of the major sialoglycoprotein of human T lymphocytes (= CD43, GPI15, leukosialin, sialophorin) (5, 6) and the...
posttranslational modifications may be involved in the altered expression of CD43 in WAS T lymphocytes.

CD43 is a heavily glycosylated integral membrane protein with 70-80 O-linked carbohydrate chains (6, 12-14). The structure of the O-glycans varies among different cell types (15) and changes during T lymphocyte activation (16). The carbohydrate structures have been shown to strongly influence the migration of this glycoprotein in SDS-PAGE indicating that in WAS T lymphocytes a change of the carbohydrate chains could be responsible for the aberrant behavior of CD43 on SDS-PAGE.

In the present study we undertook the analysis and characterization of the carbohydrate structures of CD43 from the PBL of patients with WAS and compared the structures and the biosynthesis of the glycans with those obtained from CD43 of normal resting and activated lymphocytes.

**Materials and Methods**

**Materials.** The rabbit anti-CD43 antiseraum and the mAb T305 (mouse IgG1) were generous gifts of Drs. S. Carlsson (Umeå, Sweden) and R.I. Fox (La Jolla, CA), respectively. The unlabeled and FITC- or PE-labeled anti-CD3, anti-CD25, and anti-CD71 antibodies were obtained from Ortho Diagnostics (Raritan, NJ) and from Becton Dickinson and Co. (Mountain View, CA). Unlabeled IOB8 (mouse IgG1, anti-CD23) was from Immunotech (Mar-selles, France). Human recombinant IL-2 was from Jansen Biochemicals (Beere, Belgium). PHA was from Wellcome (Darford, England). Tritiated sodium borohydride, UDP-[14C] N-acetyl-D-galactosamine (GalNAc), UDP-[3H]Gal, UDP-[3H]GlcnNAc, CMP-[3H] N-acetyl neuraminic acid (NeuNAc), and [methyl-14C] mol wt standards were from New England Nuclear (Boston, MA); GalNAc, GlcNAc, Galβ1-→3GalNAcα1-→O-Benzyl, bovine sub-maxillary mucin, asialo-bovine submaxillary mucin, and neuraminidase from Clostridium perfringens (type V) were purchased from Sigma Chemical Co. (St. Louis, MO); fixed Staphylococcus aureus (Pansorbin), neuraminidase from Vibrio cholerae and pronase were obtained from Calbiochem-Behring (La Jolla, CA). Sephadex G-50 was purchased from Pharmacia (Uppsala, Sweden), Bio-Gel P-4 from Bio-Rad Labs (Richmond, CA), Sep-Pak C-18 disposable reversed phase columns as well as a 5 μ C-18 reversed phase HPLC column (4.6 x 250 mm) were from Waters Chromatography Division (Milford, MA).

**Preparation of Peripheral Blood Lymphocytes.** Blood samples from WAS patients or healthy volunteers were collected in EDTA (12.5 mM final concentration) and mononuclear cells were prepared by centrifugation over a Ficoll-Hypaque (Pharmacia) gradient. For the biochemical analysis monocytes were removed by adherence to plastic dishes in RPMI 1640 with 10% FCS (Gibco BRL, Grand Island, NY). The monocyte deplete preparations usually contained more than 95% viable cells and between 80 and 85% CD3 positive cells. T lymphocytes were activated by incubation of PBMCs with 5 μg/ml PHA or 50 ng/ml anti-CD3 (OKT-3; Ortho Diagnostics) in RPMI 1640 supplemented with 10% FCS and 25 U/ml IL-2 for 5 or 6 d with one change of medium on day 3.

**Immunofluorescence Analysis.** For the fluorescence analysis 5 x 10^9 PBMC were incubated for 30 min on ice with the appropriate amounts of antibody as indicated by the suppliers in a total volume of 25 μl of PBS containing 2% BSA and 0.02% sodium azide. The isotype matched control antibody and T305 were unlabeled and were revealed by an affinity-purified, PE-labeled goat anti-mouse antibody (Nordic, Täby, Sweden). After three washes with PBS/BSA the cells were labeled for 30 min at 0°C with FITC-labeled anti-CD3 (Leu4). In parallel experiments all antibodies were also tested as single labels and compared with the double labeling. In both single and double labeling experiments the percentage of cells reacting with one given antibody was always found to be identical. Immunofluorescence analysis was carried out on a FACS® Star Plus (Becton-Dickinson and Co., Mountain View, CA) cytofluorimeter set up for double fluorescence analysis. Side and forward angle light scatter was used to exclude dead cells and monocytes identified in separate single labeling experiments by staining with propidium iodide and OKM3, respectively. The cells were gated on the CD3 positive population and the fluorescence histograms of the second gate are presented with the relative number of cells on a linear scale plotted vs. the relative fluorescence intensity on a logarithmic scale, both in arbitrary units.

**SDS-Polyacrylamide Gel Electrophoresis.** Cells were lysed at 4°C in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, and 0.5% NP-40 and centrifuged for 15 min at 15,000 g. The lysates (25-100 μg protein) were subjected to SDS-PAGE (18) on 7% polyacrylamide gels and the separated proteins transferred to nitrocellulose membranes (Schleicher & Schüll, Mainz, Germany) (19). CD43 was revealed either with the rabbit anti-serum or with T305 followed by affinity-purified second antibodies coupled to alkaline phosphatase (Cappel, Cochranville, PA). The bound antibody-enzyme complexes were visualized with bromochloroindoiy-phosphate and nitroblue-tetrazolium (Sigma Chemical Co.) as described (20). In some experiments the Western blots were incubated with [125I]-labeled protein G (New England Nuclear) followed by autoradiography. Immunoprecipitated samples were subjected to SDS-PAGE and gels were treated for fluorography with Enlightning (New England Nuclear). [Methyl-14C]methylated mol wt standards were myosin (200 kD), phosphorylase B (97.4 kD), BSA (69 kD), ovalbumin (46 kD), and carbonic anhydride (30 kD).

**Separation of O-linked Oligosaccharides Isolated from Leukosialin by Gel Filtration on Bio-Gel P-4 and HPLC.** Cell surface carbohydrates were labeled (21, 22) and CD43 analyzed as described (23). Glycopeptides were prepared from immunoprecipitated CD43 by pronase digestion at 45°C for 72 h. After preincubation of pronase (10 mg/ml) for 30 min at 37°C in 500 μl of 0.15 M Tris-HCI, pH 8.1, 1 mM CaCl2, three aliquots of 100 μg of pronase were added at 20 h intervals to each immunoprecipitate. Pronase-digested material was fractionated by passage over a column (1.0 x 5.50 cm) of Sephadex G-50 (superfine) equilibrated with 0.1 M NH4HCO3, at a flow rate of 5 ml/h. The radioactive fractions corresponding to the elution position of glycopeptides were pooled, concentrated and incubated in 50 mM NaOH, 1 M NaBH4, for 16 h at 45°C. The samples were then evaporated three times under nitrogen, after addition of 1 volume of 100 mM acetic acid in methanol, β-eliminated products were applied onto a Bio-Gel P-4 column (1.0 x 110.0 cm) equilibrated in 0.1 M NH4HCO3. The flow rate was 5 ml/h and each fraction contained 1 ml. An aliquot of each fraction was used for scintillation counting. The oligosaccharides separated by Bio-Gel P-4 were then analyzed by HPLC on a Lichrosorb-NH2 column (Merck, Darmstadt, Germany) as described previously (16). A Varian HPLC apparatus (Model 5000; Varian Associates, Inc., Palo Alto, CA) was used. Standard oligosaccharides were obtained from human glycoprotein (24) and from CD43 of HL-60 cells (15).

**Glycosyltransferase Assays.** Microsomes were prepared from 2-5 x 10^9 cells by suspending the cells in 15 mM Tris-HCl, pH 8.1, containing 350 mM sucrose, 5 mM EDTA and 2 mM PMSF and 1502 CD43 Glycosylation in Wiskott-Aldrich Syndrome
passing them repeatedly through a 23-gauge needle. Incompletely disrupted cells and nuclei were removed by centrifugation at 5,000 g for 10 min and the microsomes were pelleted at 100,000 g. The membranes were suspended in the homogenization buffer at a protein concentration of 10 mg/ml and stored at −20°C until used. α-N-acetyl-galactosaminyl transferase, β1→3 and β1→4 galactosyl transferases and α2→3 and α2→6 sialyl transferases were assayed as described (25–29). The β1→6 N-acetyl-galactosaminyl transferase assays followed the published procedures with minor modifications (30). The incubation mixture contained in a total volume of 25 µl 100 mM MES, pH 6.5, 10 mM MnCl₂, 0.05% Triton X-100, 100 mM GlcNAc, 2 mM ATP, 2.8 mM UDP-[3H]GlcNAc (20,000 cpm/nmol), 2 mM Galβ1→3GalNAcα1→O-Benzyl and 50, 100, or 150 µg microsome protein. After incubation for 30 min or 120 min at 37°C the reaction was stopped by the addition of water and the mixture passed over a column of Dowex 1 × 8 (Cl⁻). The flow through fraction was collected, diluted with an equal volume of 100 mM NH₄HCO₃, and applied to a Sep-Pak C-18 column. The column was washed with 100 mM NH₄HCO₃ followed by water and the bound benzylated oligosaccharides were eluted with 1.5 ml methanol and either counted directly in a liquid scintillation counter or the methanol was evaporated, the sample taken up in 100 µl of water containing 0.1% trifluoro-acetic acid and applied to a C-18 reversed phase HPLC column. The column was eluted at 1 ml/min with a mixture of acetonitrile and 17% water and the fractionstaken at 1-min intervals were counted for radioactivity.

### Results

**Immunofluorescence Analysis of Peripheral Blood Lymphocytes.** The patients analyzed in this study were diagnosed as having WAS by their severe thrombocytopenia associated with a reduced platelet volume, eczema, and immunodeficiency. Some patients had been splenectomized at the time of the analysis (Table 1) and some patients showed signs of autoimmunity. In the flowcytometric analysis the amount of T305 positive T lymphocytes was measured for patients and control samples. The T305 antibody recognizes a carbohydrate dependent epitope on CD43 expressed on activated and not on resting human T lymphocytes (31). In normal individuals only between 5 and 15% of the CD3 positive PBLs carry the T305 epitope whereas in 10 out of the 12 WAS patients tested the amount of T lymphocytes recognized by T305 was increased to between 22 and 74% (Table 1). Since the T305 antibody recognizes activated normal lymphocytes and since most patients suffered from infections at the time of analysis the presence of activation antigens was examined on the lymphocytes from patients who showed high levels of the T305 epitope. In all five patients tested neither the IL-2 receptor α chain (CD25) nor the transferrin receptor (CD71) were expressed in higher amounts than on normal resting T lymphocytes.

Unstimulated WAS lymphocytes (patient FB, Table 1), unstimulated normal lymphocytes, and in vitro activated normal lymphocytes were analyzed with antibodies to CD3 and to the IL-2 receptor α chain (CD25), the transferrin receptor (CD71) and with T305 (Fig. 1). In the unstimulated normal CD3⁺ lymphocytes <10% of the cells reacted with the T305 antibody whereas >40% of the unstimulated WAS CD3⁺ lymphocytes were stained with the T305 antibody. As expected, the T305 antibody bound to almost all of the in vitro activated normal T lymphocytes incubated for 5 d with PHA and IL-2. Although the unstimulated WAS T lymphocytes reacted with the T305 antibody, they were negative for the receptors for IL-2 and transferrin (Fig. 1, H and K) as well as for other activation antigens such as 4F2 and HLA-DR (not shown).

**Western Blot Analysis of CD43 from WAS Lymphocytes.** PBLs from WAS patients were analyzed by immunoblotting using a specific anti-CD43 antiserum. In all five patients (Figs. 2, A and C) the anti-CD43 antiserum revealed a band at 135 kD with variable intensity. Two patients (Fig. 2 A, lanes 3 and 5) revealed detectable bands only at 135 kD while two other patients (Fig. 2 A, lanes 2 and 4) showed additional bands of equal intensity at 100 kD and 80 kD, respectively. The fifth patient (Fig. 2 C, lane 1) had bands at 135 kD and at 120 kD. The PBLs of normal donors showed only a single band at 120 kD which corresponds to the molecular mass of CD43 in normal resting T lymphocytes (16). The proteins which are stained at 200 kD and 70 kD are not related to CD43 (12). To determine the molecular mass of CD43 on normal T lymphocytes activated in vivo, lymphocytes from the synovial fluid of a patient with rheumatoid arthritis were analyzed and revealed a broad CD43 band from 120 to 135 kD (Fig. 2 A, lane 6).

The murine mAb T305 has been shown to be specific for the high mol wt form (135 kD) of CD43 found in activated

### Table 1. Flowcytometric Analysis of Lymphocytes from WAS Patients and Normal Individuals

| Patient | Age (yr) | SE* | CD3* | T305* | CD25* |
|---------|---------|-----|------|-------|-------|
|         |         | (%) | (%) of | (%) of |       |
|         |         | CD3* cells | CD3* cells |       |       |
| FB      | 4       | +    | 65   | 40    | 1.5   |
| TC      | 2       | +    | 57   | 33    | 1.2   |
| FD      | 9       | -    | 65   | 47    | 2.0   |
| RH      | 2.5     | +    | 64   | 55    | -     |
| BL      | 4.5     | +    | 62   | 53    | -     |
| FM      | 0.2     | -    | 59   | 46    | -     |
| AO      | 4       | -    | 54   | 60    | -     |
| NO      | 10      | +    | 87   | 74    | 1.0   |
| AP      | 4       | -    | 51   | 15    | -     |
| MSB     | 1       | -    | 58   | 5     | -     |
| JS      | 3       | +    | 71   | 22    | -     |
| AY      | 4       | +    | 78   | 40    | 1.0   |
| Control | (N = 14)| -    | 60-80| 11 ± 6| <3   |

* SE: Spleenectomy.
Figure 1. Immunofluorescence analysis of WAS lymphocytes. Cells were analyzed by double immunofluorescence as described in Materials and Methods. The lymphocytes were gated for the CD3 positive cell population and the second fluorescence histograms are shown. Isotype matched control IOB8 (A, B, C), T305 (D, E, F), anti-CD25 (G, H, I), and anti-CD71 (J, K, L).
Figure 2. Western blots of total cell lysates of WAS lymphocytes. (A) anti-CD43 antiserum; Lane 1: normal lymphocytes, lanes 2-5: WAS lymphocytes, lane 6: synovial fluid lymphocytes from a patient with rheumatoid arthritis panel. (B) T305 (clone G9), same samples as in (A). (C) WAS lymphocytes, lane 1: anti-CD43, lane 2: T305.

T-lymphocytes (12, 31). As shown in Fig. 2, B and C, the T305 antibody reacted with the 135 kD band of CD43 of lymphocytes from WAS patients and of the synovial fluid lymphocytes from the patient with rheumatoid arthritis. The T305 antibody did not recognize the lower molecular mass forms of CD43, i.e. the band at 120 kD.

Immunoprecipitation of CD43 Labeled in its Carbohydrate Part. Normal and WAS PBLs were labeled in their carbohydrate portions through the oxidation of sialic acid or after removal of the sialic acid residues through the oxidation of galactose followed by the reduction with tritiated sodium borohydride. Immunoprecipitation of normal lymphocytes with the rabbit anti-CD43 antiserum gave a labeled band at 120 kD while the WAS lymphocytes gave a band of 135 kD (Fig. 3, lane 3). After removal of the sialic acid residues the apparent molecular mass of CD43 from the WAS patient shifted to about 170 kD (Fig. 3, lane 4) whereas normal asialo-CD43 migrated at 150 kD (Fig. 3, lane 2). Following carbohydrate labeling, immunoprecipitation of CD43 from WAS T lymphocytes revealed only one band from both native and sialidase-treated lymphocytes indicating that the material of lower molecular mass present on Western blots of lysates from some WAS patients (reference 9 and Fig. 2) is not present in the patient tested or that these lower molecular mass forms of CD43 do not contain sufficient amounts of carbohydrate to be detectable.

Figure 3. Immunoprecipitation of CD43 from normal and WAS lymphocytes. The intact cells were labeled by periodate oxidation of sialic acid and reduction with [3H] sodium borohydride or, after removal of sialic acid, by oxidation with galactose oxidase and reduction with [3H] sodium borohydride according to the published procedures (21, 22). CD43 was immunoprecipitated with the carbohydrate independent antiserum and analyzed on 7% polyacrylamide gels. Lanes 1 and 2 show immunoprecipitates from native and sialidase treated normal lymphocytes, and lanes 3 and 4 native and asialo CD43 from WAS lymphocytes.
Carbohydrate Analysis of CD43 from WAS Lymphocytes. The labeled oligosaccharides released from cell surface-labeled WAS lymphocytes were analyzed by gel filtration on Bio-Gel P-4 and by HPLC on a Lichrosorb-NH₂ column. Irrespective of whether the labeling occurred through the sialic acid residues of after removal of the sialic acid through galactose or N-acetylgalactosamine, the oligosaccharides released by alkaline borohydride treatment from immunoprecipitated CD43 from WAS lymphocytes displayed a different elution profile (Fig. 4) than the oligosaccharides obtained from normal lymphocytes. The major oligosaccharide from native or sialidase-treated WAS CD43 eluted in gel filtration earlier than the predominant oligosaccharide from normal CD43 indicating a higher molecular mass for the patient's oligosaccharide. In the HPLC system the sugars released from CD43 of WAS lymphocytes eluted later than those from normal lymphocytes indicating

Figure 4. Carbohydrate analysis of WAS CD43. Lymphocytes were labeled in their cell surface carbohydrates, CD43 immunoprecipitated, the oligosaccharides were released by alkaline/borohydride treatment and analyzed by gel filtration on Bio-Gel P-4 (A-C) and by HPLC on aminobonded silica (Lichrosorb-NH₂) (D-F). Oligosaccharide profiles from unstimulated normal (A and D) and WAS lymphocytes (B and E) and from normal lymphocytes cultured for 5 d in the presence of 50 ng/ml OKT 3 and 50 U/ml IL-2 (C and F) are shown. (•) Oligosaccharides from native and (○) from neuraminidase treated cells. Fractions marked by the bars (●) in A-C were pooled and analyzed by HPLC (D-F). The numbered arrows indicate the elution positions of oligosaccharide standards: (1) NeuNAcα2→3Galβ1→3(NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAcOH; (2) NeuNAcα2→3Galβ1→3(NeuNAcα2→6)GalNAcOH; (3) NeuNAcα2→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→6)GalNAcOH and Galβ1→3(NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAcOH; (4) NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAcOH and Galβ1→3(NeuNAcα2→6)GalNAcOH; (5) Galβ1→3(Galβ1→4GlcNAcβ1→6)GalNAcOH; (6) Galβ1→3GalNAcOH; (7) GalNAcOH.
again a more complex structure for the carbohydrates derived from WAS lymphocytes. In both analytical systems the elution profile of saccharides from WAS CD43 resembled closely the pattern of those obtained from activated normal T lymphocytes.

The major structure released from native WAS CD43 co-eluted from the gel filtration as well as from the HPLC column with the standard oligosaccharide NeuNAcα2→3Galβ1→4GlcNAcβ1 (69.0% of the relative amount). After removal of the sialic acid residues and labeling in the galactose moiety, the major oligosaccharide from WAS CD43 eluted at the same position as Galβ1→4GlcNAcβ1 (7.5%) in both gel filtration and HPLC. The relative amounts of labeled oligosaccharides released from CD43 from WAS as well as normal resting and activated lymphocytes are summarized in Table 2.

Biosynthesis of O-linked Carbohydrates in WAS Lymphocytes. To study the biosynthetic mechanisms underlying the change of O-glycan structure in WAS T lymphocytes, microsomal preparations of normal and WAS lymphocytes were analyzed for the enzymatic activities of six glycosyltransferases. These enzymes are known to be involved in the biosynthesis of O-linked carbohydrate structures and specific assay protocols have been published for each of the six transferases (25-30). The enzymatic activities were calculated from the radioactivity transferred from [14C]-labeled sugar nucleotide donor substrates to specific well defined acceptor substrates. The transfer rates were corrected for non-specific degradation and for the transfer to endogenous substrates in the enzyme preparations by subtracting the values obtained with control incubations from which the acceptor substrates were omitted. The mean transfer rates of two protein concentrations are shown in Table 3. Five of the six enzymes displayed comparable activities in resting normal and WAS lymphocytes. In contrast, the β1→6GlcNAc transferase was only barely detectable (0.05 nmol/h mg protein) in normal lymphocytes whereas its activity was comparable to that of other glycosyltransferases (0.4 nmol/h mg protein) in WAS lymphocytes. The labeled product of the β1→6 GlcNAc transferase reaction was characterized by HPLC analysis on a reversed phase column. The elution time of the product (22 min) corresponded to that

| Oligosaccharides                          | Resting normal PBL | WAS PBL | Activated normal PBL |
|------------------------------------------|--------------------|--------|----------------------|
| NeuNAcα2→3Galβ1→4GlcNAcβ1                | (%)                | (%)    | (%)                  |
| NeuNAcα2→3Galβ1→3GalNAcOH                | 4.9                | 63.0   | 48.0                 |
| Galβ1→4GlcNAcβ1                          | 7.5                | 10.6   | 20.0                 |
| Galβ1→3GalNAcOH                          | 18.5               | 8.0    | 13.0                 |

Table 2. Relative Amounts of Oligosaccharides Released from CD43

| Enzymes                                  | Lymphocytes        |
|------------------------------------------|--------------------|
|                                          | Normal | WAS  | (nmol/h mg protein) |
| Polypeptide: αGalNAc transferase         | 0.33   | 0.2  |
| GalNAc: β1→3 Gal transferase             | 0.17   | 0.26 |
| Galβ1→3GalNAc: β1→6 GlcNAc transferase  | 0.05   | 0.4  |
| GlcNAc: β1→4 Gal transferase             | 2.38   | 1.49 |
| GalNAc: α2→6 NeuNAc transferase          | 3.08   | 2.56 |
| Galβ1→3GalNAc: α2→3 NeuNAc transferase  | 0.17   | 0.2  |

Glycosyltransferase activities were assayed in duplicates at two different protein concentrations. Incubations were carried out at 37°C for 30 min. The acceptor substrates were as described in reference 16.
of the standard compound Galβ1→(GlcNAcβ1→6)GalNAcα1→O-benzyl (32) which is the structure synthesized by the β1→6GlcNAc transerase in the assay system used.

Discussion

The only molecular defect reported in the severe X-linked immunodeficiency, WAS, is the abnormal expression of CD43. Recently the cDNA coding for CD43 has been cloned and unambiguously localized on chromosome 16 (7, 8), indicating that a structural defect of the protein is unlikely to be the primary cause of the observed abnormalities of CD43 found in WAS T lymphocytes. CD43 is the heavily glycosylated major sialoglycoprotein of human T lymphocytes. Most mAbs developed against this glycoprotein recognize carbohydrate dependent epitopes on the molecule (33). The mAb T305 is unique since it recognizes specifically a sialic acid dependent epitope which is absent from normal resting T lymphocytes but is expressed on activated T lymphocytes (31, 34). The appearance of the T305 epitope on activated T lymphocytes and on several T cell lines coincides with the presence of a specific carbohydrate structure on the CD43 molecule (15). The large amounts of sialylated O-linked glycans on CD43 have a marked influence on its mobility in SDS-PAGE and the glycans changes observed between resting and activated normal T lymphocytes result in an increase in its apparent mol wt from 120 kD in resting T lymphocytes to 135 kD in activated T lymphocytes (16). Among the differently glycosylated CD43 molecules the mAb T305 recognizes only the high molecular mass form of CD43 and on all cell types tested the reactivity of T305 is associated with the presence of the structure NeuNAcα2→3Galβ1→3 (NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAccα1→Ser/Thr on human CD43 (15).

The immunofluorescence analysis of WAS lymphocytes revealed, in 10 out of the 12 patients analyzed, an increased percentage of T305+ T lymphocytes as compared to normal T lymphocytes. Although the T305 epitope on CD43 can be considered an activation antigen, the lymphocytes of the WAS patients were negative with other activation markers notably the receptors for IL-2 (CD25) and transferrin. The amount of T305 positive T cells in WAS patients is highly variable. However a majority of the patients studied showed significantly higher than normal levels of T305 reactive T cells. This heterogeneity of T305 levels is in agreement with earlier reports on CD43 in WAS lymphocytes (5, 6, 9). In addition, the Western blot analysis confirmed the previous reports (6, 9) showing abnormally migrating CD43 molecules in lymphocytes from WAS patients. Although most patients examined in these studies showed low Mr forms of CD43 a number of the patients exhibited also a band at 135 kD which was thought to be related to CD43. However, in this study all lysates from the five WAS patients analyzed showed one isoform of CD43 at 135 kD whereas only a single band of CD43 at 120 kD was revealed on blots from control cells. In all samples, only the high molecular mass form of CD43 reacted with the T305 antibody confirming the immuno fluorescence data from WAS lymphocytes and demonstrating that the T305 antibody recognizes an epitope on the CD43 molecule present on WAS T lymphocytes as well as on activated normal T lymphocytes.

One patient was studied in greater detail; his lymphocytes were isolated and the cell surface carbohydrates labeled, CD43 immunoprecipitated and the O-linked glycans isolated after alkali/borohydrate cleavage. The carbohydrate independent anti-CD43 antiserum immunoprecipitated only a single band from WAS lymphocytes which migrated in SDS-PAGE at 135 kD. Normal lymphocytes gave also a single band with a Mr of 120 kD. The difference in mobility between the immunoprecipitated CD43 molecules from WAS and normal T lymphocytes was not due to different amounts of sialic acid as had been suggested in a previous report (9) since the treatment of the cells with sialidase and the labeling of the underlying galactose residues did not abolish the difference in migration.

In the experiments described above the CD43 from WAS lymphocytes behaved at least in part like the CD43 from activated T cells of normal individuals indicating strongly that carbohydrate changes may be responsible for the altered behavior of CD43 in WAS lymphocytes. The structural analysis of the O-linked carbohydrates of CD43 confirmed this hypothesis. The native or desialylated O-glycans released from WAS CD43 eluted at the same positions as the oligosaccharides from CD43 from activated T lymphocytes and very little material could be found which corresponded to the oligosaccharides present on normal resting lymphocytes. From these results and from the comparison with the chromatographic behavior of well defined O-glycan standards it can be concluded that CD43 of resting WAS T lymphocytes carries increased amounts of the carbohydrate structure NeuNAcα2→3Galβ1→3(NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAcα1→Ser/Thr which is found on normal T lymphocytes only after they are activated.

In normal T cells the change from the simpler structure NeuNAcα2→Galβ1→3(NeuNAcα2→6)GalNAcα1→Ser/Thr on resting cells to the more complex saccharides NeuNAcα2→3Galβ1→3(NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAcα1→Ser/Thr on activated cells is brought about by the induced expression of a β1→6GlcNAc transerase and by the simultaneous decrease of the a2→6 NeuNAc transerase activity which competes with the β1→6GlcNAc transerase for the same acceptor substrate Galβ1→3GalNAcα1→Ser/Thr (16). The analysis of the glycosyltransferases involved in the biosynthesis of O-glycans in WAS T cells revealed that compared to normal resting T lymphocytes the amount of β1→6GlcNAc transerase was increased eight-fold. However, the a2→6 sialyltransferase showed about the same level of activity in WAS and normal lymphocytes. The results suggest that in WAS patients the increase of β1→6GlcNAc transerase alone is responsible for the change in the carbohydrate structures of CD43. A recent report describing the glycosyltransferase activities in T lymphocytes from seven WAS patients confirms that increased levels of β1→6GlcNAc transferase activities are associated with the WAS. Interestingly, in contrast to normal lymphocytes, WAS T cells appear to loose the β1→6 GlcNAc transerase activity during activa-
tion (35). This is in agreement with an earlier finding showing that EBV transformed lymphocytes from two WAS patients express lower levels of this enzyme than EBV transformed normal B lymphocytes (36).

The results described in this study present evidence that the O-linked carbohydrate structures on CD43 from WAS lymphocytes are altered as compared to O-glycans from normal resting T cells. The abnormal glycosylation of CD43 is the only molecular alteration presently defined in WAS. However, in addition, CD43 from WAS lymphocytes may be more rapidly degraded than normal CD43 since lower molecular mass forms of CD43 could be easily detected in WAS lymphocytes. Together these modifications could result in a complete absence of normally glycosylated CD43 on resting WAS T lymphocytes. The precise role of CD43 or of its carbohydrate is not known but several recent reports (37-40) indicate that CD43 may be involved in T cell activation.

The gene responsible for WAS has not yet been identified but the results presented in this study suggest that the primary defect in WAS may affect a gene which is involved in the regulation of O-glycosylation. The altered O-linked carbohydrate structures of CD43 as well as the increased levels of β1→6 GlcNAc transferase responsible for these structural changes found in resting WAS lymphocytes are present in normal T lymphocytes only after activation (16). Although activation antigens other than the O-glycan dependent T305 epitope on CD43 were absent from WAS lymphocytes it is conceivable that the glycan changes may indicate a partial or incomplete activation for WAS lymphocytes. Patients with WAS show evidence for defective T cell function (1) and normal activated T lymphocytes are transiently refractory to further stimulation through the T cell antigen receptor (41). The mechanism for the unresponsiveness of activated T lymphocytes is not known. The similarity of the CD43 glycosylation pattern in WAS lymphocytes and in normal activated T cells raises the possibility that their unresponsiveness is mediated through the glycosylation of CD43 or of another membrane glycoprotein by the α1→6GlcNAc transferase. Such abnormal glycosylation may result in or may be an indicator of a state of pseudoactivation, in which specific T lymphocyte responses are not possible.

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