The HB-6, CDw75, and CD76 Differentiation Antigens are Unique Cell-Surface Carbohydrate Determinants Generated by the \(\beta\)-Galactoside \(\alpha\),6-Sialytransferase

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Abstract. Expression of the \(\beta\)-galactoside \(\alpha\),6-sialyltransferase (\(\alpha\),6-ST) was shown to regulate the generation of multiple cell-surface differentiation antigens (Ags) that may be necessary for lymphocyte function. A new mAb was produced, termed HB-6, that was shown to identify a novel neuraminidase-sensitive cell-surface Ag expressed by subpopulations of human lymphocytes and erythrocytes. In attempting to isolate a cDNA encoding the HB-6 antigen by expression cloning, a cDNA encoding the \(\alpha\),6-ST (EC 2.4.99.1) was obtained. Since expression of the \(\alpha\),6-ST protein was shown to be limited to the Golgi apparatus, the cell-surface HB-6 Ag was demonstrated to be the product of \(\alpha\),6-ST activity. Interestingly, \(\alpha\),6-ST expression also generated two other neuraminidase-sensitive lymphocyte cell-surface differentiation Ags, CDw75, and CD76. The HB-6, CDw75, and CD76 mAb identified distinct Ags that were differentially expressed by different B cell lines and exhibited different patterns of expression in tissue sections. These results indicate that \(\alpha\),6-ST expression is a critical regulatory step in the formation of the Ags that are recognized by these mAb, and that an \(\alpha\),6-linked sialic acid residue is an essential component of each Ag. Thus, expression of a single ST can result in the generation of multiple distinct antigenic determinants on the cell surface which can be distinguished by mAb and may have regulatory roles in lymphocyte function.

The identification of leukocyte cell-surface structures, delineation of their primary amino acid sequences, and elucidation of their potential functions has been a major focus in the field of immunology during the last decade. Each cell-surface protein has been found to have a unique primary sequence that presumably directs function of the molecule. However, these proteins may also serve as scaffolds for the presentation of cell-surface carbohydrates. It is likely that cell-surface carbohydrates are also functional moieties with a broad potential for diversity not only in the proteins upon which they may appear, but also in the complexity of carbohydrate side chains possible (46, 49, 51). In this study, we have examined some of the potential diversity in three leukocyte cell surface antigens (Ag)\(^1\), HB-6, CDw75, and CD76.

The CDw75 Ag is a cell surface molecule expressed by the majority of blood B cells and a subpopulation of blood T cells (9). Four mAb were used to define CDw75 (see Table I): EBU-141 (9), LN1 (12), OKB-4 (39), and HH2 (55). LN-1 also reacts with red blood cell precursors (12). These CDw75 mAb are likely to identify spatially related structures since binding inhibition studies have shown that binding of each CDw75 mAb blocks the binding of the other CDw75 mAb (40). Several studies indicate that CDw75 is expressed only on mature sIg\(^+\) B-cells, with expression occurring later than sIg and ceasing during terminal differentiation into plasma cells (reviewed in 9). The CDw75 mAb also react weakly with about 30\% of peripheral blood T cells. A unique feature of CDw75 is its predominant expression by germinal center B cells (9, 12, 29, 42), with weaker expression by follicle mantle-zone B cells (9). The OKB-4 mAb immunoprecipitated a surface Ag of 53,000 \(M_\text{r}\) (40) in one study, but of 87,000 \(M_\text{r}\) in another (39), while other CDw75 mAb fail to precipitate defined structures. However, a cDNA as-

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1. Abbreviations used in this paper: \(\alpha\),6-ST, \(\beta\)-galactoside \(\alpha\),6-sialyltransferase; Ag, antigen; E\(^+\) PBMC, T cells isolated from nonadherent peripheral blood mononuclear cells by sheep erythrocyte rosette formation; E\(^-\) PBMC, nonadherent mononuclear cells that do not rosette with sheep erythrocytes.
associated with CDw75 expression was recently cloned by Stamenkovic et al. (56), who suggested that CDw75 is a cell surface β-galactoside α2,6-sialyltransferase (α2,6-ST).

The CD76 Ag is identified by two mAbs, HD-66 and CRIS-4, which react strongly with mature B cells and moderately with mature T lymphocytes (8, 36, 41). Within the B cell lineage, CD76 is not expressed on early B-cells, but is expressed on the majority of slgM+ B cells (8). Within the T cell lineage, the CD76 Ag is predominantly expressed on mature cells (8, 36). In contrast to the CDw75 mAb, the CD76 mAb show a different characteristic staining pattern in lymphoid tissues, being reactive exclusively with mantle-zone B-cells within secondary follicles (8). The same (or spatially related) epitope on the CD76 molecule is detected by both mAbs (40). While the HD66 mAb immunoprecipitates protein bands of 85,000 and 67,000 M, (8), most recently, the two CD76 mAb have been shown to identify a carbohydrate Ag present on gangliosides (27). However, the two CD76 mAb may actually have different ligand specificities, with the CRIS-4 mAb identifying many gangliosides of higher polarity than the HD66 mAb (27). This is consistent with the observation that while similar, the HD66 and CRIS-4 mAb differ in their reactivities. The CRIS-4 mAb stains a portion of granulocytes, the myeloid cell line HL-60, and the T cell line CEM, while the HD66 mAb does not stain these cells at detectable levels (8).

The HB-6 mAb reacts with a heterogeneous uncharacterized surface molecule which is unique in that it is one of the few lymphocyte cell surface molecules expressed by "spotted" cells, which are thought to represent the precursor for multiple myeloma plasma cells (34). In this study, the cellular expression of the HB-6 Ag was examined, revealing similarities with the CDw75 and CD76 Ag. In addition, cDNAs that generate the HB-6 Ag were isolated, revealing that a sialyltransferase is responsible for HB-6 Ag production. Moreover, expression of this sialyltransferase in COS cells was sufficient to generate cell surface expression of the CDw75 and CD76 differentiation Ag.

### Materials and Methods

#### Antibodies

The HB-6 mAb described in this report was produced in the laboratory of Dr. Max D. Cooper (University of Alabama, Birmingham, AL). Hybridomas were generated by fusion of spleen cells (from female Balb/c mice immunized with the BJAB lymphoblastoid cell line) with P3X63Ag8.653 myeloma cells as described (26). The HB-6 mAb was of the IgM isotype as determined by indirect immunofluorescence staining with mouse Ig isotype specific reagents (Southern Biotechnology Associates, Birmingham, AL). Optimal indirect immunofluorescence staining was obtained using a 1/500 dilution of HB-6 ascites fluid. The HB-6 mAb was also conjugated to FITC and used at a concentration of 5 µg/ml. Other mAbs used include the CDw75 mAb, EBU-141 (9), LN1 (12), OKB-4 (39), and HH2 (55), and the CD76 mAb HD66 and CRIS-4 (8), all of the IgM isotype and obtained from the Fourth International Workshop on Human Leukocyte Differentiation Antigens. The HB-10 (CD45RA, IgM) mAb was as described (61), the CD20 mAb HB-13a (AP-291; IgM) was as described (59), CD24 using the anti-UM4D4 mAb (IgM) (38), CD60 using the anti-UM4D4 mAb (24), and B7 was identified using an anti-B7 mAb (IgM) (16). CD3, CD4, and CD8 were identified using the Leu-4, Leu-3, and Leu-2 mAbs, respectively (Becton-Dickinson, Sunnyvale, CA).

The polyclonal rabbit antibody specific for the α2,6-ST was produced by immunization with purified rat liver α2,6-ST as described (68) and was affinity purified using recombinant protein as an immunosorbent.

### Cell Samples

Mononuclear cells were isolated from blood and tissues by Ficoll-Hypaque density gradient centrifugation of heparinized single cell suspensions. Cells were obtained by protocols approved by the Human Protection Committee of Dana-Farber Cancer Institute and the University of Alabama in Birmingham. Cells were kept at 4°C and examined immediately after isolation. Monocytes were isolated from PBMC by plastic adherence (adherent cells). T lymphocytes (E' PBMC) were isolated from the nonadherent mononuclear cell population by rosette formation with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide and subsequent density gradient centrifugation (44). The remaining fraction of cells (E" PBMC) was fractionated into Neutrophils (80% B cells that expressed the surface Ag, CD19, or CD20. Neutrophils were isolated from blood samples at 20°C by centrifugation for 20 min at 1,000 g on a cushion of Mono-Poly Resolving Medium (Flow Laboratories, McLean, VA) followed by lysis of the red blood cells with ice-cold hypotonic 0.2% (wt/vol) NaCl solution.

All human lymphoblastoid cell lines were grown in RPMI 1640 medium containing 10% FCS and antibiotics. Malignant leukocytes were examined as freshly isolated cell samples where >90% of the cells were determined to be tumor cells by morphological and cell surface marker analysis. Cases were considered positive if ≥20% of the cells were reactive with the HB-6 mAb by indirect immunofluorescence with flow cytometry analysis.

### Immunofluorescence Analysis

Indirect immunofluorescence analysis was carried out after washing the cells three times. Suspensions of viable cells were analyzed for surface Ag expression by incubation for 20 min on ice with the appropriate mAb as ascites fluid diluted to the optimal concentration for immunostaining. After washing, the cells were treated for 20 min at 4°C with TRITC- or FITC-conjugated goat anti-mouse Ig antibodies (Southern Biotechnology Associates). Single-color immunofluorescence analysis was performed on an Epics Profile flow cytometer (Coulter Electronics, Hialeah, FL) or a FACS IV (Becton-Dickinson, Mountain View, CA). 10,000 cells were analyzed in each instance and all histograms are shown on a three decade log scale. Fluorescence staining was also visualized using a Leitz Orthoplan fluorescence microscope equipped with Ploem epi-illumination and discriminating sets of excitation and barrier filters. For two-color immunofluorescence analysis, the cells were stained with mouse mAb and counterstained with TRITC- or FITC-conjugated goat anti-mouse Ig heavy chain-specific antibodies (Southern Biotechnology Associates).

### Characterization of the HB-6 Ag

To assess cell surface HB-6 Ag sensitivity to enzyme treatment, cells (5 x 10⁶/ml) were washed in PBS and incubated in normal saline (pH 7.4) containing trypsin (2.5 mg/ml) or Vibrio cholerae neuraminidase (Calbiochem, La Jolla, CA; 0.1 U/ml) for 30 min at 37°C. Similar procedures were carried out for CD45RA as a control using the HB-10 mAb (61). After treatment, the cell preparations were washed with RPMI 1640 medium containing 15% FCS and were stained for indirect immunofluorescence analysis.

### Table 1. mAbs Used in These Studies

| Antigen | mAb     | Isotype | Reference |
|---------|---------|---------|-----------|
| Unknown | HB-6    | IgM     | 60        |
| CDw75   | EBU-141 | IgM     | 9         |
| CDw75   | LN1     | IgM     | 12        |
| CDw75   | OKB-4   | IgM     | 39        |
| CDw75   | HH2     | IgM     | 55        |
| CD76    | HD-66   | IgM     | 8         |
| CD76    | CRIS-4  | IgM     | 8         |
| CD2    | anti-T1110 | IgM | 38        |
| CD12   | MNA     | IgM     | 22        |
| CD20   | HB-13a  | IgM     | 59        |
| CD24   | HB-9    | IgM     | 60        |
| CD45RA | HB-10   | IgM     | 61        |
| CD57   | HNK-1   | IgM     | 1         |
| CDw60  | anti-UM4D4 | IgM | 24        |
| B7     | anti-B7 | IgM     | 16        |
cDNA Isolation

Human spleen cells were activated as described (17) and their RNA was isolated by a modification of the guanidine thiocyanate/oligo(dT) gradient centrifugation technique (54). Poly(A)\(^+\) RNA was purified by two cycles of oligo(dT) selection and used to generate cDNA that was inserted into the pCDM8 vector (52) for library construction. Plasmid DNA was isolated from a 500-ml culture of the original transformation of the spleen cDNA library and purified by the alkaline lysis procedure followed by banding in CsCl gradients twice (35). In further procedures, DNA was isolated using Qiagen pack-500 columns (Qiagen, Studio City, CA) according to the manufacturer's instructions.

In the first round of library screening, sixteen 100-mm dishes of 50% confluent COS cells were transfected with 50 ng of plasmid per ml using the DEAE-dextran method (53). The cells were trypsinized and replated after 48 h. Half of the dishes were incubated with the HB-6 mAb, washed and distributed into panning dishes coated with affinity purified goat anti-mouse IgM antibody (Southern Biotechnology Associates), and allowed to attach at room temperature. Cells were washed four times for 5 min with 500 \(\mu\)l PBS at room temperature. A combination of a 1:100 dilution of TRITC-conjugated goat anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) and a 1:100 dilution of FITC-conjugated F(ab\(^\prime\)), goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking buffer was added and the incubation allowed to continue for 45 min. Cells were again washed four times for 5 min each with 500 \(\mu\)l PBS and mounted on microscope slides. Cells were visualized and pictures taken using a Nikon Microphot-FXA fluorescence microscope.

RNA and DNA Blot Analysis

Poly(A)\(^+\) RNA was isolated as described (54). For Northern blot analysis, \(<5\) \(\mu\)g of poly(A)\(^+\) RNA was denatured with formaldehyde, fractionated by electrophoresis through a 1.2% agarose gel and transferred to nitrocellulose (63). The cDNA inserts used as probes were isolated, twice gel purified, nick-translated (48), and hybridized with the filters as described (66).

Immunohistochemistry

Blocks of hyperplastic tonsils from four individuals were snap-frozen in liquid nitrogen and cryostat sections cut. These were immunohistochemically stained using an avidin-biotin-peroxidase technique with the chromogen diaminobenzidine as described (47).

Results

Production and Characterization of the HB-6 mAb

The HB6 mAb-producing hybridoma was generated with spleen cells obtained from mice immunized with the human B lymphoblastoid cell line, BJAB. The reactivity of the HB-6 mAb with subpopulations of blood cells was assessed by indirect immunofluorescence analysis (Fig. 1). Approximately half of blood mononuclear cells (53 \(\pm\) 12\%, \(n = 7\)) expressed the HB-6 Ag with three distinct populations of cells observed; a negative to weakly positive population, a subpopulation of moderately bright cells, and a small subpopulation of intensely bright cells (Fig. 1). After cellular enrichment for B and T lymphocytes and monocytes, the brightest population of cells segregated with the B cells (E-PBMC), while the moderately bright cells were mostly T cells (E\(^+\)PBMC), and monocytes (adherent cells) were negative. In addition, CD57\(^+\) natural killer cells (73 \(\pm\) 15\%, \(n = 3\)) and erythrocytes were HB-6 positive and CD15\(^-\) neutrophils did not express detectable HB-6 Ag. Similarly, most pre-B and B cell lines (PB-207, NALM-6, BJAB, Daudi, SB, G5-K, Jijo, Raji, Namalu, Ramos, Akata, Aren), some T cell lines (Molt-4, Rex, HPB-ALL), and an erythroleukemia cell line (K562) were HB-6 positive. However, one pre-B cell line (PB-697), some T cell lines (Molt-3, H-SB2, Hut-7B), and a monocytic cell line (U-937) were HB-6 negative.

Two-color immunofluorescence assays were carried out to further characterize the cells identified by the HB-6 mAb. Essentially all blood IgM\(^+\) B cells and a large subpopula-
Expression of the HB-6 Ag (solid line) examined by indirect immunofluorescence staining with flow cytometry analysis. The HB-6 mAb was reactive with PBMC, the E⁺ PBMC fraction enriched for B cells (48% IgM⁺), and a T cell-enriched (E⁺ PBMC) fraction (96% CD3⁺). The mAb was unreactive with adherent monocyctic cells (92% CD15⁺). The dashed lines represent background staining with unreactive mouse ascites fluid and the FITC-conjugated anti-mouse Ig antibodies used as developing agents.

Expression of the HB-6 Ag by malignant leukocytes was similar to that found in their normal counterparts. Most B cell malignancies were HB-6⁺, two of four cases of pre-B acute lymphocytic leukemia (ALL) were positive, two of three cases of B-ALL, nine of nine cases of chronic lymphocytic leukemia, two of two cases of hairy cell leukemia, five of six cases of Burkitt's lymphoma, and five of six cases of B cell lymphoma. Among malignant T cells, one of one case of Sezary cell lymphoma and one of three cases of T-ALL were HB-6 positive. One of four null cell leukemias was HB-6⁺ positive. None of five cases of myeloid leukemia expressed the HB-6 Ag.

**Table II. Tissue Distribution of T Cells Expressing the HB-6 Antigen**

| Cell source (no. of samples) | % of cells that were HB-6⁺ (mean ± SD) |
|-----------------------------|--------------------------------------|
|                            | all cells   | CD3⁺ | CD4⁺ | CD8⁺ |
| Thymus: adult (3)          | 26 ± 7      | 93   | ND   | ND   |
| Bone marrow:               |            |      |      |      |
| fetal (3)                  | 5 ± 2       | 85 ± 9| 97 ± 5| 73 ± 14|
| adult (3)                  | 6 ± 5       | 55 ± 22| 81 ± 12| 45 ± 26|
| Blood:                     |            |      |      |      |
| newborn (3)                | 69 ± 10     | 99 ± 1| 98 ± 2| 97 ± 1 |
| adult (4)                  | 53 ± 12     | 74 ± 12| 90 ± 5| 68 ± 14|
| Tonsil: adult (4)          | 62 ± 5      | 35 ± 19| 23 ± 17| 41 ± 24|
| Spleen: adult (3)          | 61 ± 14     | 51 ± 16| 72 ± 12| 35 ± 26|

Cells were labeled with HB-6 mAb followed by TRITC-conjugated antirabbit Ig heavy chain-specific antibodies. T cell populations were identified using the indicated mAb and FITC-conjugated antirabbit Ig heavy chain-specific reagents. Reactivity was determined by two-color indirect immunofluorescence microscopy.

**Table III. Tissue Distribution of B Cells Expressing the HB-6 Antigen**

| Cell source (number of samples): | % of cells that were HB-6⁺ (mean ± SD) |
|----------------------------------|--------------------------------------|
| Pre-B cells: fetal liver (3)     | 43 ± 5                                |
| IgM⁺ B cells:                    |                                      |
| Fetal liver (3)                  | 83 ± 6                                |
| bone marrow (3)                  | 52 ± 16                               |
| spleen (4)                       | 94 ± 1                                |
| Newborn blood (4)                | 98 ± 2                                |
| Adult bone marrow (4)            | 99 ± 2                                |
| blood (4)                        | 99 ± 1                                |
| spleen (3)                       | 96 ± 5                                |
| tonsil (3)                       | 93 ± 5                                |
| Plasma cells:                    |                                      |
| Spleen, tonsil, and bone marrow (8)| 42 ± 24                             |

HB-6 reactivity was assessed using FITC-conjugated antimouse μ heavy chain-specific antibodies. B cells were identified with TRITC-conjugated antihuman μ heavy chain-specific antibodies. Plasma cells were identified by the presence of large amounts of cytoplasmic Ig using TRITC-conjugated antihuman F(ab)₂ specific antibodies. Reactivity was determined by two-color indirect immunofluorescence microscopy.

**Biochemical Characterization of the HB-6 Ag**

The HB-6 mAb was used to immunoprecipitate the HB-6 Ag from surface iodinated or metabolically labeled PBMC and B cell lines. However, no specific protein or proteins were identified. Following trypsin treatment of PBMC a substantial amount of the HB-6 Ag was still present, while the CD45RA Ag was completely removed (Fig. 2). In contrast, treatment of PBMC with neuraminidase removed essentially all of the HB-6 Ag and had little effect on reactivity of the HB-10 mAb. These results suggest that HB-6 mAb binding is dependent on the presence of sialic acid residues and that only a portion of the Ag was trypsin sensitive.

**Isolation of cDNAs That Encode the HB-6 Ag**

The nature of the HB-6 Ag was further examined by expression cloning of a cDNA that generated the HB-6 epitope on COS cells. A cDNA library was constructed in the pCDM8 vector using mRNA from activated splenic B cells. This library was introduced into COS cells, the cells were treated with the HB-6 mAb and cells expressing the HB-6 Ag were isolated by panning as described (53). After three rounds of selection, the isolated plasmids were used to transform bacteria and the resulting colonies were analyzed for the presence of an intact vector with a labeled oligonucleotide homologous with the region 5' of the cDNA insertion site. 6% of the colonies hybridized, indicating that this portion of the plasmid had not been deleted in these colonies during selection and that these plasmids were likely to also contain a cDNA insert. Positive clones were harvested and their plasmids were isolated and transfected into COS cells. Three

**Figure 1.**
percent of the plasmids contained a similar cDNA insert of 3.2 kb which induced high level expression of the HB-6 Ag in COS cells as determined by indirect immunofluorescence analysis using the HB-6 mAb (Fig. 3). This plasmid was termed pHB-6. COS cells transfected with pHB-6 were reactive with the HB-6 mAb, but were not reactive with other IgM isotype mAb that identify CD24, CD2, B7, CD45RA, CDw60, and CD20 (Fig. 3).

That the isolated pHB-6 cDNA specifically induced expression of the HB-6 Ag and that transfection alone did not induce HB-6 Ag expression, was further verified by transfecting COS cells with pHB-6 or cDNAs encoding CD2 (53), B7 (17), CD19 (58), and CD20 (62). Cells transfected with each cDNA were stained with each of the murine IgM mAb followed by indirect immunofluorescence staining and fluorescence microscopy analysis. Only pHB-6 cDNA transfected COS cells reacted with the HB-6 mAb, whereas only CD2, B7, CD19, and CD20 transfected COS cells reacted with their respective mAb (data not shown). Vector transfected COS cells demonstrated no significant staining with any of the mAb.

Structure and Sequence of the pHB-6 cDNA

A restriction map was generated for the pHB-6 cDNA and the nucleotide sequence was determined using the strategy shown in Fig 4A. An open reading frame was identified that could encode a protein of 406 amino acids with a single hydrophobic domain near the putative amino terminal end of the protein (Fig. 4B). Comparison of the predicted HB-6 protein sequence with known proteins revealed that the HB-6 Ag was homologous, if not identical to, the α2,6-ST (EC 2.4.99.1) encoded by cDNAs isolated from rat liver (68), human submaxillary gland (32), and human placental (20) libraries. Subsequently, a human α2,6-ST cDNA has been isolated from a human B lymphoblastoid cell line, Daudi, library (56). Comparison of the nucleotide sequences of the cDNAs revealed that the pHB-6 cDNA and published Daudi cDNA contained a stretch of sequence at the 5' end that is different from that found at the 5' end of the placental cDNA (underlined in Fig. 4B), suggesting that different transcription initiation sites are used in the different cell lineages or that differential mRNA splicing occurs. Comparison of the nucleotide sequences between the pHB-6 cDNA and the
placental cDNA revealed identical nucleotide and amino acid sequences. In contrast, the pHB-6 and Daudi nucleotide sequence identity (one base mismatch). Furthermore, 95.6% identical, while comparison of the overlapping sequence of the pHB-6 cDNA and the published Daudi cDNA sequence indicated that they are 95.6% identical, while comparison of the overlapping sequences between the pH6-6 and placenta cDNA indicated 95.5% sequence identity (one base mismatch). Furthermore, comparison of the coding region of the pH6-6 cDNA and the placental cDNA revealed identical nucleotide and amino acid sequences. In contrast, the pH6-6 and Daudi nucleotide sequences in this region were only 99.5% identical, resulting in changes in three amino acids. Therefore, it is likely that the pH6-6 cDNA encodes the same α2,6-ST as has previously been described (20, 32, 56, 68).  

### α2,6-ST mRNA Expression

Northern blot analysis was carried out with the pH6-6 cDNA insert to examine lymphocyte mRNA size and expression. α2,6-ST mRNA was expressed by every cell line examined, but the levels of expression varied widely. Blood T and B cells, activated or resting, and the B cell line Namalwa expressed high levels (+++) on a relative scale, B cell lines including Ramos, Raji, and Daudi were (+++), while the K562 erythroleukemia line, a T cell line, Rex and chronic myelogenous leukemia, and multiple myeloma cells expressed little (+) mRNA (Fig. 5, A and B, and data not shown). In addition, slight differences in the sizes of mRNA species were observed between different cell sources, Rex α2,6-ST mRNA was ~4.2 kb, K652 was ~4.1 kb, while mRNA from Raji or activated lymphocytes was smaller ranging from ~3.9 to ~4.0 kb (Fig. 5 A and data not shown). These minor differences in size were observed in multiple mRNA samples and in at least three different Northern blots. Therefore, Southern analysis was carried out to determine whether a single gene product was being examined or whether multiple genes were being represented. In all cases examined, probing endonuclease digested genomic DNA with the pH6-6 cDNA insert revealed that the α2,6-ST gene was likely to be single copy (Fig. 5 C) as has been shown for the rat α2,6-ST gene (57). These results suggest that different cell types use different transcription initiation sites for mRNA production from a single gene or process the mRNA differently. Nonetheless, the level of α2,6-ST mRNA
Figure 5. Northern and Southern blot analysis using the pHb-6 cDNA as probe. (A) Poly(A)+ RNA isolated from cell lines, activated blood T cells (30 min to 24 h, left; 36 to 72 h, right) and activated spleen B cells. 5 µg of each RNA was used per lane. Autoradiography was with an intensifying screen for 1 h. (B) The same filter as in A autoradiographed for 26 h. (C) Southern blot analysis of DNA digested with BamHI, EcoRI and HindIII, electrophoresed in 0.8% agarose, transferred to nitrocellulose, and hybridized with the labeled HB-6 cDNA probe. DNA was isolated from: (lane 1) normal splenocytes; (lane 2) the DHL-4 B cell line; malignant leukocytes from a patient with diffuse histiocytic lymphoma, lymph node (lane 3) and peripheral blood (lane 3'); leukocytes from a second patient's lymph node (lane 4') and blood (lane 4).

expressed by different cells was consistent with the intensity of HB-6 surface Ag expression.

Expression of the α2,6-ST in the Golgi Apparatus Results in Expression of the HB-6 Ag on the Cell Surface

That lymphocytes express α2,6-ST as the cell surface CDw75 differentiation Ag has been previously suggested (56). However, an overwhelming body of information indicates that terminal glycosyltransferases are localized within the Golgi apparatus (reviewed in 43). Therefore, to determine whether expression of the α2,6-ST correlated with expression of the HB-6 Ag on the cell surface, Cos-1 cells were transfected with cDNA encoding the rat α2,6-ST (68). 40–48 h after transfection, cells were fixed and lightly permeabilized with 0.1% Triton X-100 to view both surface and intracellular staining, or left unpermeabilized to view only cell surface staining. Untransfected and cDNA transfected cells were incubated with both rabbit anti-rat α2,6-ST antibody and mouse anti-HB-6 mAb which were subsequently detected with a TRITC-conjugated goat anti-rabbit IgG second antibody and an FITC-conjugated goat anti-mouse IgM F(ab')2 second antibody, respectively.

Neither the α2,6-ST nor the HB-6 Ag were detected intracellularly or at the cell surface in untransfected COS-1 cells (Fig. 6). In cDNA transfected COS-1 cells, the HB-6 Ag was detected on the surface of 10–20% of the unpermeabilized cells, while the α2,6-ST was never detected on the surface of these cells (Fig. 6 A). However, in cDNA transfected COS-1 cells that were permeabilized, the α2,6-ST was localized to the perinuclear Golgi region in 10–20% of the transfected cells and punctate HB-6 Ag staining was detected on the cell surface of only those cells expressing the ST in the Golgi apparatus (Fig. 6 B, third row). In addition to cell surface localization, the HB-6 Ag was also observed in the Golgi apparatus of some cells, the site of its generation. In COS cells overexpressing the ST, some ST was found in the ER as well as Golgi (Fig. 6 B, second row), but was never localized to the cell surface. These observations demonstrate that expression of the α2,6-ST in the Golgi apparatus of cells correlates with the cell surface expression of the HB-6 Ag, and indicates that the HB-6 Ag is a product of ST activity.

α2,6-ST Generates Expression of the HB-6, CDw75, and CD76 Ag

Since CDw75 was previously suggested to be the α2,6-ST (56) and the CD76 Ag has been previously shown to include sialic acid–bearing gangliosides (27), the reactivity of these mAb with pHb-6 cDNA transfected COS cells was examined. In all cases, α2,6-ST cDNA transfected COS cells were specifically stained with each of four CDw75 mAb and both CD76 mAb (Fig. 3). Staining with the HB-6 and all CDw75 and CD76 mAb was intense and none of the mAb bound untransfected COS cells or COS cells transfected with other cDNAs. This finding suggests that the HB-6, CDw75, and CD76 mAB may identify similar molecules on the cell surface.

HB-6, CDw75, and CD76 Are Distinct Neuraminidase-sensitive Ag

Binding of the HB-6 mAb and all CDw75 and CD76 mAb was completely eliminated by pretreatment of PBMC or cell lines with neuraminidase as shown for the HB-6 Ag (Fig. 2). Therefore, it is likely that an essential element of these four mAb specificities is a sialic acid residue. The specificity of
Figure 6. Expression of the HB-6 Ag on the cell surface correlates with Golgi apparatus expression of the α2,6-ST. COS cells (Control) and COS cells transfected with the rat α2,6-ST cDNA were analyzed by indirect immunofluorescence for expression and localization of the α2,6-ST and the HB-6 Ag. Cos cells (A) fixed with 1% paraformaldehyde for detection of cell surface immunofluorescence, or (B) permeabilized with detergent for detection of both cell surface and cytoplasmic immunofluorescence. HB-6 mAb staining was detected using FITC-conjugated F(ab)2 goat anti-mouse IgM second antibody. The α2,6-ST was detected using an affinity-purified rabbit anti-rat α2,6-ST polyclonal antibody and a TRITC-conjugated goat anti-rabbit IgG second antibody. The second panel in B shows COS cells that overexpress the α2,6-ST, while in the third panel normal Golgi expression is demonstrated. The bar in the bottom left panel represents 10 μM.
Figure 7. Binding of the CDw75 and CD76 mAb does not block HB-6 mAb binding. SB cells, which express similar levels of HB-6, CDw75, and CD76 Ag, were stained with FITC-labeled HB-6 mAb after treatment with the indicated mAb: Cont., control unreactive mouse ascites fluid; HB-6, mAb ascites diluted 1:10; CDw75, the EBU-141 mAb as diluted ascites fluid (1:10); CD76, the HD66 mAb as diluted ascites fluid (1:10). Reactivity of HB-6 mAb was examined by flow cytometry. The HH2, LNI, OKB-4, and CRIS-4 mAb also failed to block HB-6 mAb binding.

Discussion

The HB-6 mAb was shown to react with a novel cell-surface Ag present on a subpopulation of leukocytes. The HB-6 Ag was highly expressed by most B cells (Fig. 1, Table III), and at lower levels by a major subpopulation of T cells (Fig. 1, Table II), and was also present on erythrocytes. Among the B and T cell lineages, the HB-6 Ag was expressed by most lymphocytes from fetal, newborn, and adult tissues (Tables II and III). Malignant counterparts of these cells were also generally HB-6+. In contrast, while most B cell lines expressed the HB-6 Ag, many T cell lines were HB-6- or expressed only low levels. This pattern of expression was similar to that reported for the CDw75 and CD76 Ags, with some exceptions. The CDw75 and CD76 Ags are expressed late during B cell development (9), while the HB-6 Ag was expressed during even the earliest stages of pre-B cell development (Table III). Also, it appears that the HB-6 Ag was more broadly distributed among T cell populations than has been reported for either the CDw75 or CD76 Ag (8, 9). The HB-6 Ag was further distinguished from the CDw75 and CD76 Ags by its patterns of expression among cell lines (Fig. 8), distribution among cell populations within tonsil (Fig. 9), and because the HB-6 mAb did not block the binding of either CDw75 or CD76 mAb (Fig. 7). Therefore, the HB-6 mAb identifies a newly characterized leukocyte cell-surface Ag.
Figure 9. Immunohistochemistry on parallel frozen sections of human tonsil (chromogen diaminobenzidine and hematoxylin counterstain). (A) The HB-6 mAb stained mantle zone and germinal center B cells and, unlike other mAb studied, large numbers of extrafollicular cells. (B) LNl (CDw75) mAb predominantly stained follicular B cells. (C) CRIS-4 (CD76) mAb bound to mantle zone B cells and scattered extrafollicular cells. Bar, 100 μM.

Isolation and sequencing of cDNA from a human B cell library that induced HB-6 Ag expression in COS cells revealed that these cDNA encoded a specific lymphocyte isoform of human α2,6-ST cDNA (Fig. 4). α2,6-ST catalyzes the transfer of sialic acid to the terminal Galβ1,4GlcNAc structures present on the oligosaccharides of glycoproteins and glycolipids (21, 49). cDNAs that encode α2,6-ST have been previously cloned from rat liver and human placenta, submaxillary gland, and a B cell line (20, 32, 56, 68). The lymphocyte α2,6-ST cDNA nucleotide sequence differed from the nonlymphoid α2,6-ST cDNAs in the 5′ untranslated region (Fig. 4 B), suggesting that there is differential regulation of this gene by different cell lineages. The α2,6-ST has previously been shown to be localized to the trans-cisternae of the Golgi and trans-Golgi network (4, 43). In this study, the protein product of the α2,6-ST cDNA was shown to remain localized within the Golgi apparatus of cDNA transfected COS cells, while the HB-6 Ag was expressed on the cell surface (Fig. 6). In addition, cell surface expression of the HB-6 Ag was completely sensitive to neuraminidase treatment and was only partially decreased after protease treatment (Fig. 2). These findings are consistent with the notion that the HB-6 Ag is a carbohydrate structure that is expressed on cell surface glycoproteins and glycolipids and one that contains an α2,6-linked sialic acid residue as a critical component of the Ag. The isolation of a cDNA which encodes a glycosyltransferase by the selection of transfected cells which express the product of the glycosyltransferase at the cell surface is not without precedent as the cDNA for several terminal glycosyltransferases have been isolated by expression cloning (13, 30, 31). Most recently, a cDNA encoding an α-1,3-fucosyltransferase, which acts in the synthesis of a carbohydrate moiety that constitutes the ELAM-1 ligand, was isolated by expression cloning (19).

Another interesting finding was that expression of α2,6-ST not only generated the Ag identified by the HB-6 mAb, but also the cell surface Ag identified by the CDw75 and CD76 mAb (Fig. 3). This result suggests that the CDw75 and CD76 Ag are also carbohydrate determinants generated by the expression of the α2,6-ST. Other data concerning the nature of these Ag also support this premise. First, the HB-6, CDw75, and CD76 Ag were all highly susceptible to neuraminidase treatment, as was previously shown for CDw75 with the LN-1 mAb (12). In addition, the CD76 Ag is resistant to formalin-fixation and paraffin-embedding, procedures which often denature cell-surface proteins (9, 12, 42). Because all four mAb used to define CDw75 appear identical in reactivity and all recognize a single epitope (40), it is likely that they recognize a single carbohydrate determinant. Second, others have shown that CD76 mAb react with sialylated glycolipids with a NeuAcα2,6Galβ1,4GlcNAcβ-terminal structure (27). Both CD76 mAb also react with paraffin-embedded tissue sections and cross-block each other's binding (8). Although the activities of the two characterized CD76 mAb are overlapping, they are distinct since the HD66 and CHRS-4 mAb show major differences in reactivities among granulocytes, the myeloid line HL-60 and the T-cell line CEM (8). Thus, expression of a single ST is required for the generation of multiple distinct antigenic determinants which can be distinguished by mAb. The antigenic diversity of these cell surface determinants may reside in their core carbohydrate structures, the synthesis and distribution of which is further controlled by the regulated tissue- and cell type-specific expression of the appropriate glycosyltransferases, and/or in the structures of their carrier proteins or lipids which may also be critical for generation of the appropriate antigen. This also indicates that the core carbohydrate (or protein sequence) components of the HB-6, CDw75, and CD76 Ag are expressed by COS cells and that all that is required for Ag expression is the addition of an
α2,6-linked sialic acid. Therefore, the regulated expression of different combinations of glycosyltransferases could generate considerable diversity in both protein- and lipid-bound carbohydrate chains, further increasing the diversity and antigenic nature of cell surface molecules.

Recently, Stamenkovic et al. (56) reported the cloning of the B cell CDw75 Ag and identified it as a cell surface α2,6-ST. However, the α2,6-ST had previously been recognized to localize exclusively in the Golgi apparatus of cells (4, 43). In this study (Fig. 6) and others (Colley, K. J., E. U. Lee, and J. C. Paulson, manuscript submitted for publication), it was further demonstrated that when the ST was overexpressed in transfected COS or CHO cells, it remained localized within the ER as well as the Golgi apparatus, but was never detected on the cell surface. Additionally, the results of this study suggest that the CDw75 Ag is a carbohydrate structure which is generated by the α2,6-ST. So, although the 3 amino acid changes noted between this B cell α2,6-ST (56) and other cloned human ST (Fig. 4, references 20, 32) could lead to mislocalization of the enzyme to the cell surface, it is more likely that Stamenkovic et al. (56) have cloned the same α2,6-ST from cells expressing the cell surface, sialylated carbohydrate Ag recognized by the CDw75 mAb.

The biological significance of expression of the HB-6, CDw75, and CD76 Ag for lymphocyte function is unknown. However, there are at least two possibilities. One possibility is that expression of these surface carbohydrate determinants may regulate interactions between cell-surface carbohydrates and their specific receptors. One of the best examples of this type of interaction is found within the selectin family of endothelial-leukocyte adhesion receptors where ligand binding is dependent on terminal sialic acid residues.

Many other cell surface molecules with C-type lectin domains have been identified which may also serve as receptors for the HB-6, CDw75, and CD76 Ag (11). The second possible function is that the sialic acid residues added to surface carbohydrate determinants may serve as biological masks. Examples of this include the regulation of homotypic interactions of N-CAM (46).

Many immunologically important cell surface molecules are glycosylated and many immunological recognition events appear to be influenced by sialic acid. The ability of B cells to induce an allogeneic response and to present Ag can be markedly enhanced by pretreatment with sialidase (6, 18). Cell surface sialic acid also influences tumor cell recognition in the mixed lymphocyte reaction presumably involving α2,6-linked sialic acids (45). Mitogen stimulation, cell lineage commitment and differentiation, and malignant transformation are all events that can dramatically alter glycosylation patterns of proteins, lipids, and glycosaminoglycans on leukocytes (21, 23, 67). For example, the least mature thymocytes have the lowest levels of ST and surface sialic acid (7, 64). While thymocytes express little sialic acid, T cells express more and B cells express even more (69). Activation of lymphocytes also leads to early increases of ST activities which precede proliferation (2). These findings correlate well with surface expression of the HB-6 (see Fig. 1, Tables II and III), CDw75, and CD76 Ag. However, despite previous observations that malignant transformation of leukocytes alters expression of cell-surface glycoconjugate expression (21, 67), expression of the HB-6 Ag was remarkably similar to that of normal leukocytes. Undoubtedly, further advances in understanding the functional relevance of those differences will be required before the roles of these carbohydrate moieties will be understood.

Many of the cell surface Ags that were originally characterized as CD Ag based on their selective expression by discrete leukocyte subpopulations are now known to be carbohydrate moieties including: CD15 (Lewis X); CD17 (lectotylerceramide); CDw52; CDw60; CDw65 (ceramide-dodecasaccharide 4c); CD57; and CD77 (Gb). This study contributes two additional members to this list, CDw75 and CD76. That sialic acids are capable of contributing to novel Ag should not be unexpected since they are a heterogeneous group of at least 30 structural variants of neuraminic acid, differing in N- and O-acetylation and other substituents. They are predominantly linked α2,6, α2,4, or α2,3 to galactose, α2,6 to GalNac, α2,6 to GlcNac, and α2,8 to sialic acid. Therefore, given the complex biochemical nature of cell-surface glycoconjugates and the high degree of diversity that can be achieved, it is likely that carbohydrate modification of cell surface structures offers an additional level of antigenic specificity that will contribute to the control of lymphocyte interactions and function. In addition, sialic acids are known moieties that generally occupy terminal positions on oligosaccharides. These factors make them ideal targets for immune recognition, as evidenced in this report. Thus, expression of the α2,6-ST described here is likely to play a central role in determining the expression of multiple leukocyte cell-surface Ags.

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