# MONOCLONAL ANTIBODIES SPECIFIC FOR T CELL–ASSOCIATED CARBOHYDRATE DETERMINANTS REACT WITH HUMAN BLOOD GROUP ANTIGENS CAD AND SDA

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The mAbs CT1 and CT2 were obtained in a screen for antibodies reacting with the murine CTL clone B3.3 and are able to block specific target cell lysis by this and other CTL clones (1). The analysis of a large panel of cytotoxic and helper T cell clones showed that Lyt-$2^+$ CTL clones invariably expressed very high levels of CT antigens, whereas all helper T cell clones either did not express or only weakly expressed the CT determinants (1, 2). In vivo, the only mature T cells that express CT antigens are Lyt-$2^+$ intraepithelial lymphocytes of the intestinal mucosa (3, 4), thus suggesting that these antigens may be restricted to the Lyt-$2^+$ subset. However, the CT determinants are expressed on fetal thymocytes in a developmentally regulated fashion with high levels of CT antigen expressed early in fetal thymus ontogeny, followed by a gradual decrease in CT antigen expression as birth approaches (5). The relationship between the presence of CT antigens in the fetal thymus and peripheral T cell populations is not yet known. It is also evident from the differing tissue and cell specificities of the CT1 and CT2 mAbs that these reagents recognize similar but not identical determinants. Although a functional role for the CT carbohydrate determinants has not yet been described, recent results in other systems indicate an intrinsic role for cell type–specific oligosaccharides during development (reviewed in references 6, 7).

Biochemical analysis showed that CT1 immunoprecipitates surface glycoproteins of the T200 family and additional, unrelated glycoproteins of 140 and 85 kD, as well as a secreted glycoprotein of 155 kD from biosynthetically labeled CTL clones (2, 8). Although forms of the T200 glycoprotein are found on all hematopoietic cells except cells of the erythroid lineage, the CT1 antigen was expressed only on the T200 glycoprotein of activated CTL in vitro (1, 2). The CT1 and CT2 antibodies bind to distinct but overlapping epitopes since CT1 blocks binding of CT2 to clone B3.3 efficiently, but the reverse is not true (2). The fact that the CT antigens are present on several unrelated glycoproteins and are destroyed by sodium periodate, coupled with the finding that during biosynthesis of T200, the CT1 antigen is acquired late, i.e., after the *N*-linked oligosaccharides become resistant to endoglycosidase H, suggested that the CT

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determinants required carbohydrate for expression (8). Moreover, the data suggested that *O*-linked carbohydrates could carry CT1 antigen since the secreted 155-kD glycoprotein synthesized in the presence of tunicamycin was immunoprecipitated by the CT1 antibody. However, the precise carbohydrate residues required for CT antigen expression have not yet been defined.

Previously, the GalNAc-specific lectin from *Vicia villosa* (VV)[1] was shown to react specifically with CTL but not helper-type lymphocytes (9–11). The biochemical analysis of the murine CTL clone B6.1 shows that it binds the $VVB_4$ isolectin and that the binding sites consist of a variety of *O*-linked structures which after desialylation, range from di- to hexasaccharides and carry β-linked GalNAc residues at their nonreducing termini (12). The smallest of these structures was found to be related to a structure previously described on glycophorin A from erythrocytes of the rare human blood Cad, namely GalNAcβ1-4(NeuAcα2-3)Galβ1-3(NeuAcα2-6)-GalNAcα1-Ser/Thr (13). B6.1.VV6, a mutant selected for resistance to the toxic effects of the VV lectin is deficient in VV binding sites, lacks these terminal GalNAc residues, and is deficient in a UdP-GalNAc: β-galactose β1,4-*N*-acetylgalactosaminyltransferase (11, 12, 14). Using this cell line as well as several other criteria we have defined the requisite oligosaccharide configuration necessary for CT antigen expression. We find that the CT determinants are similar to the human blood group antigens Cad and Sda.

## Materials and Methods

*Cells.* The origin of B6.1, B6.1.VV6, and B3.3 cell lines have been described (1, 11). Fresh Cad and thawed Tn erythrocytes were kindly donated by Dr. J. P. Cartron (Centre National de Transfusion Sanguine, Paris, France). The Cad erythrocytes were from the original Cad individual whose glycophorin A contains β1,4-linked GalNAc on 12 of 15 of its *O*-linked oligosaccharides (15). $A_1$ and $A_2$ erythrocytes (16) were obtained through the local blood bank and O, A, AB, and B erythrocytes were taken from healthy volunteers using citrate for anticoagulation. All cells were washed four times in PBS before they were used in agglutination assays.

*Agglutination Assays.* Agglutination assays were performed in Nunclon Microwell plates (72 × 10 μl, Gibco Laboratories, Basel, Switzerland). Antibodies and cells were diluted in PBS, 1 mM $CaCl_2$, and 2 mg/ml BSA. We added 2 μl of cell suspension containing about $10^3$ cells or $10^4$ erythrocytes to 4 μl of diluted antibody. The plates were then incubated for 45 min at 6°C in a humidified atmosphere on a clinical rotator operating at 80 rpm. The plates were scored under the inverted microscope. Purified $VV_4$ lectin was the kind gift of Dr. S. Tollefsen, Washington University, St. Louis, MO.

*Solid-Phase Radioimmunoassay.* Purified $Sda^+$ or $Sda^-$ Tamm-Horsfall glycoproteins were kindly given to us by Dr. W. Watkins, Clinical Research Center, Harrow, United Kingdom. The glycoproteins were diluted in PBS and added to 96-well polyvinyl microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) at 30 μl per well; after incubation for 30 min at room temperature in a humidified atmosphere, the remaining sites were blocked by adding 200 μl/ml of PBS with 2 mg/ml BSA. This buffer was used through the following steps. After incubation at room temperature for 5 min, the wells were washed two times (200 μl/well/wash), received 50 μl of antibody containing ascites diluted as indicated, were incubated at room temperature for 30 min or as indicated, washed three times, and were subsequently incubated with affinity-purified rabbit anti–mouse IgG radiolabeled with $^{125}I$ by the Iodogen method. Each well received 3 × $10^5$ cpm of antibody with a specific activity of ~0.1 μCi/μg in 80 μl. After incubation at 4°C for 1 h

[1] *Abbreviation used in this paper:* VV, *vicia villosa.*

or as indicated, plates were washed seven times and individual wells were cut from the plate with scissors to be counted in a gamma counter. Desialylation of bovine submaxillary mucin (Sigma Chemical Co., St. Louis, MO) was performed as described (17) and the release of sialic acid was followed by the method of Warren (18).

*Glycosidase Treatment.* CTL clones were lysed with ice-cold 1% NP-40 in PBS containing 100 Kallikrein units of aprotinin/ml (Sigma Chemical Co.) and 2 mM PMSF (Behring Diagnostics, La Jolla, CA). Extracts were centrifuged at 50,000 *g* for 30 min. 10 μl of the lysate equivalent to $10^6$ cells was then incubated for 18 h at 37°C with or without the addition of glycosidases. Buffers and enzyme concentrations used were as follows; endoglycosidase F (*N*-glycanase; Genzyme Corp., Boston, MA) 50 U/ml, 0.17% SDS, 0.2 M sodium phosphate, 10 mM 1,10-phenanthroline hydrate, 1.25% NP-40, pH 8.6; neuraminidase (*V. cholerae*, Gibco Laboratories; *Cl. perfringens Type X*, Sigma Chemical Co.), 0.05 M sodium-acetate, pH 5.5, with 9 mg NaCl/ml and 1 mg $CaCl_2$/ml. In some cases the neuraminidase inhibitor 2-3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added from 1 to 10 mM.

*Western Blotting.* After treatment of lysates with glycosidases or with buffer alone, the samples were subjected to acetone precipitation at −20°C for 24 h. The samples were centrifuged at 13,000 *g* for 30 min, the pellets were resuspended in 50 μl of PBS containing aprotinin and PMSF, and duplicate samples (25 μl each) were dot-blotted onto nitrocellulose. The nitrocellulose was dried and nonspecific binding was blocked by overnight incubation in BLOTTO (19). The strip was then incubated for 2 h at room temperature with 20 ml of a 1:200 dilution in BLOTTO of CT1 ascites fluid. The strip was washed three times over a 1 h period followed by incubation for 1 h with 20 ml of a 1:500 dilution of rabbit anti–mouse Ig (Cappel Laboratories, Cochranville, PA). After washing, $2 \times 10^5$ cpm/ml $^{125}$I-labeled protein A from *Staphylococcus aureus* (ICN Biomedicals, Irvine, CA) was added for 1 h followed by washing and drying of the strip. The blots were cut out and the amount of radioactivity bound was measured in a gamma counter.

*Periodate Treatment and Fluorescence Analysis.* Intact CTL clones were incubated for 45 min at 4°C in the dark with increasing concentrations (1–4 mM) of sodium-*m*-periodate (Sigma Chemical Co.) dissolved in PBS (20). The cells were then washed with PBS containing 1 mg/ml BSA and 0.1% $NaN_3$. The cells were incubated for 30 min with CT1, CT2, or 13/2.3 (a rat mAb reactive with the mouse T200 glycoprotein [21]). After washing, the cells were reacted with fluorescein-labeled goat anti mouse-Ig (for CT1 and CT2) or a goat anti rat-Ig (Cappel). Single cells were analyzed by fluorescence flow cytometry on a FACS IV (Becton Dickinson & Co., Sunnyvale, CA).

## Results

*Interaction of CT1 and CT2 with the CTL Line B6.1 and its VV-resistant Mutants.* We wished to determine whether B6.1 expressed the CT antigens and whether the CT antigens were retained on the cell surface after VV selection. Since the CT mAbs are of the IgM isotype, we used their ability to agglutinate $CT^+$ cells to determine expression. CT1 and CT2 mAbs both strongly agglutinated the CTL line B6.1 but did not agglutinate the B6.1.VV6 mutant line. This line had 100-fold less binding sites for VV than the B6.1 line when assayed with a crude VV preparation containing both the A and B isolectins (Fig. 1) (11). Two other VV-resistant mutants, B6.1.VV3 and B6.1.VV4, that still expressed high numbers of VV binding sites (11) were agglutinated by CT1 and CT2 to the same extent as the parental line. As expected, $VVB_4$ lectin only agglutinated B6.1 but not the B6.1.VV6 mutant. These findings are consistent with the idea that the terminal β-1,4-linked GalNAc residues that are lacking in the B6.1.VV6 mutant are an essential component of the determinant(s) recognized by CT1 and CT2.

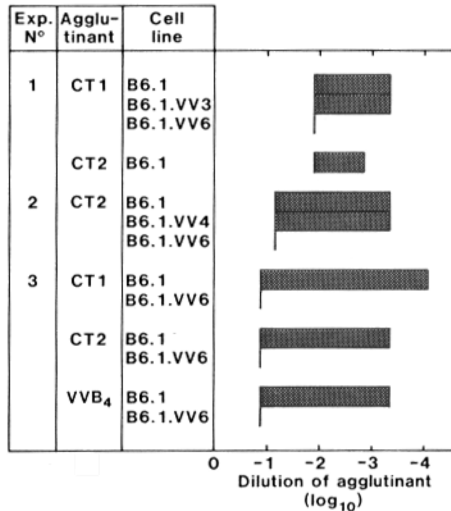


FIGURE 1. Agglutination of CTL line B6.1 and VV-resistant mutants. For CT1 and CT2, we used ascites fluid as stock solution for the dilutions (ascites usually contains 1–5 mg/ml of antibody). The stock of $VVB_4$ lectin was 500 µg/ml. Bars range from the lowest to the highest dilution resulting in strong agglutination. In the absence of agglutination, the highest antibody concentration tested is indicated by a vertical line.

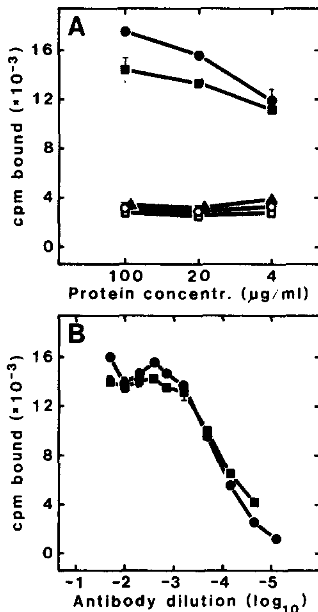


FIGURE 2. Binding of CT1 and CT2 to Tamm-Horsfall glycoprotein in solid-phase RIA. (*A*) $Sda^+$ (*closed symbols*) or $Sda^-$ (*open symbols*) Tamm-Horsfall glycoproteins were used at varying concentrations to çoat polyvinyl plates. Subsequently, a fixed amount of antibody (ascites, diluted 1:100) was added. Bound CT antibody was then detected with radiolabeled second antibody. (*B*) plates were coated with $Sda^+$ Tamm-Horsfall glycoprotein at 10 µg/ml and subsequently incubated with varying dilutions of antibody. Points represent the mean of duplicate assays. Standard deviations are indicated by bars if they amounted to more than 2%. CT1 (○, ●); CT2 (□, ■); F.F6 (▲); F.F6 is an unrelated mAb used as a control.

*Binding of CT1 and CT2 to Human Blood Group $Sda^+$ Antigen.* Although found in the ganglioside series of glycolipids, GalNAc-linked β1,4 to galactose is not commonly found on glycoproteins. Glycophorin A from red blood cells of the rare human blood group Cad contains terminal β1,4-linked GalNAc on some of its *O*-linked oligosaccharides (Table I) (13, 15). The determinant is also present on Tamm-Horsfall urinary glycoprotein of $Sda^+$ individuals whereas the same glycoprotein lacks GalNAc residues in $Sda^-$ individuals (22–24). Thus, binding of CT1 and CT2 to Tamm-Horsfall glycoproteins was assessed by solid-phase RIA. As shown in Fig. 2, both antibodies bound to the $Sda^+$ but not to the $Sda^-$ glycoprotein. The specific binding was dependent on the amount of $Sda^+$

TABLE I
*Reactivity of CT mAb with Human Blood Group Structures*

| Structure | Origin | CT reactivity | $VVB_4$ reactivity |
|---|---|---|---|
| GalNac$\alpha$1-3Gal$\beta$1-3/4GlcNAc <br> \| $\alpha$1-2 \| $\alpha$1-4 <br> Fuc Fuc | Blood group A | − | − |
| Gal$\alpha$1-3Gal$\beta$1-3/4GlcNAc <br> \| $\alpha$1-2 \| $\alpha$1-4 <br> Fuc Fuc | Blood group B | − | − |
| Gal$\beta$1-3/4GlcNAc <br> \| $\alpha$1-2 \| $\alpha$1-4 <br> Fuc Fuc | Blood group O | − | − |
| Gal$\beta$1-3GalNAc$\alpha$1-Ser/Thr <br> \| $\alpha$2-3 \| $\alpha$2-6 <br> SA SA | Main *O*-linked structure of glycophorin A | − | − |
| GalNAc$\alpha$1-Ser/Thr | Main O-linked structure of glycophorin A in Tn erythrocytes (32) | − | + |
| GalNAc$\beta$1-4Gal$\beta$1-3GalNAc$\alpha$1-Ser/Thr <br> \| $\alpha$2-3 \| $\alpha$2-6 <br> SA SA | Main O-linked structure of glycophorin A in Cad erythrocytes (8) | + | + |
| GalNAc$\beta$1-4Gal$\beta$1-4GlcNAc$\beta$1-3Gal-. . . <br> \| $\alpha$2-3 <br> SA | Sda + pentasaccharide from Tamm-Horsfall glycoprotein (13) | + | NT |

NT, not tested.

glycoprotein attached to the plate, whereas no such dose-dependent binding could be detected with the $Sda^-$ glycoprotein. Titration curves of both antibodies were similar and binding was detectable at 50,000-fold diluted ascites (Fig. 2*B*). At plateau levels, ~100 ng of $^{125}$I-labeled rabbit anti–mouse Ig were specifically bound. This binding could not be blocked by the addition of 0.2 M GalNAc to the reaction. Thus, it was likely that the CT1 and CT2 mAb recognized the GalNAc$\beta$1,4[SA$\alpha$2,3]Gal determinant. Moreover, the determinant appears to be recognized independently of the protein core to which it is attached and suggests that the CT determinants can be carried by *N*-linked oligosaccharides, since Tamm-Horsfall glycoproteins are believed not to contain *O*-linked oligosaccharides (22).

*Interaction of CT1 and CT2 with Cad Erythrocytes.* CT1 and CT2 were tested as agglutinins of erythrocytes of type A, B, O, Cad, and Tn which carry the antigenic carbohydrate determinants depicted in Table I. As illustrated by Fig. 3, we did not observe any agglutination of A, B, O, or Tn erythrocytes whereas Cad cells were strongly agglutinated. ~95% of Cad cells were agglutinated indicating that the CT1 and CT2 determinants were carried by most cells. The $VVB_4$ isolectin agglutinated Cad as well as Tn, but not A, erythrocytes (25). To ensure that the nonagglutinated erythrocytes were not binding subagglutinating concentrations of mAbs, type A, B, or O erythrocytes were used to absorb a limiting amount of CT1 mAb. After absorption, the unbound antibody was quantitated by the solid-phase RIA on $Sda^+$ Tamm-Horsfall glycoprotein. Erythrocytes from A, B, or O donors did not absorb any CT1 antibody at $3 \times 10^9$

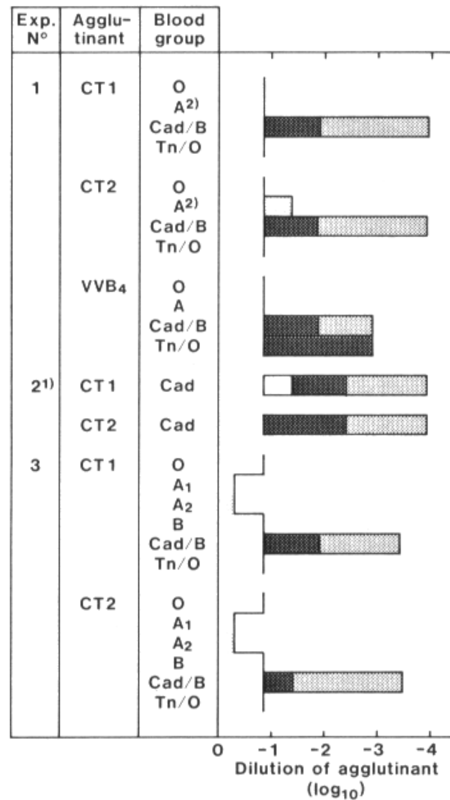


FIGURE 3. Agglutination of erythrocytes by CT1 and CT2. (*Dark bars*) heavy agglutination; (*light bars*) agglutination of part of the cells into small clumps (two to six erythrocytes). (*Stippled areas*) loose aggregates observed. (*1*) Experiment was done at room temperature. (*2*) The A donor in Exp. 1 was not typed as $A_1$ or $A_2$ (17).

cells/ml, whereas Cad erythrocytes absorbed the antibody completely (data not shown). Furthermore, significant binding of the CT mAbs to type A, B, or O erythrocytes was not detected using fluorescence flow cytometry (data not shown).

The fact that CT1 and CT2 did not agglutinate Tn erythrocytes suggested that they were unable to bind to GalNAc$\alpha$1-Ser/Thr structures (Fig. 3). This was confirmed by using native and desialylated bovine submaxillary mucin as an inhibitor in the RIA on $Sda^+$ Tamm-Horsfall glycoprotein. Bovine submaxillary mucin contains SA$\alpha$2–6GalNAc $\alpha$1-Ser/Thr as the predominant *O*-linked oligosaccharide structure. 50% inhibition of the binding of CT1 and CT2 to $Sda^+$ Tamm-Horsfall glycoprotein by the $Sda^+$ glycoprotein was achieved at $3.8 \times 10^{-7}$ and $10^{-6}$ M, respectively (molarities in terms of GalNAc residues), whereas the desialylated bovine submaxillary mucin required 1,400-fold (CT1) or 350-fold (CT2) higher concentrations (data not shown). Moreover, since there was no difference in the inhibitory capacity between native and desialylated mucin we conclude that the CT1 antibody does not react with the GalNAc$\alpha$1-Ser/Thr structure. The results thus far strongly suggested that the CT determinants were contained within the unique oligosaccharide structure found on Cad erythrocytes (Table I). This was confirmed by the demonstration that the CT mAbs react with purified $Cad^+$ gangliosides (Cartron, J. P., personal communication).

*Requirement for Sialic Acid in CT Determinants.* To determine if sialic acid was

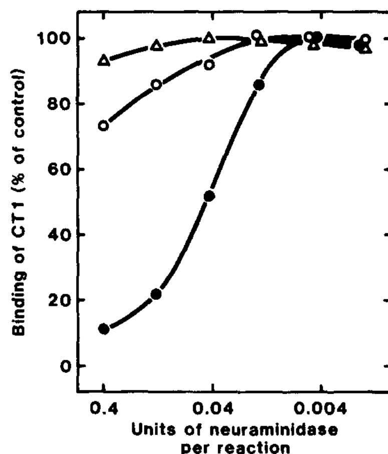


FIGURE 4. Requirement for sialic acid in CT1 determinants. Aliquots of detergent lysate made from CTL clone B3.3 (equivalent to $10^6$ cells) were incubated for 18 h at 37°C with neuraminidase at the indicated concentrations (●). The neuraminidase inhibitor, 2-3-dehydro-2-deoxy-*N*-acetylneuraminic acid, was added at 1 mM (○) or 10 mM (△). After incubation, the samples were acetone precipitated and the resulting material was dot-blotted onto nitrocellulose followed by detection using CT1 mAb, rabbit anti-mouse IgG and $^{125}$I-labeled protein A.

TABLE II
*Effect of Sialidase Treatments on CT1 Reactivity*

| Enzyme | Cells | Control binding | | |
|---|---|---|---|---|
| | | Exp. 1 | Exp. 2 | Exp. 3 |
| | | | % | |
| Neuraminidase (*V. cholerae*) (10 U/ml) | B3.3 | 86 | 82 | |
| Neuraminidase (*Cl. perfringens*) (5 U/ml) | B3.3 | 11 (95) | 11 (93) | 8 |
| | B6.1 | 2 (89) | 3 | |
| | Cad | 20 (94) | 15 | |

Aliquots of detergent lysate (100 µl, equivalent to $10^6$ lymphocytes or $2 \times 10^7$ erythrocytes) were incubated for 18 h at 37°C with or without the addition of sialidases. After acetone precipitation, the resulting material was dot-blotted onto nitrocellulose followed by detection using CT1, rabbit anti-mouse IgG and $^{125}$I–protein A. Numbers in parentheses indicate the binding of CT1 after enzyme treatments performed in the presence of the neuraminidase inhibitor, 2-3-dehydro-2-deoxy-*N*-acetylneuraminic acid, added to 10 mM.

involved in the antigenic determinant recognized by the CT antibodies, we treated cell lysates from CTL clone B3.3 with neuraminidase and quantitated the remaining CT1 reactivity using a dot-blot assay. The CT2 antigen could not be assessed because the CT2 mAb is extremely inefficient in Western blotting. Initially, neuraminidase from *V. cholerae* was used and proved ineffective at altering CT binding (see Table II). However, when neuraminidase from *C. perfringens* was used, nearly complete abrogation of CT1 binding was achieved (Fig. 4; Table II). To insure that neuraminidase activity was the activity being measured in these assays, the neuraminidase inhibitor, 2-3-dehydro-2-deoxy-*N*-acetylneuraminic acid was added to the reaction. As is shown in Fig. 4, a concentration of 1 mM of this inhibitor greatly reduced the removal of the CT1 determinant and a concentration of 10 mM of the inhibitor completely blocked removal of CT1 antigen by *C. perfringens* neuraminidase. Thus, sialic acid appears to be an integral part of the CT1 antigenic determinant in clone B3.3.

It has previously been demonstrated that terminal, unsubstituted sialic acid

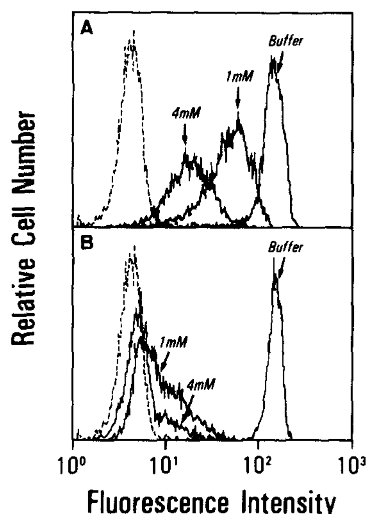


FIGURE 5. Positions 7 to 9 of sialic acid are important for CT determinants. Intact CTL clones were incubated at 4°C in the dark with 1 mM or 4 mM sodium-*m*-periodate dissolved in PBS. The cells were then reacted with CT1 (*A*) or CT2 (*B*) and subsequently stained with FITC-labeled goat anti–mouse IgG. Individual cells were analyzed by fluorescence flow cytometry. Stippled line: untreated cells with secondary antibody only.

TABLE III
*Periodate Sensitivity of CT Antigens on CTL Clone B6.1 and Cad Erythrocytes*

| Cells | Periodate pretreatment | CT1 | CT2 |
|---|---|---|---|
| | *mM* | | |
| B6.1 | None | 122 | 185 |
| | 1 | 116 | 29 |
| | 4 | 48 | 5 |
| Cad | None | 11 | 11 |
| | 1 | 0.7 | 0.5 |
| | 4 | 0.3 | 0.16 |

Cells were periodate treated as described in Fig. 5 and stained with CT1 and CT2 mAb followed by FITC-labeled rabbit anti–mouse IgG. Individual cells were analyzed by fluorescence flow cytometry. Numbers represent mean fluorescence intensity per cell on an arbitrary linear scale.

can be selectively oxidized between the exocyclic 7-,8- and 9-hydroxyl positions by low concentrations of periodate under mild conditions (20, 26, 27). However, if the sialic acid has an acetyl group at positions 8 or 9, this reaction is blocked (28, 29). We used mild periodate treatment to find out if positions 7 to 9 of sialic acid were necessary for the binding of the CT antibodies. Results for clone B3.3 are shown in Fig. 5 and for clone B6.1 in Table III. Treatment of intact CTL clones with 1 mM periodate significantly reduced CT1 binding and nearly completely abrogated the binding of CT2, indicating that sialic acid was also required for formation of the CT2 determinant. Increasing the concentration of periodate to 4 mM further reduced CT1 binding, while CT2 exhibited little binding above background after this treatment. In Cad erythrocytes binding of both antibodies was nearly completely abolished by treatment with 1 mM periodate. The binding of mAbs against the T200 glycoprotein (which bears the CT determinants of CTL clones) was not affected by these treatments (data not

shown). This result indicates that positions 7 to 9 of the sialic acid involved in the CT determinant are important for the binding of these antibodies. In addition, periodate treatment affected CT2 binding more readily than CT1 binding. Endoglycosidase F, when added to a lysate of B3.3 cells did not affect the subsequent binding of the CT1 antibody, although immunoprecipitation of endoglycosidase F–treated [$^{35}$S]methionine-labeled material showed a significant reduction in apparent molecular weight of the CT1-reactive proteins (data not shown). This suggested that the CT1 determinant is not present on *N*-linked glycans on B3.3 cells.

## Discussion

This study demonstrated that the CT1 and CT2 mAbs react with the GalNAc$\beta$1,4-[SA$\alpha$2,3]Gal carbohydrate determinant. Three independent sources containing $\beta$1,4-linked GalNAc residues were recognized by the CT mAb: the murine CTL-clone B6.1, the human blood group Cad antigen, and the Tamm-Horsfall urinary glycoprotein from Sda$^+$ individuals. The corresponding sources that lacked the GalNAc residue (B6.1.VV6, non-Cad erythrocytes, Sda$^-$ glycoprotein) did not bind these antibodies. Sialic acid was an integral part of the antigenic structure as well, since sialidase from *C. perfringens* destroyed the CT1 antigens of B3.3 cells, whereas sialidase from *V. cholerae* was ineffective. It appeared likely that the antigenic sialic acid was attached to the subterminal galactose residue (see Table I), since sialic acid in this position is resistant to *V. cholerae* but not *C. perfringens* sialidase (30) and since on Sda$^+$ Tamm-Horsfall glycoprotein no other sialic acid is present in the vicinity of the $\beta$1,4-linked GalNAc residue. The destruction of CT antigens by mild periodate oxidation further demonstrated the importance of sialic acid for the CT determinants. The structures recognized by the CT mAb were periodate-sensitive both in CTL clones and Cad erythrocytes. Since the conditions used here for periodate treatment have been shown to result in oxidation only between the exocyclic 7-, 8-, and 9-hydroxyl positions of terminal, unsubstituted sialic acid residues (19, 25, 26), it appeared that these positions were important for CT mAb binding.

It is possible that, on CTL clones, the CT mAbs also recognize other *O*-linked oligosaccharides, since on the B6.1 CTL line the Cad oligosaccharide was only found as one component among several *O*-linked structures that all carried $\beta$-linked GalNAc residues at their nonreducing terminus (12). Although isolated in their desialized form, all these structures probably carried sialic acids in their native form since the UdP-GalNAc:$\beta$-galactose $\beta$1,4-*N*-acetylgalactosaminyltransferase is only functional with sialylated acceptors (14, 31).

It is also interesting to note the cellular and tissue distribution of this enzyme. Similar to the expression of the CT antigens, the activity of the $\beta$1,4-GalNAc transferase is much higher in CTL clones than in T helper cell clones (32). Morever, the enzyme is found at high levels in the small intestine, where the CT antigens are found on intraepithelial lymphocytes and the CT2 antigen is found on the majority of intestinal epithelial cells (3, 4). The enzyme is also detectable in kidney extracts, while the CT2 but not the CT1 determinant is also found on discrete structures within the kidney (Lefrancois, L., unpublished data). Although the precise differences between the CT1 and CT2 determinants is not known,

the distribution of the β1,4-GalNAc transferase and the results presented here strongly suggest that this enzyme is involved in the formation of the oligosaccharide moieties required for expression of both the CT1 and CT2 antigens.

A substrate for the β1,4-GalNAc transferase in the immune system is the T200 glycoprotein, which bears the CT determinants in $CT^+$ lymphocytes (1, 2, 5). Furthermore, IL-2 can induce posttranslational modifications of the T200 glycoprotein resulting in the expression of the CT determinants on cytotoxic T cells and thus may be regulating CT antigen expression at the level of this enzyme (8, 33). In addition, recent results indicate that lymphocyte subset-specific expression of portions of the T200 protein occurs via variable usage of exons encoding regions near the $NH_2$-terminus of the protein (34–37). These regions are thought to contain the majority of *O*-linked glycans of T200 (35) and may be a second level of regulation of CT determinant expression since CTL express a larger T200 mRNA than T helper cells and CTL clones are $CT^+$ while T helper clones are not (38).

A functional role for the CT determinants has yet to be demonstrated. However, the restricted expression of these antigens suggest a possible role in differentiation of lymphocyte subsets. During fetal development, high levels of the CT antigens are present on nearly all early fetal thymocytes with a decrease in the number of positive cells and a concomitant decrease in expression levels of the CT antigens as birth approaches (5). The CT1 antigen is found only on a subset of $Lyt\text{-}2^-$,$L3T4^-$ fetal thymocytes while the CT2 antigen is expressed on these cells as well as some $Lyt\text{-}2^+$,$L3T4^+$ fetal thymocytes. The only mature peripheral T cells that have been found to express the CT antigens are a subset of intraepithelial lymphocytes in the small intestine (3, 4). All of these cells are $Lyt\text{-}2^+$,$L3T4^-$ and a proportion are $Thy\text{-}1^-$. We can speculate that the CT determinants may play a role in the development of this branch of the mucosal immune system, perhaps by influencing differentiation during thymus ontogeny or by being involved in the homing of specific cells to the gut mucosa. Current studies are centered around a functional assignment of the CT antigens and the T200 glycoproteins.

## Summary

The CT antigenic determinants have previously been shown to be present on the T200 glycoproteins and other proteins of murine cytotoxic T cell clones but not of T helper clones or nonactivated lymphocytes (1, 2). Two determinants recognized by mAbs CT1 and CT2 are also expressed on thymocytes in a developmentally regulated fashion during fetal thymus ontogeny and are found in a subset of $Lyt\text{-}2^+$ intraepithelial lymphocytes in the intestinal mucosa (3–5). Previous studies of the biosynthesis of $CT^+$ proteins suggested that these determinants were composed of carbohydrate (8). We now demonstrate that the anti-CT mAbs react with a carbohydrate determinant at the nonreducing terminus of *O*-linked oligosaccharides that has the configuration GalNAcβ1,4[SAα2,3]-galactose. The CT antibodies detected this determinant not only on CTL clones but also in the human blood group antigens Cad and $Sda^+$. Variant CTL lines, non-Cad erythrocytes, and $Sda^-$ glycoproteins that lacked the GalNAc residue did not bind the CT mAb. Sialic acid was essential for CT antigen expression

since neuraminidase or mild periodate treatment abrogated CT antibody binding. In addition, other carbohydrate structures with terminal GalNAc residues such as the A or Tn blood group antigens were not recognized. The CT antibodies thus define GalNAc and sialic acid containing carbohydrate antigens that are expressed on discrete subsets of T lymphocytes and may also be useful reagents for the detection of Cad and $Sda^+$ blood group antigens.

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## References

1. Lefrancois, L., and M. J. Bevan. 1985. Functional modifications of cytotoxic T-lymphocyte T200 glycoprotein recognized by monoclonal antibodies. *Nature (Lond.).* 314:449.
2. Lefrancois, L., and M. J. Bevan. 1985. Novel antigenic determinants of the T200 glycoprotein expressed preferentially by activated cytotoxic T lymphocytes. *J. Immunol.* 135:374.
3. Klein, J. R. 1986. Ontogeny of the Thy-$1^-$,Lyt-$2^+$ murine intestinal intraepithelial lymphocyte. Characterization of a unique population of thymus-independent cytotoxic effector cells in the intestinal mucosa. *J. Exp. Med.* 164:309.
4. Lefrancois, L. 1987. Carbohydrate differentiation antigens of murine T cells: expression on intestinal lymphocytes and intestinal epithelium. *J. Immunol.* 138:3375.
5. Lefrancois, L. 1987. Expression of carbohydrate differentiation antigens during ontogeny of the murine thymus. *J. Immunol.* 139:2220.
6. Edelman, G. M. 1985. Cell adhesion and the molecular processes of morphogenesis. *Annu. Rev. Biochem.* 54:135.
7. Feizi, T. 1985. Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. *Nature (Lond.).* 314:53.
8. Lefrancois, L., L. Puddington, C. E. Machamer, and M. J. Bevan. 1985. Acquisition of cytotoxic T lymphocyte-specific carbohydrate differentiation antigens. *J. Exp. Med.* 162:1275.
9. Kimura, A., H. Wigzell, G. Holmguist, B. Ersson, and P. Carlsson. 1979. Selective affinity fractionation of murine cytotoxic T lymphocytes (CTL). Unique lectin specific binding of the CTL associated surface glycoprotein, T145. *J. Exp. Med.* 149:473.
10. Kimura, A., and H. Wigzell. 1978. Cell surface glycoproteins of murine cytotoxic T lymphocytes. I. T145, a new cell surface glycoprotein selectively expressed on Lyt-$2^+$ cytotoxic T lymphocytes. *J. Exp. Med.* 147:1418.
11. Conzelmann, A., R. Pink, O. Acuto, J. P. Mach, S. Dolivo, and M. Nabholz. 1980. Presence of T145 on cytolytic T cell lines and their lectin-resistant mutants. *Eur. J. Immunol.* 10:860.
12. Conzelmann, A., and S. Kornfeld. 1984. $\beta$-linked N-acetylgalactosamine residues present at the nonreducing termini of O-linked oligosaccharides of a cloned murine cytotoxic T lymphocyte line are absent in a Vicia villosa lectin-resistant mutant cell line. *J. Biol. Chem.* 259:12528.
13. Blanchard, D., J. P. Cartron, B. Fournet, J. Montrevil, H. Van Halbeek, and J. F. G.

Vliegenthart. 1983. Primary structure of the oligosaccharide determinant of blood group Cad specificity. *J. Biol. Chem.* 258:7691.

14. Conzelmann, A., and S. Kornfeld. 1984. A murine cytotoxic T lymphocyte cell line resistant to Vicia villosa lectin is deficient in UDP-GalNAc:β-Galactose B1,4-N-acetylgalactosaminyltransferase. *J. Biol. Chem.* 259:12536.
15. Blanchard, D., C. Capon, Y. Leroy, J. P. Cartron, and B. Fournet. 1985. Comparative study of glycophorin A derived O-glycans from human Cad, $Sd(a^+)$ and $Sd(a^-)$ erythrocytes. *Biochem. J.* 232:813.
16. Bird, G. W. G. 1952. Relationship of the blood sub-groups $A_1$, $A_2$ and $A_1B_1A_2B$ to hemagglutinins present in the seeds of Dolichos biflorus. *Nature (Lond.).* 120:674.
17. Elhammer, A., and S. Kornfeld. 1984. Two enzymes involved in the synthesis of O-linked oligosaccharides are localized on membranes of different densities in mouse lymphoma BW5147 cells. *J. Cell Biol.* 99:327.
18. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* 234:1971.
19. Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* 1:3.
20. Cheresh, D. A., R. A. Reisfeld, and A. P. Varki. 1984. O-acetylation of disialoganglioside GD3 by human melanoma cells creates a unique antigenic determinant. *Science (Wash. DC).* 225:844.
21. Trowbridge, I. S. 1978. Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. *J. Exp. Med.* 148:313.
22. Soh, C. P. C., W. T. J. Morgan, W. M. Watkins, and A. S. R. Donald. 1980. The relationship between the N-acetylgalactosamine content and the blood group $Sd^a$ activity of Tamm and Horsfall urinary glycoprotein. *Biochem. Biophys. Res. Commun.* 93:1132.
23. Donald, A. S. R., C. P. C. Soh, W. M. Watkins, and W. T. J. Morgan. 1982. N-acetyl-D-galactosaminyl-β-(1,4)-D-galactose: a terminal non-reducing structure in human blood group $Sd^a$-active Tamm-Horsfall urinary glycoprotein. *Biochem. Biophys. Res. Commun.* 104:58.
24. Donald, A. S. R., A. D. Yates, C. P. C. Soh, W. T. J. Morgan, and W. M. Watkins. 1983. A blood group $Sd^a$-active pentasaccharide isolated from Tamm-Horsfall urinary glycoprotein. *Biochem. Biophys. Res. Commun.* 115:625.
25. Tollefsen, S. E., and R. Kornfeld. 1987. The $B_4$ lectin from *Vicia villosa* seeds interacts with N-acetyl galactosamine residues α-linked to serine or threonine residues in cell surface glycoproteins. *J. Biol. Chem.* 258:5172.
26. Van Lenten, L., and G. Ashwell. 1971. Studies on the chemical and enzymatic modification of glycoproteins. *J. Biol. Chem.* 246:1889.
27. Mclean, R. L., M. Suttajit, J. Beidler and R. J. Winzler. 1971. N-acetyl neuraminic acid analogues. I. Preparation of the 8-carbon and 7-carbon compounds. *J. Biol. Chem.* 246:803.
28. Shukla, A. K., and R. Schauer. 1982. Fluorimetric determination of unsubstituted and 9(8) O-acetylated sialic acids in erythrocyte membranes. *Hoppe-Seyler's Z. Physiol. Chem.* 363:255.
29. Varki, A., and S. Kornfeld. 1980. An autosomal dominant gene regulates the extent of 9-O-acetylation of murine erythrocyte sialic acids. A probable explanation for the variation in capacity to activate the human alternate complement pathway. *J. Exp. Med.* 152:532.
30. Corfield, A. P., and R. Schauer. 1982. *In* "Sialic acids, chemistry, metabolism and function," R. Schauer, editor. Springer-Verlag New York Inc., New York 229–230.

31. Piller, F., D. Blanchard, M. Huet, and J. P. Cartron. 1986. Identification of a α-neuAc (2 → 3)-β-D-galactopyranosyl N-acetyl-β-D-galactosaminyltransferase in human kidney. *Carbohydr. Res.* 149:171.
32. Conzelmann, A., and C. Bron. 1987. Expression of UDP-GalNAc:β-galactose β1,4-N-acetylgalactosaminyltransferase in functionally defined T cell clones. *Biochem. J.* 242:817.
33. Lefrancois, L., and O. Kanagawa. 1986. Coordinate expression of cytolytic activity and cytotoxic T cell-specific carbohydrate antigens in a T cell hybridoma. *J. Immunol.* 136:1171.
34. Ralph, S. J., M. L. Thomas, C. C. Morton, and I. S. Trowbridge. 1987. Structural variants of human T200 glycoprotein (leukocyte-common antigen). *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1251.
35. Barclay, A. N., D. I. Jackson, A. C. Willis, and A. F. Williams. 1987. Lymphocyte specific heterogeneity in the rat leukocyte common antigen (T200) is due to differences in polypeptide sequences near the $NH_2$-terminus. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1259.
36. Thomas, M. L., P. J. Reynolds, A. Chain, Y. Ben-Neriah, and I. S. Trowbridge. 1987. B-cell variant of mouse T200 (Ly-5): evidence for alternative mRNA splicing. *Proc. Natl. Acad Sci. USA.* 84:5360.
37. Saga, Y., J. S. Tung, F. W. Shen, and E. A. Boyse. 1987. Alternative use of 5′ exons in the specification of Ly-5 isoforms distinguishing hematopoietic cell lineages. *Proc. Natl. Acad. Sci. USA.* 84:5364.
38. Lefrancois, L., M. L. Thomas, M. J. Bevan, and I. S. Trowbridge. 1986. Different classes of T lymphocytes have different mRNAs for the leukocyte-common antigen, T200. *J. Exp. Med.* 163:1337.