ACQUISITION OF CYTOTOXIC T LYMPHOCYTE-SPECIFIC CARBOHYDRATE DIFFERENTIATION ANTIGENS

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The mechanism by which cytotoxic T lymphocytes (CTL)[1] recognize and subsequently destroy target cells remains obscure. This process occurs in three discrete steps: (*a*) recognition and binding of the CTL to the target cell, (*b*) programming for lysis, and (*c*) target cell destruction. One requirement for proper recognition by CTL is the simultaneous expression on the target cell plasma membrane of a specific foreign antigen and a particular histocompatibility molecule. At the CTL level, not only is the T cell receptor for antigen necessary for target cell recognition (1, 2) but a number of other molecules have been implicated in CTL-induced lysis. These include T3, which is noncovalently associated with the T cell antigen receptor (3, 4); Lyt-2 (5, 6) and L3T4 (7), which may confer specificity for a particular class of histocompatibility molecule; and the leukocyte function–associated antigens (8). In each of these cases the binding of antibodies specific for these proteins to the CTL cell surface results in the inhibition of CTL-mediated lysis. However, since all of these molecules are present on naive, noncytolytic CTL precursors, activational processes must occur after a primary encounter with antigen which allow the subsequent expression of cytolytic capability by CTL in an antigen-specific manner.

Recently (9, 10), we have described activation antigens that are associated with CTL (termed CT determinants). Monoclonal antibodies (mAb) specific for these antigens inhibit the function of CTL expressing high levels of CT antigen on their surface. Biochemical analyses revealed that the CT determinants are present on the T200 molecules of murine CTL and on two other unidentified cell surface proteins. Although forms of T200 are found on all T cells and hematopoietic cells except cells of erythroid lineage (11), the CT antigens are expressed only on the T200 glycoprotein of activated CTL. T200 has been previously implicated in natural killer (NK) cell–mediated lysis (12, 13) but its role in the lytic mechanism of CTL remains controversial. The Ly-5 alloantigenic determinants

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; EGF, epidermal growth factor; endo H, endoglycosidase H; HP, *Helix pomatia*; IL-2, interleukin 2; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; NK, natural killer; PBS, phosphate-buffered saline; rIL-2, recombinant IL-2; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Th, T helper; VV, *Vicia villosa*.

are present on the T200 molecule (14, 15), and anti-Ly-5 sera has been shown in some cases (5, 16), but not others (17, 18), to inhibit CTL-mediated lysis. mAb specific for T200 have generally been unable to inhibit CTL function (9, 19) but this result may be due to the epitope specificity of the individual mAb (20). Although involvement of T200 in other immunological mechanisms including B cell differentiation has been suggested (21), the precise function of the T200 glycoprotein remains undefined. A recent report (22) indicated that the T200/Ly-5 molecule may have an autoproteolytic activity.

These findings led us to investigate the maturational alterations of CTL T200 during activation that lead to CT antigen expression. Our previous results (10) indicated that, during in vitro activation of CTL, progressive modifications of the T200 glycoprotein occur that result in an increase in the apparent molecular weight and the number of different T200 molecules present on the cell surface. During this process the CT determinants appear early after activation at relatively low levels and gradually increase in concentration during prolonged culture in the presence of soluble factors. This increase in expression occurs concomitant with the apparent molecular weight changes observed in the T200 glycoproteins. We now report that the CT determinants are carbohydrate in nature and that their expression appears to be regulated by interleukin 2 (IL-2). Furthermore, the character of the carbohydrate present on the cell surface of CTL appears to be dramatically different than that of T cells of the T helper (Th) lineage, particularly in regard to O-linked glycans. Thus, our results demonstrate the presence of cell-type–specific oligosaccharides whose expression is hormonally regulated and functional in nature.

Materials and Methods

Mice. C57BL/6J (B6, H-2^b) and DBA/2 (H-2^d) were obtained from the vivarium at Scripps Clinic and Research Foundation.

Monoclonal Antibodies. mAb used for these studies were: 13-4, anti-Thy-1.2 (23); anti-Lyt-2 (24); and I3/2.3, anti-T200 (25; generously provided by Dr. Ian Trowbridge, The Salk Institute). The production and characterization of the CT mAb have been previously described (9, 10). CT1 and CT2 have been typed as IgM, κ light chain type.

Cell Lines. The CTL clones used here have been previously described (10). CTL clone H7 was derived from a C57BL/6J mouse and is specific for a BALB minor antigen recognized in conjunction with H-$2K^b$. The noncytolytic Th clone BB5, which is Mls reactive, was kindly provided by Dr. Andrew Glasebrook, Eli Lilly Research Co., La Jolla, CA. Other cell lines used were P815 (H-2^d, mastocytoma) and EL4 (H-2^b, thymoma).

Mixed Lymphocyte Cultures (MLC). Primary in vitro cultures were established by coculturing 5×10^6 spleen cells with 5×10^6 irradiated (2,000 rad from a ^{137}Cs source) stimulator spleen cells in 2 ml of RPMI 1640 medium with 5% fetal calf serum with or without the addition of purified recombinant human IL-2 (rIL-2) (Cetus Corp., Emeryville, CA) in 24-well tissue culture plates (Costar, Cambridge, MA). The IL-2 concentration was equivalent to the level of IL-2 in 5% supernatant from concanavalin (Con A)-stimulated rat spleen cells (66 U/ml). Long-term cultures were propagated by culturing 5×10^5 to 1×10^6 cells from primary MLC with 5×10^6 irradiated stimulator cells in 2 ml cultures and restimulating every 7 d.

Immunofluorescent Staining. Viable cells were obtained by passage of cell populations over Ficoll-Isopaque (26). After washing, the cells were resuspended in phosphate-buffered saline (PBS)–0.1% bovine serum albumin (BSA)–0.1% NaN_3 (containing 1 mM $CaCl_2$ and $MgSO_4$) at a concentration of 1×10^7/ml. 1×10^6 cells were incubated with 100 μl of a 1:100 dilution of ascites fluid containing the primary mAb at 4°C followed by incubation

with an appropriate dilution of the secondary fluorescein-conjugated antibody that was either an affinity-purified $F(ab')_2$ rabbit anti-mouse Ig reagent (Cappel Laboratories, Cochranville, PA) or a goat anti-rat Ig reagent (Cappel Laboratories). The cells were then washed three times and resuspended at 1×10^6 cells/ml for cytofluorimetric analysis. For staining with lectins, fluorescein isothiocyanate-labeled lectins were used (E-Y Laboratories, San Mateo, CA). Relative fluorescent intensities of individual cells were measured using the FACS IV (Becton Dickinson Immunocytometry Systems, Mountain View, CA) equipped with a 5 W argon laser. Forward angle light scatter was used to exclude dead and aggregated cells. The results are presented as fluorescence histograms with the relative number of cells on a linear scale plotted vs. the relative fluorescence intensity on a logarithmic scale, both in arbitrary units.

Cell Surface Periodate Treatment. Viable CTL clone H7 cells were fixed with 3% (wt/vol) paraformaldehyde in PBS and subsequently washed in PBS–0.1% glycine. The fixed cells were then incubated for 2 h at 37°C in 0.05 M sodium acetate buffer, pH 5.5, with or without the addition of 0.08 M sodium *m*-periodate. The cells were then washed and stained as described above.

Radioimmunoprecipitation. Cell surface proteins were radioiodinated by the lactoperoxidase method (27). The cells were first centrifuged over Ficoll-Isopaque to remove residual stimulator cells and dead cells and, in some cases, again centrifuged over 30% Percoll to further remove debris (28). For iodination, cells were suspended at 1×10^7/ml in PBS/5 mM glucose. $Na^{125}I$ (1 mCi/10^7 cells), lactoperoxidase (20 μg/ml), and glucose oxidase (0.1 IU/ml) were added in succession and the mixture was incubated for 20 min at room temperature. For metabolic labeling, CTL clones (1×10^7/ml) were incubated at 37°C in methionine-free RPMI for 30 min before addition of [^{35}S]methionine (250 μCi/ml). For pulse labeling, the cells were harvested after 5 min, an aliquot taken and placed on ice, and the remainder of the cells incubated at 37°C in medium containing 10 mM methionine for the indicated times. For inhibition of N-linked glycosylation, the cells were incubated at 37°C in medium containing 5 μg/ml tunicamycin for 2 h before depletion of methionine for 30 min and the addition of [^{35}S]methionine (250 μCi/ml) for an additional 2 h incubation. After washing, the cells were resuspended in RIPA buffer (10 mM Tris, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml BSA, and 100 Kallikrein units of aprotinin per milliliter) and incubated on ice for 30 min. The lysates were centrifuged at 50,000 *g* for 20 min and precleared twice with fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA). Aliquots of the supernatant were incubated with CT1 or I3/2.3 or, as a control, bovine gamma globulin coupled directly to Sepharose 4B. After incubation at 4°C the immune complexes were washed extensively with RIPA buffer. For removal of high mannose oligosaccharides the washed precipitates were boiled in 50 mM Tris-HCl, pH 6.8, and 1% SDS, followed by the addition of 0.15 M sodium citrate, pH 5.5, with or without the addition of endoglycosidase H (endo H) (Miles Laboratories, Inc., Naperville, IL) at a concentration of 2 μg/ml. Digestion was carried out for 16 h at 37°C. The precipitates were disrupted in sample buffer (40% glycerol, 4% SDS with 5% 2-mercaptoethanol) and analyzed by SDS/polyacrylamide gel electrophoresis (SDS-PAGE) (29), using as acrylamide stock a 30% (wt/vol) acrylamide, 0.17% *bis*-acrylamide solution, followed by fluorography (30). After drying, the gels were subjected to autoradiography at −70°C using Kodak XAR-5 x-ray film and Dupont Cronex intensifier screens.

Limited Proteolysis and Peptide Mapping. Partial proteolysis of proteins was carried out with *S. aureus* V8 protease (Miles Laboratories, Inc.) by the method of Cleveland et al. (31). Immunoprecipitated proteins were separated on a 7.5% acrylamide gel and, after drying of the gel and autoradiography, the labeled proteins were excised and rehydrated in 125 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS, 1 mM 2-mercaptoethanol, and 30% glycerol. The rehydrated gel slices were placed in wells of a 15% polyacrylamide gel and overlaid with buffer containing *S. aureus* V8 protease (2 μg/slice). The samples were run into the stacking gel, electrophoresis was stopped, and, after 30 min, electrophoresis was started. The gels were processed as described above.

Results

Proteins Precipitated by CT mAb Are Not Related at the Peptide Level. Our initial characterization of the two CT antigen-reactive mAb, CT1 and CT2, indicate that these mAb bind to nonidentical but overlapping epitopes (10). CT2 also differs from CT1 in that it functions poorly in immunoprecipitation whereas CT1 is very efficient. Therefore, we used CT1 in all of the biochemical characterizations described here. We have previously shown (9) that the CT1 mAb immunoprecipitates from ^{125}I surface-labeled CTL clones five major proteins with apparent molecular weights (M_r) of 240,000 (240 K), 220, 200, 140, and 85 K. A minor protein of M_r 190 K is also sometimes observed (Fig. 1*A*). Sequential immunoprecipitation revealed that the proteins of M_r 240, 220, and 200 K bore determinants recognized by a T200-specific mAb, while the 140 and 85 K proteins did not (9). Western blot analysis indicated that all five proteins contained antigenic determinants recognized by CT mAb. When the CTL clone, H7, was labeled with [^{35}S]methionine and immunoprecipitation was carried out with the CT1 mAb, three high molecular weight proteins (M_r 240, 220, and 200 K) were precipitated, as well as a protein of M_r 155 K (gp155) that was not

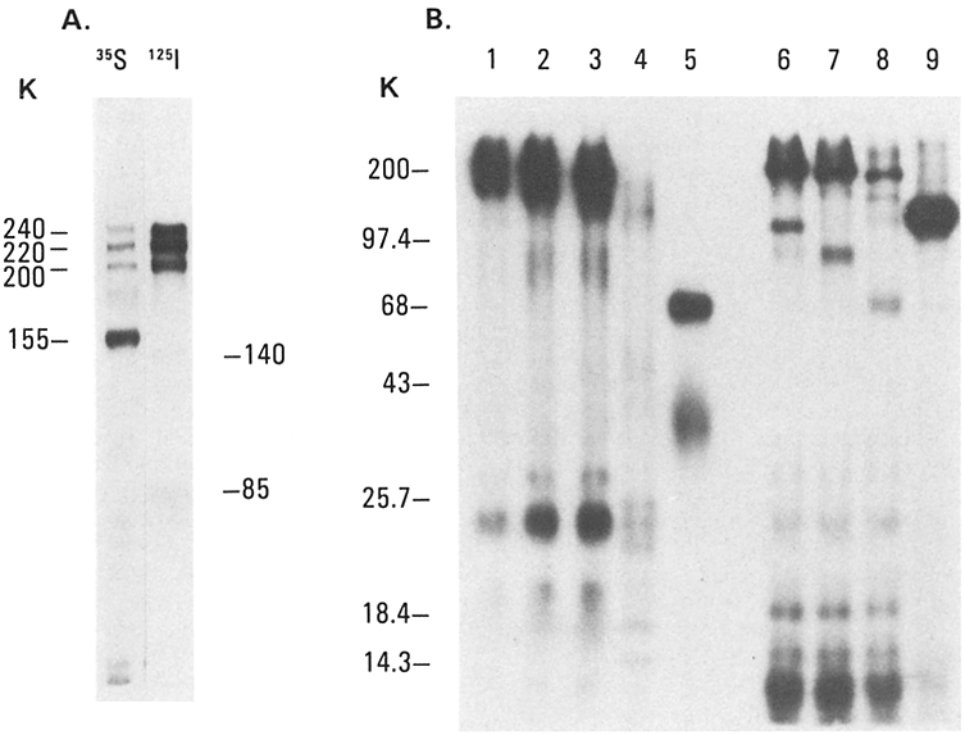

FIGURE 1. Limited proteolysis of CT reactive proteins. CTL clone H7 was surface labeled with ^{125}I or metabolically labeled with [^{35}S]methionine for 2 h as described in Materials and Methods. Immune precipitation was carried out with mAb CT1 coupled directly to Sepharose 4B. The precipitated proteins were separated on a 7.5% SDS-polyacrylamide gel (*A*), excised from the gel, and digested with 2 μg per lane of *S. aureus* V8 protease. (*1–5*) ^{125}I-labeled proteins: (*1*) M_r 240 K; (*2*) 220 K; (*3*) 200 K; (*4*) 140 K; (*5*) 85 K. (*6–9*) [^{35}S]methionine-labeled proteins: (*6*) 240 K; (*7*) 220 K; (*8*) 200 K; (*9*) 155 K.

observed in precipitates from surface-labeled cells (Fig. 1 *A*). In some cases, after prolonged exposure we observed the M_r 85 K protein but not the 140 K protein in precipitates from [^{35}S]methionine-labeled cell extracts. To determine if the CT-reactive proteins were closely related at the protein level and thus perhaps contained a crossreactive determinant, we obtained peptide maps. Both the ^{125}I surface-labeled (Fig. 1 *A*) and the [^{35}S]-labeled proteins were digested using *S. aureus* V8 protease and the resulting peptides separated by SDS-PAGE. The ^{125}I-labeled T200 proteins of M_r 240, 220, and 200 K gave virtually identical maps, with a number of low molecular weight peptides evident (Fig. 1 *B*, *1–5*). This agrees well with previously published peptide maps of the T200 glycoprotein (33). Digestion of the ^{125}I-labeled, M_r 140 K protein (Fig. 1 *B*, *4*) resulted in the appearance of peptides that comigrated with peptides of the T200 proteins but also in the appearance of new peptides. Thus, it was possible that this protein was related to the T200 glycoprotein. However, digestion of the ^{125}I-labeled M_r 85 K protein (Fig. 1 *B*, *5*) yielded a single large peptide of M_r ~35 K, suggesting that this protein was not related to T200. In addition, although the [^{35}S]-methionine-labeled T200 proteins gave similar peptide maps, gp155 was virtually undigested under identical conditions (Fig. 1 *B*, *6–9*). These results suggest that the antigenic determinants recognized by the CT mAb may not be protein in nature, since proteins unrelated at the peptide level are CT reactive. It is interesting to note that unique high molecular weight peptides are also observed upon digestion of the ^{35}S-labeled, M_r 240, 220, and 200 K proteins. These differences could be accounted for by heterogeneous glycosylation, proteolytic processing, or primary amino acid differences.

Carbohydrate Is Required for the Expression of CT Determinants. Because some of the proteins reactive with the CT mAb were not related at the peptide level, it was possible that the CT antigens were the result of other modifications of the CT$^+$ proteins, such as the addition of oligosaccharides during protein processing. This possibility was examined by treating the cell surface of CTL clone H7 with sodium *m*-periodate, which preferentially oxidizes carbohydrate beginning with sialic acid residues (34), and then determining the level of CT mAb binding by fluorescence flow cytometry (Fig. 2). Periodate treatment of the cell surface did not significantly affect the binding of the anti-T200 mAb, indicating that the T200 protein was not destroyed by periodate treatment (Fig. 2, *A* vs. *B*). In contrast, while the CT mAb bound at very high levels to the control-treated CTL clone (Fig. 2 *A*), >90% of their reactivity was lost after cell surface periodate treatment (Fig. 2 *B*), suggesting that carbohydrate was necessary for CT antigen expression.

Further evidence for the necessity of carbohydrate in CT determinant expression was obtained by performing immunoprecipitations from [^{35}S]methionine-labeled, tunicamycin-treated CTL clone H7. Clone H7 was pretreated for 2 h with tunicamycin, incubated in methionine-free medium for 30 min, and incubated for a further 2 h in medium containing [^{35}S]methionine with or without the addition of tunicamycin. Anti-T200 mAb precipitated the three high molecular weight proteins (M_r 240, 220, and 200 K) from lysates of untreated cells (Fig. 3, lane *1*) while three proteins of M_r 165, 155, and 150 K were precipitated by anti-T200 from lysates of tunicamycin-treated cells (Fig. 3, lane *2*). As seen

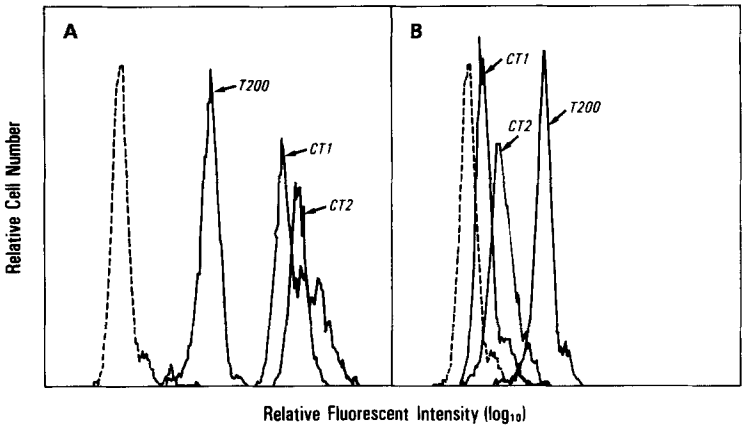

FIGURE 2. Periodate treatment of the cell surface abrogates CT mAb binding. CTL clone H7 was fixed in 3% (wt/vol) paraformaldehyde and incubated at 37°C for 2 h in buffer (0.05 M sodium acetate, pH 5.5) without (*A*) or with (*B*) the addition of 0.08 M sodium *m*-periodate. The cells were then processed for fluorescence analysis as described in Materials and Methods, using 13/2.3 (anti-T200), CT1, or CT2. Dotted lines indicate staining of H7 cells with the fluorescein isothiocyanate–labeled second antibody alone. The cells were then analyzed by fluorescence flow cytometry.

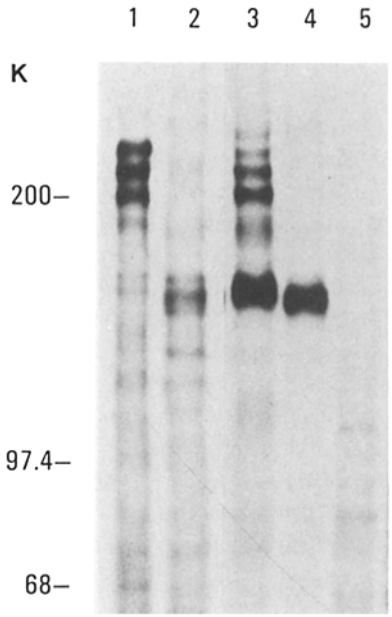

FIGURE 3. Blocking of N-linked glycosylation by tunicamycin prevents CT1 binding to T200 proteins. CTL clone H7 was grown in the presence (*2*, *4*) or absence (*1*, *3*, *5*) of tunicamycin and labeled with [^{35}S]methionine. Immune precipitation was then carried out with 13/2.3 (anti-T200) (*1*, *2*), CT1 (*3*, *4*), or bovine gamma globulin (*5*), coupled to Sepharose 4B, and analyzed on a 7.5% SDS-polyacrylamide gel.

in Fig. 1, CT1 precipitated proteins of M_r 240, 220, 200, and 155 K from untreated cells (Fig. 3, lane *3*). An additional protein of M_r 250 K was also precipitated and is variably present in CT1 precipitates (Fig. 3, lane *3*; see Fig.

4). When precipitates were obtained with CT1 from tunicamycin-treated cells, only a single protein of M_r 150 K was observed and none of the T200 precursor molecules were apparent. Although the T200 precursor proteins could be obscured by the M_r 150 K protein, pulse chase experiments indicated that this was not the case (see below). Therefore, inhibition of N-linked glycosylation of the T200 proteins by tunicamycin results in the loss of CT determinants. Because gp155 still bears CT determinants after blocking of N-linked glycosylation, the CT antigens may be dependent on the addition of O-linked oligosaccharides to glycoproteins. Indeed, when the tunicamycin experiments were repeated using clone H7 grown in [^{3}H]glucosamine, only the gp150 was labeled and not the T200 precursors (data not shown). These results suggest that gp155 is transported normally from the rough endoplasmic reticulum through the Golgi complex despite the lack of N-linked glycosylation, and that T200 is blocked in transport as a result of the loss of N-linked glycans. Examples of both of these situations have been previously observed for a number of proteins (35).

Synthesis of gp155 Suggests CT Determinants Require O-linked Oligosaccharides for Expression. Since gp155 retains the carbohydrate antigens required for CT mAb binding regardless of the addition of N-linked glycosylation, the protein must still be transported within the cell to obtain the glycans necessary for CT antigen expression. We asked whether gp155 was a secreted protein, and, if so, whether it was secreted in the presence of tunicamycin. Culture supernatants from H7 cells labeled with [^{35}S]methionine in the presence or absence of tunicamycin were clarified by centrifugation at 50,000 *g* and immune precipitation was performed. As immunosorbents, CT1, anti-T200, and an *N*-acetyl galactosamine (GalNAc)-binding lectin from *Vicia villosa* (VV) were used. If gp155 receives carbohydrate modifications after blocking of N-linked glycosylation, they may be expected to be of the O-linked type. Since GalNAc has been shown to occur primarily on O-linked glycans, VV could detect these moieties (36, 37). For comparison, a CT1 immunoprecipitate from ^{35}S-labeled H7 cell extracts is shown (Fig. 4, lanes *1*, *2*). Lanes *3* and *4* are precipitates obtained using a control immunosorbent. Precipitation by CT1 of culture supernatant from untreated H7 cells revealed a major protein species of M_r 150 K, indicating that gp155 is secreted (lane *5*). Precipitation by CT1 from supernatants of tunicamycin-treated cells yielded a single protein of M_r 145 K (Fig. 4, lane *6*), showing that the gp155 precursor is still secreted after blocking of N-linked glycosylation. The secreted proteins, in the absence or presence of tunicamycin, are of a slightly lower molecular weight (150 K, 145 K) than that obtained from cell lysates from tunicamycin-treated cells (155K, 150 K), perhaps due to further processing during secretion. When VV was used for precipitation from supernatants of untreated cells, a number of proteins were obtained including one comigrating with gp150 and a protein of M_r 105 K, while VV precipitates from tunicamycin-treated cells contained a single major protein of M_r 145 K. Immunoprecipitation by anti-T200 from H7 supernatants (Fig. 4, lane *9*) or by CT1 from supernatants from a Th cell clone grown under identical conditions (lane *10*) did not result in the isolation of any protein species. Thus, gp155 is a secretory protein that is transported in the absence of N-linked glycosylation and probably contains O-linked glycans that are involved in CT determinant expres-

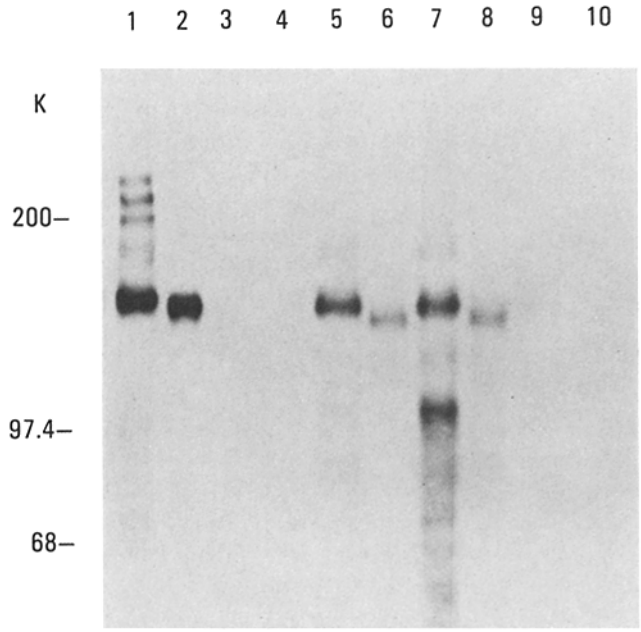

FIGURE 4. Reactivity of secreted proteins with CT1. CTL clone H7 (*1–9*) or noncytolytic Th clone BB5 (*10*) were incubated in medium containing [^{35}S]methionine (250 μCi/ml) for 4 h. Cell lysates (*1–4*) or supernatants (*5–10*) were subjected to immunoprecipitation by CT1 (*1, 2, 5, 6, 10*), *Vicia villosa* (*7, 8*), I3/2.3 (anti-T200) (*9*) or bovine gamma globulin (*3, 4*). Cells were incubated in the presence (*2, 4, 6, 8*) or absence (*1, 3, 5, 7, 9, 10*) of tunicamycin.

sion. The characteristics of gp155 are very similar to a recently described sulphated glycoprotein (38, 39), called entactin, which is associated with the extracellular matrix and basement membrane. In fact, the gp155 described here is also heavily sulphated (data not shown). The significance of this secretory protein for CTL function in vivo is an intriguing issue to be addressed.

CT Carbohydrate Antigens Are Acquired Late in T200 Biosynthesis. Our evidence thus far suggests that CT determinants are the result of the addition of O-linked glycans to T200 and at least three other glycoproteins. To determine the point in protein biogenesis at which the CT determinants were acquired, we performed pulse chase experiments using CTL clones (Fig. 5). The H7 CTL clone was pulsed for 5 min with [^{35}S]methionine and incubated for the indicated times in medium containing an excess of unlabeled methionine. Precipitates obtained with CT1 or anti-T200 were treated with endo H, which removes high mannose type oligosaccharides (40). Since resistance to endo H is acquired after synthesis in rough endoplasmic reticulum during transit to the Golgi complex, this experiment should give an indication of the point at which CT antigens are acquired. Precipitation with anti-T200 after 5 min of labeling gave three proteins of M_r 187, 180, and 173 K (Fig. 5*A*, lane *1*). After treatment of this precipitate with endo H, three proteins of M_r 165, 157, and 150 K were seen, indicating that all of the T200 oligosaccharides at this time are sensitive to removal with endo H (Fig. 5*A*, lane *2*). After 10 min of chase, ~20% of the oligosaccharides were resistant to cleavage by endo H, while 50% were resistant to removal at 20 min

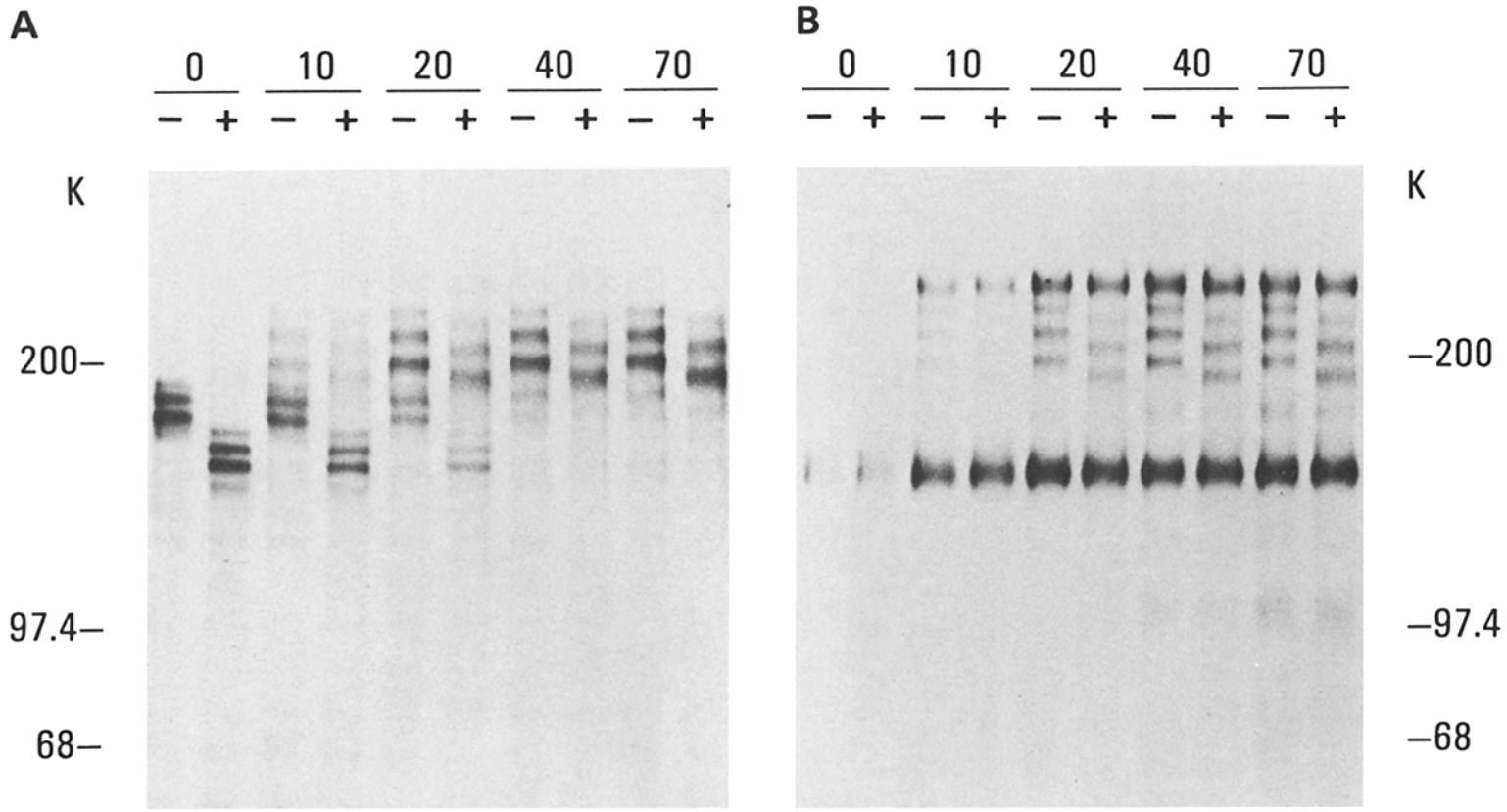

FIGURE 5. CT determinants are acquired late in T200 biosynthesis. CTL clone H7 cells were pulse labeled for 5 min with 250 μCi/ml of [^{35}S] methionine. Cells were harvested immediately or after a chase with medium containing 10 mM unlabeled methionine of 10, 20, 40, or 70 min. Immunoprecipitates (I3/2.3, anti-T200 [*A*]; CT1, [*B*]) from each lysate were divided in half and treated with endo H (+) or mock treated (−) for 16 h at 37°C, followed by analysis on a 7.5% SDS-polyacrylamide gel.

of chase. At later times (40 min, 70 min) a minor decrease (~5 K) in molecular weight of the high molecular weight proteins was observed after endo H treatment. This decrease was also observed when the ^{125}I surface-labeled T200 proteins were treated with endo H (data not shown), indicating that one to two oligosaccharides in the mature form of the protein contain high mannose carbohydrate moieties.

The same lysates used for anti-T200 precipitation were subjected to precipitation with CT1. After 5 min of labeling (Fig. 5*B*, time 0) only a minor amount of gp155 was precipitated with CT1 and its oligosaccharides were resistant to removal with endo H. None of the lower molecular weight precursor forms of T200 were precipitated by CT1 (compare *A* and *B*, time 0, Fig. 5). After 10 min of chase, minor amounts of the mature high molecular weight forms of T200 glycoproteins and none of the earlier precursors were precipitated. The intensity of gp155 was significantly increased at this point and a protein of M_r 250 K was also precipitated (as mentioned, this protein is of unknown identity and is only occasionally observed in precipitates from surface-labeled cells). After 20, 40, and 70 min of chase, all of the high molecular weight forms of T200 were precipitated by CT1 and, again, none of the less mature forms were observed. Thus, the CT determinants appear to be acquired relatively late in T200 biogenesis, concomitant with or after the acquisition of endo H–resistant oligosaccharides. This result agrees with our contention that O-linked glycans constitute the CT antigen, since the terminal stages of O-linked glycosylation are thought to occur in the Golgi complex, at which time most N-linked oligosaccharides are resistant to removal by endo H (41).

Differences in Cell Surface Carbohydrate Between Cytotoxic and Th Cells. Since the CT antigens require carbohydrate for expression and the CT mAb bind only to CTL and not to Th phenotype T cells, we wished to determine if more generalized differences in cell surface oligosaccharides are evident in comparisons between CTL and Th. To address this question, saturating concentrations of fluorescein-conjugated lectins were bound to the surface of CTL or Th clones and the degree of binding was measured by fluorescence flow cytometry (Fig. 6). The lectins used were VV and *Helix pomatia* (HP), both of which are specific for D-GalNAc, and Con A, which is specific for alpha-D-glucose and alpha-D-mannose. As we have previously observed, CTL clones (in this case H7) expressed very high levels of CT antigens (Fig. 6*A*). Significant levels of T200 were also detected (it should be noted that the concentration of T200 cannot be directly compared with CT since a different second-step reagent was used). Similar to CT binding, VV, HP, and Con A bound at high levels on CTL clone H7 (Fig. 6*B*) (this binding was totally inhibited by the appropriate monosaccharides). However, the noncytolytic Th clone, BB5, expressed extremely low levels of CT antigen (Fig. 6*C*). Interestingly, VV and HP binding was also dramatically lower (~10–20-fold lower) to Th clone BB5 than to H7 (Fig. 6*D*). However, BB5 bound Con A at an equivalent level to that observed with H7. Since CT mAb do not react significantly with BB5, but VV and HP do bind at moderate levels, it is clear that the CT mAb have much stricter requirements for binding than the latter two lectins. (This has been confirmed by immunoprecipitation as well; data not shown). Furthermore, since GalNAc is found primarily on O-linked

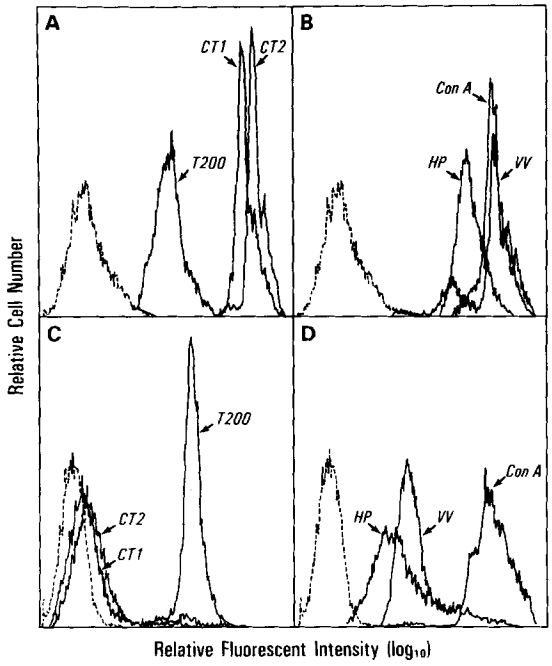

FIGURE 6. Analysis of CT mAb and lectin binding to CTL and Th. CT1, CT2, 13/2.3 (anti-T200), or lectins from VV, HP, or Con A were reacted with CTL clone H7 (*A*, *B*) or Th clone BB5 (*C*, *D*) followed by reaction with a fluorescein isothiocyanate–labeled secondary antibody in the case of the mAb (lectins were directly fluoresceinated). The binding was then measured by fluorescence flow cytometry. Dotted lines indicate staining with the fluorescein isothiocyanate–labeled secondary antibody.

glycans, the majority of carbohydrate differences between CTL and Th appear to be O-linked in character. Using a large panel of lectins, we have now confirmed that the major differences between CTL and Th are associated with increases in GalNAc-containing oligosaccharides (unpublished results).

IL-2 Induces the Expression of CT Determinants. The dichotomy in CT antigen expression observed between CTL and Th suggested an inherent metabolic difference affecting glycosylation between the two cell types. We had previously observed (10) that, during continuous culture of cytolytic mixed lymphocyte cultures (MLC), a marked shift from low to high expression of CT antigen occurred only if supernatants from Con A–induced rat spleen cells were added to the culture. These supernatants contain high levels of IL-2 and a number of other soluble factors. With the availability of highly purified recombinant IL-2 (rIL-2), we have now been able to perform these experiments in a more defined system. MLC were initiated in the presence or absence of rIL-2 and analyzed for CT antigen expression at various times. Primary MLC cells (and cells from long-term MLC cultured without rIL-2) expressed relatively low but significant levels of CT antigen (Fig. 7*A*). The level of VV, HP, and Con A binding to primary MLC cells was also determined. Low to moderate levels of VV- and HP-reactive determinants were present, while Con A bound at a high level. At 3 wk of culture, the CT determinant density had increased dramatically and was distrib-

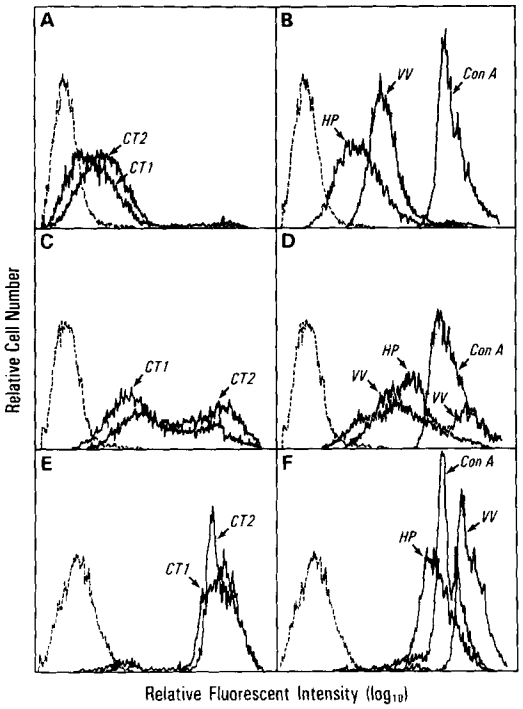

FIGURE 7. IL-2 induces marked changes in CT antigen expression and GalNAc expression in MLC. C57BL/6 spleen cells were cocultured with irradiated DBA/2 spleen cells in medium without (*A, B*) or with (*C–F*) the addition of purified rIL-2. Cultures were restimulated every 7 d for 2 wk (*A, B*), 3 wk (*C, D*), or 5 wk (*E, F*). Viable cells were stained and analyzed as in Fig. 6. The binding of VV and HP was >95% inhibited by soluble GalNAc (0.1 M) and Con A binding was >95% inhibited by α-methylmannoside (0.1 M).

uted between two major cell populations (Fig. 7*C*). (It should be noted that these cells were >95% of the CTL phenotype Thy-1$^+$, T200$^+$, Lyt-2$^+$, L3T4$^-$). A very interesting result was obtained when lectin binding to these cells was analyzed. While Con A binding levels remained high, a significant increase in VV and HP binding was noted as compared with primary MLC cells. Furthermore, VV binding was bimodally distributed in a pattern similar to CT mAb binding (Fig. 7*D*). After two additional restimulations in culture, >90% of the cells expressed very high levels of CT antigen, and bound VV, HP, and Con A at high levels as well. It is possible that IL-2 was acting on a second non-CTL in the MLC population, which was then inducing CTL to express high levels of CT antigen, or that IL-2 was causing the expansion of a small percentage of cells that expressed high levels of CT antigen. We feel that these explanations for CT antigen induction by IL-2 are not valid, since, early after activation in a primary MLC, the majority of the T200 molecules have undergone modification and 40–60% of the cells are CT$^+$ (10). That IL-2 can act directly on CTL to cause CT determinant expression is suggested by the finding that a cloned, cytolytically inducible T cell hybridoma concomitantly expresses lytic activity, CT antigens, and marked changes in cell surface carbohydrate after induction by either Con A–stimulated spleen cell supernatants, which contain many soluble factors, or

rIL-2 (Lefrancois and Kanagawa, manuscript submitted for publication). Thus, IL-2 is able to act on a single cell type to induce the expression of carbohydrate differentiation antigens.

Discussion

The importance of carbohydrate structures in protein transport and secretion and as differentiation and tumor antigens has become evident of late (42). A number of mAb specific for oligosaccharide moieties have recently been described that define oncofetal antigens, stage-specific embryonic antigens, and antigens of neuronal cell subsets (42–44). Neural cell adhesion molecules, myelin-associated glycoprotein, and human NK cells appear to share a common carbohydrate antigen (45). As yet, little is known of the function of these saccharide antigens. Our discovery of the presence of T cell subset–specific carbohydrate antigens further indicates an important role for carbohydrates in cell type–specific differentiation. Furthermore, the CT antigens appear to be of functional significance, since CT-specific mAb inhibit CTL-mediated lysis (9). Because CT mAb bind to a number of proteins at the CTL cell surface (T200, gp140, gp85), we have been unable as yet to determine if the functional inhibition of CTL is the result of CT mAb binding to one or more of these proteins. Alternatively, CT mAb may react with glycolipids at the cell surface and inhibit lysis by an unknown mechanism. Quantitatively, the T200 glycoproteins bind more CT than gp140 or gp85, and other antibodies to T200 have been shown to inhibit NK- and CTL-induced lysis, although this latter point remains controversial. A functional role for T200 in cell-mediated lysis has also been suggested by a study (46) which showed that T200 is a major cell surface protein that binds *N*-tosyl-L-lysyl-chloromethyl ketone (TLCK), an inhibitor of trypsin-like proteases. In addition, TLCK inhibits CTL-induced lysis (47). In support of this finding is the recent report (22) suggesting that the T200 glycoprotein may be an enzyme with Ca^{++}-dependent autoproteolytic activity. The authors (22) discussed the similarities of the T200-associated protease and the protease that activates protein kinase C (48) and is the endogenous enzyme that cleaves the epidermal growth factor (EGF) receptor (49). Like the EGF receptor, the T200 molecule has an unusually large intracytoplasmic domain (M_r 80 K) with several possible phosphorylation sites, although little sequence homology between T200 and the EGF receptor was noted (50). Interestingly, carbohydrate structures related to blood group–specific oligosaccharides have been described on the leukocyte common antigen of human lymphocytes (51, 52) and the EGF receptor glycoprotein (53). Thus, it seems likely that T200 is important in transmembrane signaling, and carbohydrate structures found on the protein may be involved in this process. T200, therefore, seems a probable candidate for CT-mediated inhibition of CTL-directed lysis.

In regard to the CT-reactive gp140 found at the CTL cell surface, it may be noteworthy that other investigators have previously described a Lyt-2^+ T cell–specific protein called T145 (54, 55). T145 was isolated from T cell blasts by fractionation of cell surface glycoproteins with VV. However, the functional significance of T145 in the lytic mechanism has been questioned, since cytolytic T cell populations selected on the basis of VV binding showed no difference in

the lytic ability of CTL expressing low or high levels of VV-reactive carbohydrate (56), and since T145$^-$ CTL also have lytic capabilities (57). Our preliminary studies indicate that the gp140 described here and T145 are similar.

The exact carbohydrate specificity of the CT antibodies is at present unknown. However, recent experiments by Conzelmann and Kornfeld (58, 59) suggest interesting possibilities. Based on the knowledge that CTL lines which are independent of stimulator cells but dependent on IL-2 have high levels of binding sites for VV (60), these authors isolated glycopeptides for carbohydrate analysis from VV-sensitive or -resistant CTL lines. VV has been shown (60) to be highly cytotoxic for cell lines that express high levels of VV-reactive determinants. The results indicate the presence on VV-binding CTL of O-linked oligosaccharides that contain GalNAc linked in a β1,4 configuration to galactose at their nonreducing termini. This configuration was not detected on CTL that did not bind VV. Furthermore, a VV-resistant mutant cell line was deficient in the N-acetylgalactosaminyltransferase apparently responsible for the addition of these residues. Our demonstration that CT mAb, VV, and HP cell surface binding was influenced greatly by the functional T cell phenotype suggests that the particular oligosaccharide moiety described by these authors may influence CT mAb reactivity. In fact, CT and VV have strikingly similar binding distributions (Fig. 7). However, it is clear from comparisons of CT-reactive and VV-reactive molecules isolated from the CTL cell surface that VV has specificity for a much broader array of proteins than the CT mAb (Lefrancois and Kanagawa, submitted for publication).

The finding that IL-2 influences CT determinant expression (and other cell surface carbohydrate modifications; Fig. 7) suggests for the first time that this hormone-like lymphokine may regulate glycosylation of specific proteins. IL-2 has been shown to be a proliferative signal for T cells (61) as well as for B cells (62) and also influences CTL differentiation in the absence of proliferation (63, 64). The mechanisms leading to these events after interaction of IL-2 with its membrane-bound receptor have not been clearly elucidated. Although IL-2 induces proliferation in both CTL and Th cells, the effects of IL-2 on glycosylation that result in CT antigen expression occur in a cell type–specific manner. This could be accomplished by induction or regulation of a specific glycosyltransferase (e.g., *N*-acetylgalactosaminyltransferase) or by affecting transcription and/or translation of proteins, thereby introducing new glycosylation sites.

Our results with tunicamycin-treated cells and the results of the pulse chase experiments indicated the presence of at least three precursor proteins corresponding to the three major cell surface T200 proteins of cloned CTL. This is in contrast to earlier results obtained with murine thymocytes which showed a single T200 precursor protein of M_r 160 K (33). Thus, major alterations seem to occur during activation of CTL. When CTL clones were grown in an antigen-independent manner with very high concentrations of supernatants from Con A–stimulated spleen cells, an increase in the molecular weight of the T200 protein was observed (65). Tung and coworkers (32) have also noted differences between functional T cell types and they observed two proteins of M_r 210 and 215 K at the cell surface of Lyt-2$^+$ clones; however, no studies identifying precursors to the surface forms were performed (32). Our preliminary results

indicate that Th cells also express different T200 precursor proteins than do CTL. There has been some question as to whether the observed molecular weight differences between the T200 forms of various T cell types and the B cell form of T200 (B220; 66, 67) are due to actual protein differences or posttranslational modifications. The recent demonstration (50) of mRNAs of different sizes coding for the leukocyte common antigen (i.e., T200) of rat thymocytes and B cells indicates differences at the protein level. Indeed, we have noted size differences in the T200 mRNA of CTL and Th (M. Thomas and Lefrancois, unpublished results). In our current investigations we hope to elucidate the mechanisms allowing the expression of multiple T200 forms on functionally disparate T cells and to examine the intriguing possibility that IL-2 may influence this discordant expression as well as posttranslational modifications of T200 and other glycoproteins.

Summary

Cytotoxic T cell (CTL)-specific activation antigens, termed CT determinants, have been detected by monoclonal antibodies (mAb) that inhibit CTL function. At the cell surface, the CT antigens are associated with the T200 glycoproteins and two other proteins of M_r 140,000 and 85,000 and are present on a secreted protein, gp155. Periodate treatment followed by binding analysis and immunoprecipitation experiments using tunicamycin-treated cells indicated that carbohydrate is necessary for CT antigen expression. Furthermore, gp155 is secreted in the presence of tunicamycin while retaining the CT antigens, and the CT determinants are added late in T200 biosynthesis, suggesting that the CT glycans are O-linked. Finally, interleukin 2 was shown to dramatically influence the expression of the CT mAb–reactive oligosaccharides present at the CTL cell surface.

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