

SELECTIVE AFFINITY FRACTIONATION OF MURINE
CYTOTOXIC T LYMPHOCYTES (CTL)

Unique Lectin Specific

Binding of the CTL Associated Surface Glycoprotein, T 145*

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Cell surface glycoproteins distinguishing populations of cytotoxic T lymphocytes (CTLs)¹ from both normal or noncytotoxic subsets of activated T blasts have been described in both mouse (1) and human (2) experimental systems. These qualitative differences in the plasma membrane composition which occur concomitantly with the development of cytotoxic activity may thus be directly related to the differentiated function expressed by these cells. In support of this, an extensive literature has accumulated leading to the conclusion that inherent membrane components of the CTL are of unquestionable importance in coordinating and perhaps dictating the unusual cytolytic behavior of this cell type (3-5). Thus, efforts to differentiate cells of cytolytic nature from all others through distinct cell-surface structures should permit the development of methods for the specific isolation of these cells. Furthermore, cell surface structures which so clearly distinguish CTLs from other activated or normal T cells represent intriguing prospects of biological membrane structure with possible relationship to function.

In the original work leading to the present studies, we have reported on the appearance of a characteristic new surface glycoprotein appearing on mouse T lymphocytes after *in vitro* or *in vivo* immune activation by major histocompatibility complex (MHC) alloantigens or through polyclonal activation of these lymphocytes (1). Because of its exclusive expression on certain T lymphocytes and mol wt of 145,000 daltons, we have called this distinguishing surface glycoprotein T 145. The expression of the T 145 glycoprotein could be shown to be directly correlated in time and extent to the levels of cytotoxicity generated in a variety of T-cell activations. In addition, specific enrichment procedures applied to purified T lymphoblasts isolated from mixed leukocyte culture (MLC) have demonstrated Ly 1⁺2⁻ blasts to be T 145 negative and Ly 1⁻2⁺ blasts to be strongly T 145 positive.

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¹ *Abbreviations used in this paper:* C, selected rabbit complement; Con A, Concanavalin A; BGA, blood group A substance from ovarian cysts; CTL, cytotoxic T lymphocyte; D-GalNAc, *N*-acetyl-D-galactosamine; FCS, fetal calf serum; MHC, major histocompatibility complex; MLC, mixed leukocyte culture; PBS, phosphate-buffered saline (without Ca⁺⁺ or Mg⁺⁺); PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin.

In the present article we report on the application of lectin affinity adsorbents as probes in classifying their reactivities towards solubilized CTL membrane glycoproteins, in particular towards T 145. During the course of these studies it was found that one lectin, *Vicia villosa*, displayed a unique selectivity for the carbohydrate portion of T 145 only. The highly selective carbohydrate binding properties of this lectin for T 145 at the molecular level is further correlated with its ability to specifically fractionate those cells participating in T-cell-mediated cytolytic reactions.

Materials and Methods

Mice. All strains of inbred mice reported in this article have been raised and maintained in our breeding facilities, Department of Immunology, Uppsala. Both male and female mice between 6 and 16 wk of age were used in these studies.

Conditions for the Generation and Purification of Murine Cytotoxic T Lymphocytes. In vitro conditions for the allogeneic sensitization of purified splenic T cells have been described in detail elsewhere (5). In brief, spleen T cells purified by passage through Ig-anti-Ig glass bead-coated columns (6) were responded in culture against 2,000 rads irradiated allogeneic spleen cells in Hepes buffered Eagles' Hanks' amino acid-supplemented media plus 0.5% normal mouse serum from the responding mouse strain. After 5-6 d in culture the cells were harvested and the activated T blasts were purified by either 1 g. velocity sedimentation or Ficoll density centrifugation (7).

Cell-Surface Labeling and Solubilization of the Labeled Cells. The various preparations of T blasts isolated from MLC were surface-labeled by the galactose oxidase- NaB^3H_4 method of Gahmberg et al. (8) and as previously described (1). Briefly, this method involves the selective oxidation of terminal galactose and *N*-acetyl galactosaminyl residues by galactose oxidase, which are then reduced with tritiated sodium borohydride of high sp act (>20 Ci/mM, The Radiochemical Centre Ltd., Amersham).

The labeled cells were then solubilized at a final concentration of 5×10^7 cells/ml in phosphate-buffered saline (PBS) + 0.5% NP 40 supplemented with protease inhibitors as previously described (1). After solubilization of the cells, the membrane-rich supernate was recovered after centrifugation of the lysate for 20 min at 20,400 g at 4°C. CaCl_2 and MgCl_2 were added to a final concentration of 1 mM to fulfill divalent cation requirements for the binding of certain lectins.

Preparation of the V. villosa Lectin. 100 g of dry *V. villosa* seeds were ground for 1 min in a Waring Blendor in 25-g portions. The resulting meal was extracted overnight at 4°C with 600 ml PBS, pH 7.4. The pH of the solution was then adjusted to 4.5 with 4 M acetic acid and the slurry was centrifuged for 20 min at 20,000 g. The pH of the supernate was then readjusted to pH 7.4 with 4 M NaOH and solid $(\text{NH}_4)_2\text{SO}_4$ was added to 80% saturation. The mixture was stirred for 2 h, centrifuged for 20 min at 2,000 g and the precipitate was dissolved in 200 ml of PBS. The solution was applied to an affinity column (3.2 x 10 cm) of blood group A substance coupled to Sepharose 2B (9) equilibrated in PBS at a flow rate of 80 ml/h. Nonadsorbed material was removed by passage of 200 ml PBS through the column. The A-specific lectin was then desorbed by elution with a solution of 0.01 M *N*-acetyl-D-galactosamine (D-GalNAc) in PBS. With such procedures, the yield of lectin was about 80 mg. A more detailed procedure and also the properties of the A-specific lectin will be published elsewhere.²

Radiolabeling of Lectins and Blood Group A Substance. Con A, wheat germ agglutinin (WGA) and *V. villosa* were labeled by the chloramine T iodination method (10) in the presence of 10 mM of their competitive sugar (α -D-methyl mannoside, *N*-acetyl-D-glucosamine and D-GalNAc, respectively) to protect the binding sites during the iodination procedure.

Purified blood group A substance (BGA) from human ovarian cysts was kindly provided by Dr. E. A. Kabat, Dept. of Microbiology, Columbia University, New York, and was labeled by galactose oxidase- NaB^3H_4 reduction. The purified, lyophilized BGA was first resuspended in PBS at a concentration of 10 mg/ml by gentle rocking of the tube overnight at 4°C. 1 mg of BGA in 0.1 ml was incubated with 10 U of galactose oxidase (Kabi AB, Sweden) for 45 min at 37°C. The mixture was then transferred to an 80°C water bath (10 min) for inactivation of the

² B. Ersson and P. Carlsson. Manuscript in preparation.

galactose oxidase. 1 mCi of NaB^3H_4 dissolved in 0.01 N NaOH was then added and allowed to react for 45 min at room temperature in a fume hood. The mixture was then applied to a Sephadex G-25 column for desalting of the unreacted NaB^3H_4 .

Preparation and Use of Lectin Affinity Adsorbents for

THE IDENTIFICATION AND RECOVERY OF CELL-SURFACE GLYCOPROTEINS DEMONSTRATING GROUP-SPECIFIC CARBOHYDRATE BINDING TO VARIOUS LECTINS. WGA (Pharmacia Fine Chemicals, Uppsala, Sweden), *Helix pomatia* A hemagglutinin and *V. villosa* were covalently coupled to CNBr activated Sepharose 4B particles (Pharmacia Fine Chemicals AB, Uppsala, Sweden) at a protein concentration of 10 mg/g dry gel in 0.1 M NaHCO_3 /0.5 M NaCl, pH 8.5. After overnight end-over-end rotation of the reaction tubes at 4°C, the beads were extensively washed with PBS and remaining active groups on the beads were blocked by a 2-h room temperature incubation with 0.1 M epsilon-amino caproic acid in PBS, pH 7.0. The beads were subsequently washed with PBS and stored at 4°C in PBS + 0.02% NaN_3 . The degree of substitution in all cases was between 2.5–3.0 mg lectin/ml of swollen gel. Con A-Sepharose 4B was purchased from Pharmacia Fine Chemicals.

The various lectin-Sepharose 4B adsorbents were equilibrated in the reaction-washing buffer (PBS + 0.1% NP 40 + 1 mM CaCl_2 + 1 mM MgCl_2 + 1 mM phenylmethylsulfonyl fluoride) and adjusted to a 20% vol/vol solution in the same. 0.5-ml aliquots of these adsorbents, corresponding to 0.1 ml packed beads were dispensed into conical bottom glass tubes and centrifuged for 5–10 s at 600 g. After removal of the supernate, 0.1-ml aliquots of the NP 40 lysate corresponding to $2\text{--}5 \times 10^6$ cells ($1\text{--}2 \times 10^6$ cpm) were gently mixed with the immobilized lectin adsorbents. The mixtures were then allowed to incubate for 45 min on ice and unbound material was removed by three 10-ml washes with ice-cold washing buffer. Such washing procedures were sufficient in removing greater than 99.9% of the radioactivity from control human serum albumin-Sepharose adsorbents. Labeled glycoproteins bound to the various lectin adsorbents were then nonspecifically eluted with electrophoresis sample buffer (11) containing 2% sodium dodecyl sulfate (SDS) and 0.15 M 2-mercaptoethanol by heating in a boiling water bath for 1–2 min. Glycoproteins specifically eluted from the lectin adsorbents by incubation of the beads with the competitive sugar have shown qualitatively similar electrophoretic profiles in SDS-acrylamide gels as those nonspecifically eluted in over 20 determinations. Nonspecific elutions with SDS was preferred due to the higher quantitative recoveries of the labeled glycoproteins.

CELL FRACTIONATION STUDIES. *V. villosa* lectin and twice recrystallized ovalbumin (Worthington Biochemical Corp., Freehold, N.J.) were directly polymerized to glutaraldehyde activated Biogel P-2 or P-6 beads (50–100 mesh, Bio-Rad Laboratories, Richmond, Calif.) in 0.1 M phosphate buffer, pH 7.6 according to the method of Weston and Avrameas (12) with an extended coupling period of 72 h of end-over-end rotation of the reaction tubes in the cold (4°C). The beads were then extensively washed with PBS and remaining active groups were blocked by a 2-h incubation with 0.1 M epsilon-amino caproic acid/PBS, pH 7.0. The degree of coupling was determined by radioimmunoassay by using rabbit anti-ovalbumin antibodies (13) or NaB^3H_4 -labeled purified human blood group A substance. In both cases the extent of substitution was assessed between 5–10 $\mu\text{g}/\text{ml}$ of swollen gel.

Blast cell-enriched fractions from MLCs were washed twice in PBS, counted and resuspended to a cell concentration of $10^7/\text{ml}$ in PBS + 0.1% gelatin. Ovalbumin and *V. villosa* affinity adsorbents were packed in either Bio-Rad Econocolumns (1 x 10 cm) or Pharmacia K 9/15 columns (0.9 x 15 cm) and equilibrated in PBS + 0.1% gelatin. The cells were passed at room temperature with a flow rate of 0.5–1.0 ml/min, and unbound cells were washed through the column with twice the bed volume of PBS + 0.1% gelatin. Bound cells were eluted by allowing the eluting buffer (0.1 M D-GalNAc in PBS + 0.1% gelatin) to enter the gel and incubate for 5 min at room temperature followed by gentle mechanical stirring of the beads. The bound-eluted cells were collected from the column at a flow rate of ≈ 3 ml/min, washed twice in PBS, and counted for use in the various tests.

Cell-Mediated Cytotoxicity Testing. Alloimmune T-cell cytotoxicity and PHA-mediated T-cell cytotoxicity by Con A blasts (14) were assayed in V-bottom Cooke microtitre plates as described earlier (1). In vitro maintained lines of the DBA/2 (H-2^d) mastocytoma P815, the C57BL/6 lymphoma EL-4 (H-2^b), and Con A generated blasts from appropriate strains served as target

cells in these studies. Calculation of percent specific lysis and lytic units (LU) were done as described by Cerottini and Brunner (15).

Polyacrylamide Slab Gels and Fluorographic Autoradiography. NaB³H₄-labeled cell lysates and lectin fractionated surface glycoproteins were analyzed by electrophoresis through 7.5–10% linear gradients of acrylamide containing 0.1% SDS as described by Laemmli (11). Apparent molecular weight determinations were calculated on plots of the relative mobilities versus logarithm molecular weight of radiolabeled marker proteins, prepared as previously described (1). Slab gels were fixed overnight and processed for fluorographic autoradiography as described by Bonner and Laskey and Laskey and Mills (16, 17). Quantitative measurements of the individual glycoprotein profiles were determined on a Joyce-Loeb recording densitometer.

Results

Analysis of the Lectin Binding Characteristics of T 145. In recent years, realization of the potential application of group-specific carbohydrate binding of membrane glycoproteins to immobilized lectin adsorbents has provided a mild and rapid fractionation step towards the purification and characterization of several lymphocyte cell-surface glycoproteins (18–20). Although the initial interest in such lectin adsorbents emerged from their rather broad spectrum of reactivities for membrane glycoproteins, it is becoming increasingly clear that certain lectins can as well select single glycoprotein moieties from a complex mixture of cell-surface glycoproteins (21).

Using surface-labeled, NP 40 solubilized preparations of purified MLC activated T blasts as starting material, we have examined the range in glycoprotein selectivity of a panel of over 20 lectins covalently attached to Sepharose 4B. Through radiofluorographic analysis of the bound-eluted glycoproteins by SDS-polyacrylamide gel electrophoresis we have classified the lectin binding characteristics of the CTL-associated glycoprotein, T 145.

Our initial hopes of outlining a procedure for the sequential purification of T 145 by affinity chromatography through several lectin adsorbents were circumvented by the finding that one lectin, *V. villosa*, displayed a unique and amazing selectivity for T 145. The range of glycoproteins specifically recovered by affinity fractionation of the radiolabeled cell lysate on several lectin adsorbents is shown in Fig. 1. As seen, a considerable number of labeled surface glycoproteins are retained on the WGA adsorbent (1B), yielding an electrophoretic profile broadly resembling that of the unfractionated cell lysate (1A). Although significant enrichment of several glycoprotein peaks can be achieved by this simple fractionation (notably at 130K, the 80–95K and 35–45K regions), it does not offer any selective advantage in reactivity towards T 145. This pattern of broad specificity is characteristic of other commonly used lectins such as Con A, *Lens culinaris*, *Ricinus communis*, and *Lathyrus maritimus* (A. Kimura, unpublished observations). However, in sharp contrast to the broad spectrum of glycoproteins bound to WGA, both *H. pomatia* (1C) and *V. villosa* (1D) display remarkable selectivity for single glycoprotein peaks. As shown previously (21), *H. pomatia* selectivity for mouse T lymphocytes resides in its reactivity for this 130,000 dalton glycoprotein. The finding that *V. villosa* could similarly select a single glycoprotein, in this case T 145, was unexpected but has now been reproduced in over 15 independent experiments on MLC blasts.

Preferential Binding of Radiolabeled V. villosa to MLC Blasts. Although clearly selective in reactivity for NP 40 solubilized surface labeled glycoproteins, versatility in the application of *V. villosa* for the quantitation and isolation of T 145 bearing cells would require proper spatial accessibility of T 145 in the intact cell membrane, as well as

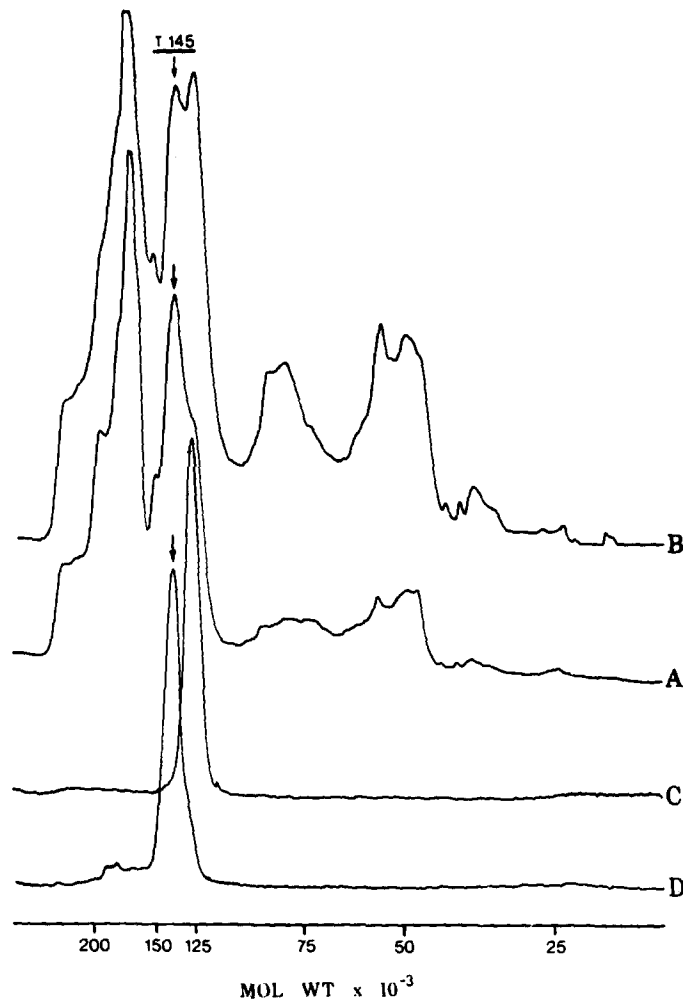


FIG. 1. SDS-PAGE profiles of the cell-surface glycoproteins recovered after interaction of NaB^3H_4 labeled, NP 40 solubilized MLC blasts with immobilized lectin adsorbents. CBA/H anti-DBA/2 MLC blasts were labeled with NaB^3H_4 and the solubilized equivalent of 5×10^6 cells were incubated with the various lectins for 45 min on ice, washed three times with PBS + 0.1% NP 40 and eluted with Tris-SDS sample buffer (11) by heating in a boiling water bath for 1.5 min. The labeled glycoproteins were separated under reducing conditions by electrophoresis through a linear 7.5–10% gradient slab gel of acrylamide + 0.1% SDS, processed for fluorographic autoradiography and the banding patterns from the X-ray film were recorded on a Joyce densitometer. (A) unfractionated labeled cell lysate, (B) glycoproteins eluted from a WGA adsorbent, (C) glycoproteins eluted from a *H. pomatia* adsorbent and (D) glycoproteins eluted from a *V. villosa* adsorbent.

some assurance that the lectin is not significantly reactive with glycolipid moieties present on other cell types.

We have approached these considerations by assaying the binding of ^{125}I labeled-*V. villosa* to purified blasts isolated from MLC and to control groups of purified thymocytes and spleen T cells. As a further control for differences in the surface areas of the blasts versus normal T cells, we have also included the binding of ^{125}I -labeled WGA and Con A whose broad reactivities would estimate differences in the total surface glycoproteins of the three cell populations.

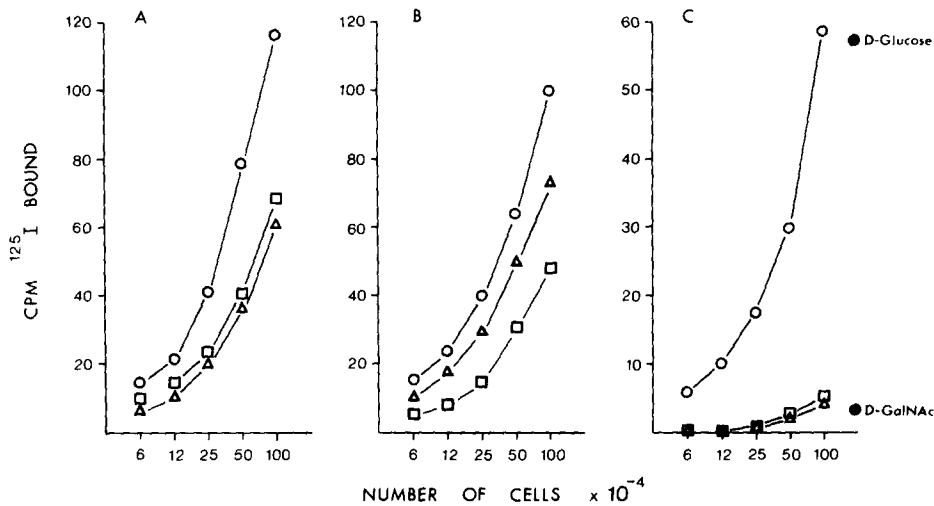


FIG. 2. Binding of radiolabeled lectins to normal and immune T-cell preparations. Ig-anti-Ig column purified CBA/H thymocytes (Δ), spleen T cells (\square) and CBA/H anti-C57BL/6 MLC blasts (\circ) were incubated with a predetermined excess of ^{125}I -labeled (A) Con A, (B) WGA and (C) *V. villosa*. Cell-bound radioactivity was determined after a 45-min incubation at 4°C and washes with ice-cold PBS. In Fig. 2C, 10^6 MLC blasts were incubated with ^{125}I -*V. villosa* in the presence of 0.1 M D-GalNAc or 0.1 M D-glucose to further establish the sugar specificity of the lectin binding. All values represent the mean of triplicate determinations with a standard error of less than 10%.

Doubling dilutions of the various cell preparations were incubated with a predetermined excess of radiolabeled lectins in a total vol of 0.1 ml PBS for 45 min on ice. After this incubation period the cells were washed four times with ice-cold PBS and transferred to tubes for gamma scintillation counting. The results of these experiments are shown in Fig. 2. As seen, both Con A and WGA (Figs. 2A and B, respectively) do in fact show an increased binding to MLC blasts when compared to normal thymocytes and spleen T cells. By comparison of the horizontal intercept of the curves a two to fourfold difference in binding to MLC blasts is seen over the normal T-cell preparations. In contrast to these differences in binding which are likely due to the differences in size, a most striking difference is seen when these three populations are compared in the binding of *V. villosa* (Fig. 2C). Here a difference of 16-fold is seen between the blast and normal populations of T cells. Evidence for the specificity of *V. villosa* binding to intact cells is seen by a blockade of its binding in the presence of 0.1 M concentration of the competitive sugar (D-GalNAc) and not by equimolar concentrations of D-glucose.

Functional and Cell-Surface Markers of MLC Blasts Separated on *V. villosa* Affinity Columns

SELECTIVE RETENTION OF CTLs. The highly selective binding of T 145 to *V. villosa* lectin as demonstrated with radiolabeled membrane glycoproteins, its accessibility for interaction with the lectin on the intact cell and preferential expression on MLC blast at once suggested its application for the physical isolation of T 145 positive-*V. villosa* binding cells for further analysis of the cytolytic potential of this unique cell population.

B10.BR anti-B10.D2 MLCs were collected at the peak of the cytotoxic response and the blast cell enriched fraction was obtained by Ficoll density centrifugation. The cells were passed through *V. villosa* and control ovalbumin affinity columns and the nonbound and bound-eluted fractions were collected, washed twice in PBS, and

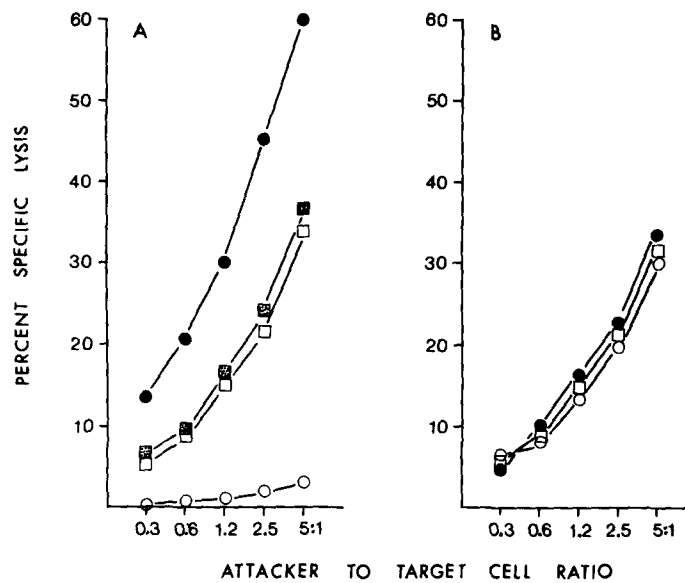


FIG. 3. Affinity fractionation of the cytolitic activity expressed by MLC blasts after filtration through *V. villosa* lectin adsorbents. The blast cell-enriched fraction from B10.BR anti-B10.D2 MLCs were passed through biogel-*villosa* and control ovalbumin-biogel columns (8 cm in height, \approx , 8 ml packed beads). Nonbound and bound-eluted fractions were washed twice in PBS and assayed for cytolitic activity against ^{51}Cr -labeled P 815 in a 4-h assay with varying ratios of lymphocytes to target cells. (■) unfractionated cells, (□) Biogel-OA passed cells, (○) *villosa* passed cells, (●) *villosa* bound-eluted cells. Fig. 3A illustrates the fractionation of cytolitic activity with cells passed in PBS + 0.1% gelatin and Fig. 3B illustrates the results obtained when cells are passed through identical columns in 0.1 M D-GalNAc in PBS + 0.1% gelatin.

titrated for cell-mediated cytolitic activity against the specific target cells. As seen in Fig. 3A, passage of this effector cell population through a *V. villosa* column results in a virtually complete elimination of cytolitic activity in the passed cells as compared to either those cells passing through the control ovalbumin column or the unfractionated cell population. Proof that the basis for the fractionation is not due to an inactivation of the CTLs during filtration through the *V. villosa* column is shown by their specific enrichment in the bound-eluted cell fraction, in this case representing a fourfold enrichment in lytic activity. As a further control to establish the sugar specificity of the lectin fractionation and possible influence of the eluting sugar on the aggressive activity of the CTLs, an identical number of the MLC generated cells were passed through a comparable *V. villosa* column in the presence of 0.1 M D-GalNAc. The results of such a column passage are shown in Fig. 3B in which no specific fractionation of cytolitic activity can be seen.

PREFERENTIAL BINDING OF Ly 2⁺ CELLS. As the Ly 1⁻2⁺ compartment of T cells arising in MLC are known to express efficient cytolitic activity and be strongly T 145 positive while the Ly 1⁺2⁻ T cells are deficient in these characteristics (1, 22), it was of interest to examine the Ly phenotype of the fractionated cell populations in parallel with the functional activity seen in Fig. 3. The results seen in Table I clearly show that a significant fractionation of Ly 2⁺ cells has occurred by passage of these B10.BR anti-B10.D2 cells through the *V. villosa* column when compared to either the control ovalbumin column, the control *V. villosa* column containing the competitive sugar or the unfractionated cell population. However, because the sum of the Ly 1⁺ and Ly 2⁺

TABLE I
Cellular and Surface Characteristics of Alloactivated T Cells Fractionated on V. villosa Affinity Columns

Column*	Cellular fraction	Percent of input cells re-covered	Percent blast cells	Percent specific cytotoxicity with:‡			Ratio of Ly 2 ⁺ : Ly 1 ⁺ cells
				Anti-Thy 1.2 + C'	Anti-Ly 1.2 + C'	Anti-Ly 2.2 + C'	
Biogel-ovalbumin	Passed	95.1	73.5	84.0	48.1	37.5	0.78:1
	Bound-eluted	0.6	75.1	ND§	ND	ND	ND
Biogel- <i>V. villosa</i>	Passed	62.4	40.8	82.6	67.3	11.9	0.17:1
	Bound-eluted	31.2	84.6	84.7	16.4	61.6	3.75:1
Biogel- <i>V. villosa</i> + D-GalNAc	Passed	82.9	70.2	80.9	42.1	32.6	0.77:1
	Bound-eluted	4.0	68.1	84.1	38.4	33.4	0.87:1
None	Unfractionated	—	77.4	82.7	45.5	32.2	0.70:1

* 2×10^7 cells from B10.BR anti-B10.D2 MLC were fractionated through the respective affinity columns at a concentration of 10^7 cells/ml in PBS + 0.1% gelatin. Packed affinity adsorbents, 8 cm in height (~8 ml bed volume) were pre-equilibrated with PBS + 0.1% gelatin before passage of the cells.

‡ 2×10^6 cells from each group were incubated with a 1:5 dilution of each sera in a total vol of 25 μ l for 40 min at 37°C, washed and further incubated with C (1:20) for 40 min at 37°C. Cell viability was immediately determined by trypan blue dye exclusion. In each case, percent-specific cytotoxicity (no. dead experimental — no. dead control/total \times 100) is based on over 250 counted cells/group. Control cytotoxicity with normal mouse serum + C' ranged from 6.8 to 10.6% for the various groups.

§ ND, not determined.

|| Cells were passed in PBS supplemented with 0.1% gelatin and 0.1 M D-GalNAc through a column pre-equilibrated with the same.

cell populations of both fractions of cells recovered from the *V. villosa* column do not significantly overlap from the value seen with anti-Thy 1.2 serum treatment it is difficult to determine what proportion of the passed or eluted cell fractions are comprised of Ly 1⁺2⁺3⁺ cells.

Other experiments have suggested that the basis for the fractionation of cytolytic activity is not strictly dependent upon the adherence of all Ly 1⁻2⁺ cells, but rather a selection of those cells within this subpopulation of cells capable of expressing cytolytic function. Such an example is in part seen in the results of exp. 1 of Table II where depletion of cytotoxicity from the *V. villosa* passed cell fraction is shown. These cells, depleted of cytotoxic activity, were typed with anti-Ly antisera + C and shown to contain 62% Ly 1⁺ cells and 33% Ly 2⁺ cells. A combined antisera treatment of the cells with both anti-Ly 1 and anti-Ly 2 sera + C was also performed to estimate the total lysable cells with these reagents and experimental conditions, which in this case was 82%. Given such a total value of lysable cells, the overlapping percentage of cells from the individual antiserum values would thus represent the percentage of Ly 1⁺2⁺ cells in this fraction. The results were interpreted to mean that of the 33% Ly 2⁺ cells in the *V. villosa* passed fraction, only 13% could be reasoned to be Ly 1⁺2⁺ cells (62% + 33% — 82%) and the remaining 20% of Ly 1⁻2⁺ phenotype.

Fractionation of the Total Cytolytic Potential from Polyclonally Activated Con A Blasts with V. villosa Affinity Columns. Activation of T lymphocytes by Con A results in the expression of a number of T lymphocyte functions autonomously effectuated by discrete subpopulations of cells (14, 23). Among these subset specified functions it has been possible to quantitate the cytotoxic activities arising from activation of antigen specific clones

TABLE II
Affinity Fractionation of MLC Generated Cytotoxicity

Exp.	Effector cell population*	Affinity column	Cell fraction†	Percent of input cells recovered	Percent blast cells	Percent specific lysis§				Percent recovery of total lytic units applied to columns
						20:1	10:1	5:1	2.5:1	
1	C57BL/6 anti-CBA/H	Ovalbumin	Passed	86.8	84.2	52.3	36.5	20.7	10.3	96.6
			Bound-eluted	2.2	78.3	N.D.	34.1	18.9	9.2	1.2
		<i>V. villosa</i>	Passed	53.4	51.2	1.1	0.8	0.3	0.3	ND¶
			Bound-eluted	34.2	85.0	86.7	57.2	37.4	18.8	75.9
2	CBA/H anti-DBA/2	Ovalbumin	Passed	88.6	83.1	76.3	52.6	25.2	14.7	93.4
			Bound-eluted	1.3	87.2	N.D.	50.3	21.4	11.8	1.3
		<i>V. villosa</i>	Passed	51.7	60.0	1.2	0.9	0.5	0.7	ND
			Bound-eluted	40.0	91.2	83.5	76.4	42.7	25.8	78.3
3	B10 anti-B10.D2	Ovalbumin	Passed	95.0	68.5	42.5	22.6	10.4	6.9	96.0
			Bound-eluted	2.0	60.0	N.D.	20.0	11.9	7.5	ND
		<i>V. villosa</i>	Passed	57.0	41.2	0.1	0.1	0.3	0.2	ND
			Bound-eluted	31.5	82.5	75.0	46.2	29.5	15.2	80.7
4	B10.G anti-B10.T (6R)	Ovalbumin	Passed	84.2	70.0	51.1	37.1	20.9	11.0	87.5
			Bound-eluted	7.3	80.0	57.9	39.4	23.6	13.8	8.7
		<i>V. villosa</i>	Passed	40.5	33.0	8.8	3.4	2.7	1.7	ND
			Bound-eluted	53.8	68.7	65.3	45.6	27.7	17.9	79.1

* Lymphocytes from the various MLCs were harvested on the 5th-day of culture and the blast-cell-enriched fraction was obtained by Ficoll density centrifugation. The cells were washed twice in PBS before use in the cytotoxicity assays.

† Ficoll purified, MLC-derived lymphocytes were suspended to a concentration of 10^7 /ml in PBS + 0.1% gelatin for fractionation through the Biogel-OA and Biogel-*V. villosa* affinity columns. The passed (non-bound) and bound-eluted fractions were collected, washed and differential cell counts made before use in cytotoxic and functional assays.

§ Percent-specific lysis at the various lymphocytes:target cell ratios was determined in a 4-h assay against 10^4 ^{51}Cr -labeled CBA/H Con A blasts (exp. 1) and the H-2^d tumor target P815 (exp. 2-4).

|| LU were calculated according to Cerotini and Brunner (15). $\text{LU}/2 \times 10^7$ unfractionated cells was arbitrarily taken as 100%. For fractions where the LU could be calculated from titrations of the specific lysis, the total LU/fraction was derived by multiplying the $\text{LU}/10^6$ cells times the percent recovery of the original 2×10^7 cells applied to the columns.

¶ ND, not determined.

(24) as well as the total cytotoxic potential of polyclonally activated CTL precursors, irrespective of their actual antigenic specificity (14). By using the latter system of analysis, we have further tested the ability of *V. villosa* adsorbents to select out the CTL compartment from polyclonally activated T lymphocytes. The results of these experiments are shown in Table III. Here all CTLs were retained and 85% of the total lytic activity present in the unfractionated cell population could be recovered in an enriched form by elution with the specific sugar.

Discussion

In the present study we have presented evidence for the specific interaction of *V. villosa* lectin and the CTL-associated glycoprotein, T 145. Our previously reported criteria of defining T 145 as a CTL-associated glycoprotein was based on a variety of T-cell activations and specific fractionation procedures in which populations enriched for cytolytic effector T cells displayed a parallel increase in T 145 expression, while purified MLC T blasts deficient in cytotoxic activity were negative in T 145 expression.

The results shown here now demonstrate that within a functionally heterogeneous population of MLC blasts, the cells actually expressing T 145 represent the only subpopulation of T lymphocytes endowed with cytolytic activity. Thus, purified MLC blasts previously being defined as T 145 positive on a population basis can now be

TABLE III
Fractionation of Polyclonally-Activated Cytotoxic T Lymphocytes

Column*	Cellular fraction	Percent of input cells recovered	Percent blast cells	Percent specific lysis†				Percent recovery of total LU applied
				50:1	25:1	12:1	6:1	
None	—	—	94.7	54.6	41.5	30.0	20.7	100
Ovalbumin	Passed	86.9	91.0	59.3	42.0	33.8	21.8	105
	Bound-eluted	2.3	94.3	ND§	ND	28.3	16.5	ND
<i>V. villosa</i>	Passed	47.6	95.1	-1.4	-0.7	-0.8	0.1	
	Bound-eluted	42.1	93.7	75.2	56.5	42.4	29.0	84.8

* The blast cell enriched fraction from day 4 Con A cultures was obtained by Ficoll density centrifugation and adjusted to a concentration of 10^7 cells/ml in PBS + 0.1% gelatin after two 20 min incubations with 0.1 M α -methyl-D-mannoside. Biogel affinity adsorbents, 7 cm in height (~7 ml bed volume) were used for the fractionation of 2×10^7 of the Con A cultured cells.

† Quantitation of T-cell-mediated cytotoxicity by Con A induced blasts was performed according to Bevan and Cohn (14) against ^{51}Cr -labeled P 815, by the addition of 0.25 μg PHA/well (2.2 $\mu\text{g}/\text{ml}$ final concentration). Percent-specific lysis was determined at the various lymphocytes to target cell ratios after 4 h of incubation at 37°C in the presence of PHA. Parallel control determinations of percent-specific lysis in the absence of PHA were in all cases below 6%.

§ ND, not determined.

further fractionated into a highly cytolytic and noncytolytic cell population by affinity fractionation through *V. villosa* adsorbent columns. In a series of over 20 such cellular fractionations, the specific retention of the CTL compartment has been achieved irrespective of all strain combinations tested or antigenic specificity of the CTL, ranging from completely allogeneic (Fig. 3, Table II) to modified-self reactions (A. Kimura, unpublished observations). The general applicability was further extended to show that the total cytolytic potential arising from polyclonal Con A activation of CTL precursors could be fractionated in an identical fashion on *V. villosa* adsorbent columns (Table III).

Recoveries of cytotoxic activity from the total input of lytic units present in the unfractionated cell population after elution and subsequent washings have been greater than 75%.

The basis for the separation of CTLs is not exclusively associated with the adherence of all Ly 1^{-2+} cells, because a variable proportion of the cells found in the noncytotoxic passed cell fraction can be shown to be Ly 1^{-2+} . These results suggest that the present procedure allows a further fractionation of the Ly 1^{-2+} subpopulation of T blasts encompassing those T lymphocytes participating in cell-mediated cytotoxicity. With these fractionation procedures it will be of considerable interest to examine the behavior of Ly 1^{-2+} suppressor T cells to further test the restricted expression of T 145. Despite the fact that a limited number of experiments have been possible for Ly typing of the fractionated cell populations, all of 4 experiments have shown a convincing enrichment of Ly 1^{-2+} cells in the *V. villosa* binding cell fractions.

Although effective displacement of *V. villosa* binding to detergent solubilized or cell-bound T 145 can be accomplished with 0.1 M concentrations of the competitive sugar (D-GalNAc), the fine specificity of this lectin for polysaccharide linkage, sequence or composition of T 145 is unknown. When compared with a number of other lectins with reported specificity for terminal GalNAc residues, the selectivity for T 145 from the total surface glycoproteins is highest with *V. villosa*. It is of interest to note that

although the present lectin has been considered to express selectivity for human A₁ erythrocytes vs. A₂, specific anti-A₁ antisera have so far failed to provide useful reagents for the identification of CTLs.

The phylogenetic conservation of *V. villosa* reactivity for the CTL compartment of a variety of species and its specified reactivity for CTL-associated glycoproteins will be dealt with in a separate communication.³ These indications of a strong preservation for a distinct polysaccharide arrangement on CTL-associated surface glycoproteins among species seems unlikely to be due to coincidence. Application of the inherent specificity of this lectin for the isolation and characterization of these surface molecules may now allow an insight as to how these lymphocytes exert their biologically intriguing function.

Summary

We have analyzed the lectin binding characteristics of cytotoxic T lymphocyte (CTL)-derived surface labeled glycoproteins by affinity chromatography of the labeled glycoproteins on a panel of immobilized lectin adsorbents. Evidence is presented for the specific interaction of the CTL-associated glycoprotein T 145 with a lectin derived from *Vicia villosa* seeds.

Conditions are described for the preparation and use of lectin affinity adsorbents for the rapid isolation of T 145 bearing cytotoxic T lymphocytes. Direct proof is given to show that T 145-positive cells arising from a variety of T-cell activations constitute the only subpopulation of cells with ability to perform cell-mediated T-cell cytotoxicity. Specific depletion of the CTLs by adherence to *V. villosa* adsorbents is shown by their depletion in the nonbound cell fraction and correspondingly enriched recovery in the sugar eluted cell fraction. Specific affinity fractionation of CTLs has occurred in every strain combination tested and irrespective of the actual antigen specificity of the effector cell population.

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