

CELL SURFACE GLYCOPROTEINS OF MURINE CYTOTOXIC T LYMPHOCYTES

I. T 145, A New Cell Surface Glycoprotein Selectively Expressed on Ly 1⁻2⁺ Cytotoxic T Lymphocytes*

BY ARTHUR K. KIMURA AND HANS WIGZELL

(From the Department of Immunology, University of Uppsala Biomedical Center, Uppsala, Sweden)

The immune system is endowed with great flexibility. It has at its disposal a large number of alternative pathways of reaction against foreign, immunogenic substances. A sizeable part of this impressive flexibility is due to the fact that a number of subsets of specialized cell types coexist and interact within this system. Among these cell types, lymphocytes have been shown to play a dominating role in both the specific induction of the immune response and in its further developments.

Within the two major groups of lymphocytes (T and B cells) there exist additional subgroups with unique functional properties. Our knowledge of T-lymphocyte subgroups stems largely from experiments using a combination of serological and functional markers to define these cells (1-4). In principle, virgin lymphocytes, although preprogrammed as to their eventual subgroup (1), normally fail to express the functional properties displayed by activated cells arising from these subgroups (5, 6). The expression of these functions associated with the activated state can be interpreted to reflect qualitative as well as quantitative differences in the surface membrane composition of the cells.

Thus, the cell surface membrane characteristics of lymphocytes are decisive factors in determining the potential activity of these cells, and although progress in this field is rapid, we still have a largely imperfect understanding of how these membrane components effect and regulate immune reactivity.

In this article we have focused our attention on T lymphocytes and their membrane glycoprotein composition after induction by immunogen or polyclonal T-cell activators. With the aid of fractionation procedures now available to enrich for murine T cells with a defined surface structure or function, in combination with procedures for selective labeling of cell surface glycoproteins, comparisons between virgin and immune T cells at different stages of differentiation have been made. As will be demonstrated, this approach has been a highly fruitful one, allowing the successful characterization of a new T-lymphocyte unique surface glycoprotein associated with a defined subgroup of cells and expressed in conjunction with the cytolytic stages of T-cell reactivity. The implications of these findings will be discussed.

Materials and Methods

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

* Supported in part by the Swedish Cancer Society, and National Institutes of Health grant AI 13485-02.

Conditions for the Generation and Purification of Cytotoxic T Lymphocytes

ALLOGENEIC SENSITIZATION: In vitro and in vivo conditions for the allogeneic sensitization of purified splenic T cells have been described in detail elsewhere (6). In brief, spleen T cells purified by passage through Ig-anti-Ig glass bead-coated columns (7) were responded in culture against 2,000 R irradiated allogeneic spleen cells, or injected intravenously into lethally irradiated (800 R) allogeneic hosts. In both systems of sensitization, responding cells were collected at the peak of the cytotoxic response (day 5-6).

CONCANAVALIN A ACTIVATION: Concanavalin A (Con A)¹ blasts were prepared by incubating Ig-anti-Ig column-purified spleen T cells with a predetermined optimal concentration of mitogen (2 μ g/ml). The cells were adjusted to a concentration of 10^6 /ml in Eagle's-Hanks' amino acid-supplemented tissue culture media plus 5% fetal calf serum (FCS) and placed in culture flasks (3024; Falcon Plastics, Div. of Bio Quest, Oxnard, Calif.). These flasks had been incubated overnight at 37°C with syngeneic spleen cells, and washed three times with culture media to obtain the adherent cell population necessary for optimal mitogen stimulation of these column-passed cells.

Isolation of the Activated Blast Cells. The various allo- and mitogen-activated blasts were isolated from the small lymphocyte fraction and cell debris by velocity sedimentation at unit gravity through a linear 15-30% FCS gradient (8). The blast cell fractions were collected, washed three times in phosphate-buffered saline (PBS) and divided for use in cell-mediated cytotoxic reactions and for cell surface labeling.

Cell-Mediated Cytotoxicity Testing. Alloimmune T-cell cytotoxicity was assayed in v-bottom Cooke microtitre plates (220 M-25 AR; Cooke Laboratory Products, Div. Dynatech Laboratories, Inc., Alexandria, Va.) at various effector:target cell ratios using a constant number (10^4) of an in vitro maintained line of the DBA/2 mastocytoma P815. The assays were performed in a total reaction volume of 0.1 ml of RPMI-1640 media supplemented with 5% heat-inactivated FCS. $2-3 \times 10^6$ target cells were labeled with 100 μ Ci of $\text{Na}^{51}\text{CrO}_4$ (Amersham/Searle Corp., Arlington Heights, Ill. sp act 366 mCi/mg) for 30-45 min at 37°C in a total volume of 100 μ l RPMI-1640 plus 5% FCS. Controlled initiation of the cytotoxic response was achieved by centrifugation of the plates at 300 g for 4 min at room temperature. Assays were carried out for 2 and 4 h at 37°C in a 7.5% CO_2 /air atmosphere. Reactions were stopped by the addition of 0.1 ml of ice-cold PBS, followed by centrifugation in the cold (4°C). 0.1-ml aliquots of the supernates were carefully removed and counted in a gamma scintillation spectrometer. Percent specific lysis was calculated according to the method of Brunner et al. (9).

Lectin-mediated T-cell cytotoxicity by Con A-generated blasts was also assayed in v-bottom plates against ^{51}Cr -labeled P815 as described above. The total cytotoxic potential of the Con A-activated blasts was determined according to Bevan and Cohn (10) by the addition of 0.5 μ g of Leukoagglutinin (Pharmacia Fine Chemicals, Uppsala, Sweden) per well and compared with identical determinations receiving the same volume of media without lectin. Spontaneous release was determined without effector cells, with and without lectin, and did not vary significantly in any case. Total isotope release was determined by sonication of the target cells at 50 Hz for 1 min. Percent cytotoxicity was calculated as above.

Enzyme-Catalyzed Cell-Surface Labeling with NaB^3H_4 . The galactose-oxidase-tritiated sodium borohydride technique of Gahmberg et al. (11) has been used for the selective radiolabeling of cell-surface glycoproteins of the various cell preparations. Briefly, this labeling procedure involves the enzyme-catalyzed oxidation of exposed terminal galactosyl and *N*-acetyl galactosaminyl residues by galactose oxidase to the corresponding C6 aldehyde, which is then reduced with tritiated sodium borohydride of high specific activity. The procedure for labeling is essentially performed according to Gahmberg (11) with a few modifications to enable the labeling of small quantities of cells. Cells to be used for surface labeling were washed twice in PBS and adjusted to a concentration of 1.5×10^7 cell/ml in RPMI-1640 media (pH 7.4) supplemented with protease-free preparations of neuraminidase (12.5 U/ml, *Vibrio cholerae*; Behringwerke AG, Marburg, W. Germany) and galactose oxidase (5 U/ml, Kabi AB, Sweden), and were incubated in a 37°C water bath

¹ *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; LPS, lipopolysaccharide; MLC, mixed leukocyte culture; Mls, M-locus; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; SD, "classical" serologically defined regions of the H-2 complex (H-2K and H-2D); SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

for 40 min. At the end of this period, the cells were washed twice in PBS (made pH 8.0 with 1 N NaOH), resuspended to the original cell concentration in PBS (pH 8) containing 1 mCi/ml NaB^3H_4 (Amersham-Searle; sp act 8–12 Ci/mM) and incubated at room temperature in a fume hood for 40 min. The labeling was stopped by the addition of ice-cold PBS (pH 8), followed by three washes in the cold. In cases where as few as 2×10^5 cells were labeled, normal syngeneic spleen or tumor cells were added with the cold PBS used to stop the reaction. The addition of cells at this point did not introduce artifactual labeling and helped considerably as carrier cells to prevent losses during the subsequent washings.

Solubilization of NaB^3H_4 -Labeled Cells. NaB^3H_4 labeled cells were solubilized at a concentration of 5×10^7 cells/ml. The cell pellet from the final wash after NaB^3H_4 labeling was first gently resuspended by the addition of one-half the final volume of ice-cold PBS, followed by an equal volume of ice-cold solubilizer (1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ soy bean trypsin inhibitor, 2% vol/vol of a saturated solution of epsilon-amino caproic acid, and 10 $\mu\text{g}/\text{ml}$ *N*- α -p-tosyl-L-lysine chloromethyl ketone HCl) in PBS. The cells were incubated for 20 min on ice and then centrifuged at 20,400 *g* for 20 min at 4°C. The membrane-rich supernate routinely contained 74–86% of the total radioactivity which was trichloroacetic acid (TCA) precipitable and ether-insoluble. Samples were immediately mixed with an equal volume of sample buffer containing 4% sodium dodecyl sulfate (SDS) and 0.3 M 2-mercaptoethanol (12), and then heated in a boiling water bath for 1.5 min. Samples were stored frozen (-70°C).

Polyacrylamide Slab Gels and Radiolabeled Marker Proteins. Solubilized NaB^3H_4 -labeled cell preparations and marker proteins were prepared with and without reduction (12), and run in linear 7.5–10% glycerol-stabilized gradient gels containing 0.1% SDS. The gel system used was the discontinuous buffer system described by Laemmli (12).

Tritiated IgM was prepared by immune precipitation of [^3H]leucine-labeled supernates from lipopolysaccharide (LPS)-stimulated blasts according to the sandwich technique of Melchers and Andersson (13). DEAE-CMC 52-purified IgG₁ from the BALB/c myeloma MOPC 21, twice-recrystallized ovalbumin (Worthington Biochemical Corp., Freehold, N. J.), human serum albumin (Kabi AB), and purified hexon from type 2 adenovirus were labeled with carrier-free ^{125}I -Na by the chloramine T method (14) and served as marker proteins for apparent molecular weight determinations for each gel.

Fluorography. Slab gels were fixed overnight in an acetic acid-isopropanol-water mixture (1.2:3.1:8), and processed for fluorographic-autoradiography using the highly sensitive method of Bonner and Laskey (15), which enables detection of ^3H by the incorporation of the fluor 2,5-diphenyloxazole (PPO) into the fixed gel. Slab gels were dried by heating and vacuum suction, overlaid with RP X-Omat x-ray film (XR-2; Eastman Kodak Co., Rochester, N. Y.), wrapped in aluminum foil and exposed for a period of 1–5 days. In some cases, the x-ray film was hypersensitized with a flash of light (16) before contact with the dried gel to ensure proportionality of image darkening such that 30 cpm in a single band could be visibly detected with 72 h of exposure. Quantitative measurements of the individual gel profiles were determined on a Joyce-Loeb recording densitometer.

Results

The Appearance of a New, Distinct Surface Glycoprotein (T 145) on T Lymphoblasts Activated Across H-2 Barriers. For our analysis of distinguishing surface markers between normal and immune T cells, populations of cytotoxic T lymphocytes were generated by both primary mixed leukocyte culture (MLC) activation across H-2 barriers and by *in vivo* sensitization in lethally irradiated allogeneic recipients. The blast cell fractions from each of these immune cell preparations were then purified from the small lymphocyte fraction by 1 *g* velocity sedimentation and then surface-labeled with NaB^3H_4 . After solubilization of the labeled cells, the glycoprotein patterns were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by fluorographic-autoradiography. Nonimmune cells labeled in a similar manner included thymocytes and normal spleen T lymphocytes. Fig. 1 shows the cell surface

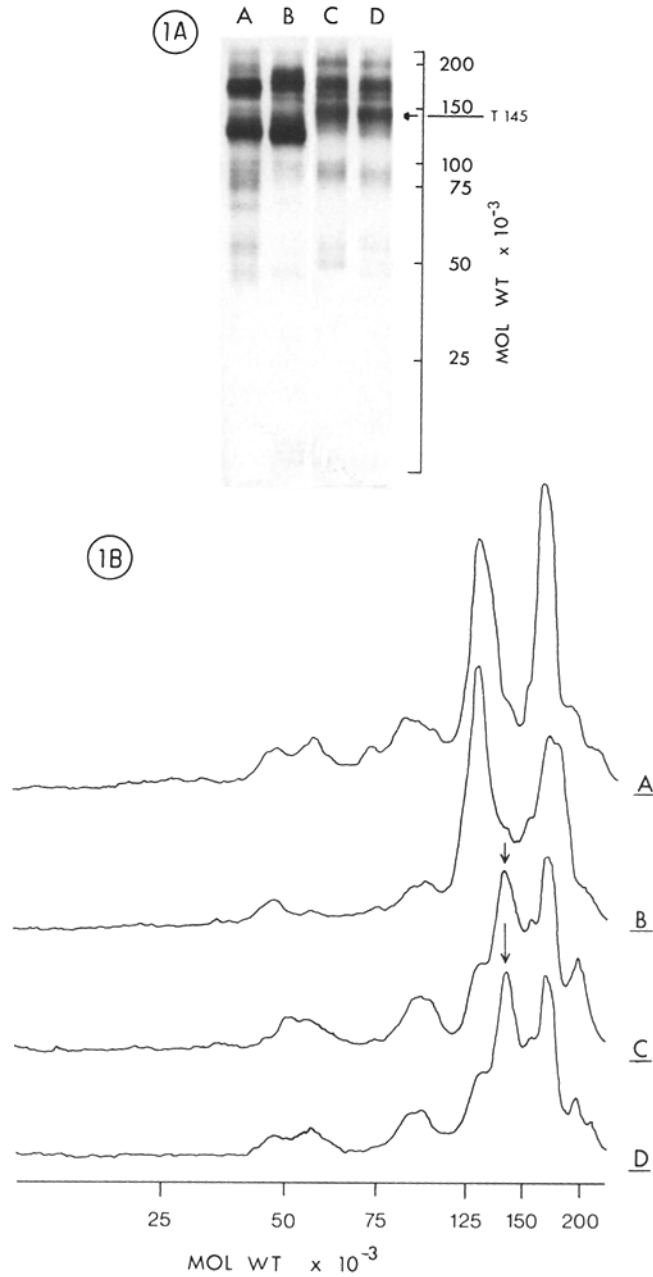


FIG. 1. Cell surface glycoprotein patterns obtained after electrophoresis of NaB³H₄-labeled normal and immune T-cell preparations. Fig. 1 A illustrates the autoradiographic glycoprotein patterns of (A) CBA/J Ig-anti-Ig column-purified thymocytes, (B) CBA/J Ig-anti-Ig column-purified spleen T cells, (C) MLC-generated CBA/J anti-DBA/2 T blasts, (D) in vivo generated CBA/J anti-DBA/2 T blasts. The quantitative relationship of the various glycoprotein bands is seen in the densitometric tracings of Fig. 1 B. Arrows indicate the position of T 145. Each preparation, corresponding to between 5 and 10 μ g of protein from the surface-labeled cell lysates were adjusted to 15,000 cpm in sample buffer (12). Electrophoresis was performed in a linear 7.5-10% gradient of acrylamide containing 0.1% SDS.

glycoprotein patterns obtained after NaB^3H_4 labeling of these normal and alloactivated T lymphocytes. Even though several glycoprotein bands appear identical between the various T-cell preparations, a number of differences are apparent. Such differences between resting and activated T cells have been previously noted by Gahmberg et al. (11). Specifically, these changes in gel patterns are seen in the appearance of large molecular weight glycoproteins at 200,000 and 145,000 daltons on activated T cells, and loss of two glycoproteins at 180,000 and 70,000 daltons. The major glycoprotein band at 130,000 daltons, which is heavily labeled on both thymocytes and peripheral T cells, can be seen here to be considerably reduced in intensity on both groups of activated T cells. As will be seen in subsequent figures, the intensity of this glycoprotein band is variable and usually less striking on activated T cells than on resting populations of T cells. This 130,000-dalton glycoprotein can, however, be clearly distinguished from the 145,000-dalton glycoprotein in that the former is apparent only after neuraminidase treatment of the cells and displays selective affinity for the T-cell lectin *Helix Pomatia*.² Furthermore, selective removal of this heavily labeled glycoprotein on *Helix Pomatia* affinity columns more clearly reveals the absence of the 145,000-dalton glycoprotein on resting T lymphocytes (data not shown).

In considering the significance of these changes in surface glycoprotein labeling patterns, one must take into account the obvious fact that the activated T cells are phenotypically blast-like in morphology, whereas resting T lymphocytes are characteristically small. Thus, the glycoprotein differences strictly related to immune activation cannot be distinguished from blast cell-associated glycoproteins. To discriminate between these possibilities we have examined the surface-glycoprotein patterns of a total of 15 different mouse T lymphomas as a representative panel of T-cell blasts functionally deficient in the effector cell activities associated with MLC-activated lymphoblasts (data not shown). In each case, the 200,000-dalton glycoprotein was a consistent feature of these cells with variable expression of labeled glycoproteins at 70,000 and 180,000 daltons, whereas none of these T lymphomas expressed the 145,000-dalton band. Thus, the only consistent cell-surface glycoprotein to display characteristics as perhaps related to immune activation of T lymphocytes has been the 145,000-dalton glycoprotein. As seen in Fig. 1, this glycoprotein is expressed with similar intensity on T lymphoblasts, regardless of whether sensitization was performed in vivo or in vitro. We call this distinguishing T lymphoblast glycoprotein T 145.

Further studies on the cellular distribution pattern of T 145 expression have shown normal spleen B cells, LPS blasts, and a number of B-cell lymphomas to all be T 145-negative. These results further emphasized the restricted expression of T 145 as a T-lymphoblast unique membrane glycoprotein and clearly not as a characteristic of any rapidly dividing T- or B-cell blast.

Different Alloantigenic Barriers Vary in their Ability to Induce T 145 on the Responding T Lymphoblasts. Gene products of the various regions of the H-2

² B. Axelsson, A. Kimura, S. Hammarström, H. Wigzell, K. Nilsson, and H. Mellstedt. Manuscript in preparation.

TABLE I
The Relative Efficiency of Various Alloantigenic Barriers to Induce T 145⁺
Lymphoblasts

Strain combinations analyzed*	Genetic difference	Relative labeling intensity of T 145‡
B10.G → B10.T(6R)	H-2D	++++
B10.S(7R) → B10.S	H-2D	+++
CBA/H-H-2 ^{ka} → CBA/H	H-2K point mutation	++++
CBA/J → C57BL/6	H-2, non-H-2	++
CBA/J → DBA/2	H-2, non-H-2	+++
C57BL/6 → DBA/2	H-2, non-H-2	+++
B10.BR → B10.D2	H-2	+++
B10.BR → B10.M	H-2	++
A.TL → B10.HTT	IA, IB, non-H-2	++
B10.S(7R) → B10.HTT	IC, H-2S, H-2G	++
B10.BR → CBA/J	non-H-2, Mls	(±)
CBA/H → CBA/J	non-H-2, Mls	+
BALB/C → DBA/2	non-H-2, Mls	+

* Ig-anti-Ig column-purified T lymphocytes were reacted in MLC against 2,000 R irradiated spleen cells of the stimulating cell genotype. Blasts were isolated on day 5 of culture by velocity sedimentation at unit gravity through a linear 15–30% FCS gradient.

‡ Purified T blasts were surface-labeled with NaB³H₄ under highly standardized conditions (see Materials and Methods) with the same batch of reagents, solubilized, and adjusted to the same total TCA-precipitable, ether-insoluble cpm. The glycoproteins of the various preparations were separated under reducing conditions by electrophoresis through a 7.5–10% gradient of acrylamide containing 0.1% SDS, fixed and processed for fluorography. The relative labeling intensity of T 145 was judged as the amount of radioactivity associated with the T 145 glycoprotein band relative to the total surface label of the individual preparations.

complex and other strong stimulating loci (Mls), have been claimed to have a differential impact as stimulating structures for various subsets of T lymphocytes (1). Using a variety of strain combinations in one-way MLCs, we have been able to select for proliferative responses against a combination of stimulating loci as well as selected regions of the H-2 complex in order to assess whether or not a specific genetic incompatibility is needed for the expression of T 145 on the responding blasts. Table I shows the relative ranking order of efficiency for the induction of T 145 expression, depending on the nature of the genetic incompatibility stimulating the MLC reaction. The most efficient incompatibility for the expression of T 145 on the responding blasts was a selective difference across the classical H-2 regions H-2K or H-2D (SD) only, with differences across the entire H-2 complex being second. T cells responding against I-region differences only do express the T 145 band, although in a lower intensity on a population basis than do the anti-SD activated T cells. MLC blasts generated in H-2 identical, Mls incompatible combinations have been the most variable in expressing the T 145 marker, and represent by far the weakest of the T blasts analyzed for the expression of T 145. As the proliferative ability of the T blasts generated against Mls and/or Ia are at least of orders of magnitude similar to

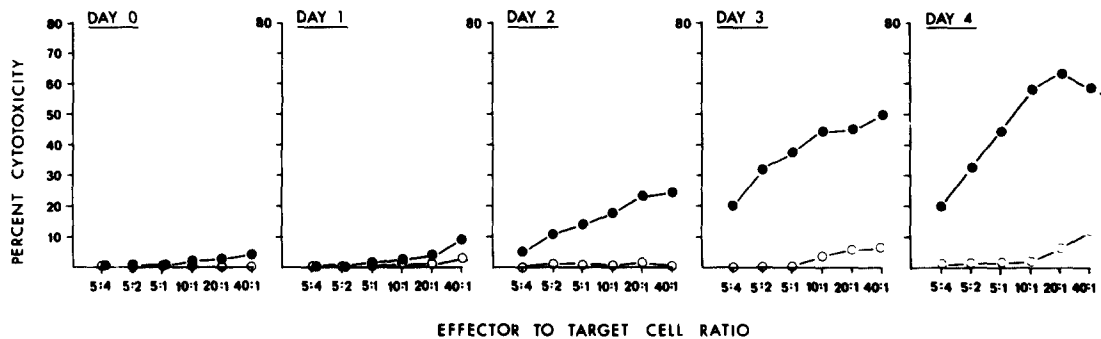


FIG. 2. Kinetics for the development of cytotoxic function by Con A-induced lymphoblasts. Cells from flask cultures were harvested at the designated times after the addition of Con A, and they were tested for cytotoxic activity in a 4-h assay against ^{51}Cr -labeled P815 tumor target cells at the indicated effector:target cell ratios. (○), cytotoxicity expressed in the absence of agglutinant; (●), total cytotoxic potential of the blast cells in the presence of 5 $\mu\text{g}/\text{ml}$ of leucoagglutinin.

those against SD differences, it would thus seem clear that the T 145 glycoprotein is not merely a marker of any proliferating T lymphoblast.

Positive Correlation in Time Between the Appearance of Cytolytic Ability and Expression of T 145 on Con A-Induced Lymphoblasts. Induction of T-cell proliferation by alloantigenic differences is a nonrandom event, as different subsets of T lymphocytes appear to respond preferentially towards the various histocompatibility antigens. Thus, it is now well recognized that the cells responding towards SD differences are to a large extent $\text{Ly } 1^{-2+3+}$, and express efficient cytolytic ability (1), whereas T cells reacting against Ia or Mls incompatibilities are dominated by $\text{Ly } 1^{+2-3-}$ cells with less impressive killing ability (1, 18, 19). The results of the preceding section would be in line with the possibility that the T 145 band may represent a differentiation antigen typical of a cell with the functions and surface markers of cytolytic nature. Using Con A as a polyclonal activator of T cells, it would then be possible to generate T blasts with a wide range of immunological activities (5), and to follow the glycoprotein profiles of the blast cells while focusing upon only one of these activities; the appearance of cytolytic function.

To obtain optimal mitogenic stimulation and expansion of the Con A-reactive cells, Ig-anti-Ig column-purified spleen T cells from CBA/H mice ($10^6/\text{ml}$) were cultured with 2 $\mu\text{g}/\text{ml}$ of Con A. The culture vessels were Falcon flasks preincubated overnight with syngeneic spleen cells and subsequently washed so that they contained only the adherent, catalyzing cell population. At the end of the 1st and 3rd day of culture, lymphocytes were diluted to a cell concentration of 3.5×10^5 cells/ml with fresh media containing Con A (2 $\mu\text{g}/\text{ml}$) to minimize cell death and allow optimal conditions for cell growth as previously described (20). At 24-h intervals cells were harvested from culture, adjusted to the appropriate cell concentration, and tested for cytolytic activity against allogeneic, ^{51}Cr -labeled target cells using phytohemagglutinin (PHA) as an agglutinant to provide efficient contact between effector and target cells (10).

Fig. 2 illustrates the kinetics and extent of cytolytic activity displayed by Con A-induced T lymphoblasts. As is seen, only minor cytolytic activity was detected

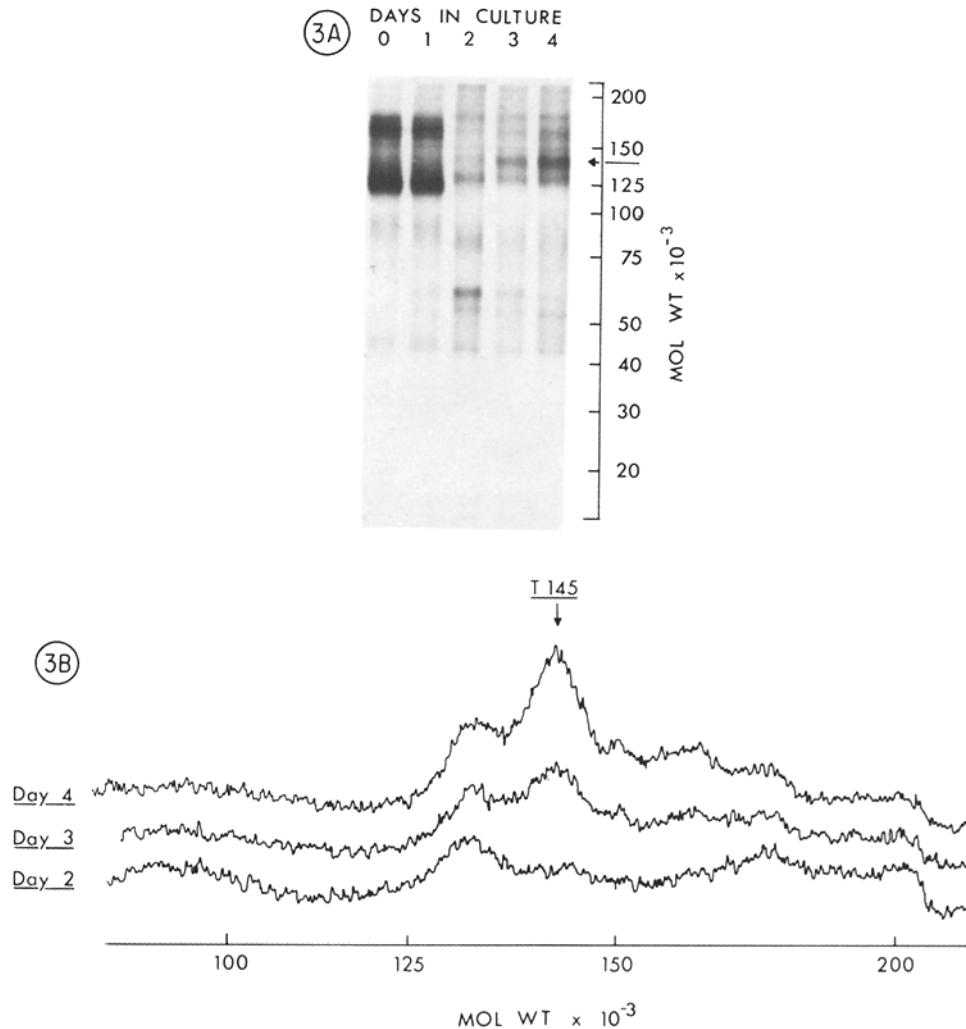


FIG. 3. Cell surface glycoprotein patterns of NaB^3H_4 -labeled blasts at various times during Con A activation. Aliquots of the same Con A cell preparations assayed for cytotoxic function in Fig. 2 were washed three times in 0.05 M α -methyl-D-mannoside, surface-labeled with NaB^3H_4 , solubilized, and examined for the expression of T 145 at various times after the initiation of the cultures. Fig. 3A illustrates the fluorographic patterns of cell surface glycoproteins at the indicated days in culture. Quantitative differences in the expression of T 145 are seen in the densitometric tracings in Fig. 3B. Each sample was adjusted to 12,500 cpm in electrophoresis sample buffer (5–10 μg protein) and separated in a linear 7.5–10% gradient of acrylamide containing 0.1% SDS.

on day 2, despite the fact that 96% of the viable cells were large blasts at this time. On days 3 and 4, the blasts were morphologically indistinguishable from those seen on day 2, and yet the killing ability of these Con A blasts increased in a drastic manner. The results of experiments carried out in parallel on the cell-surface glycoprotein patterns during Con A activation are shown in Fig. 3. As is shown, T 145 was virtually absent on days 1 and 2, and it first appeared in detectable quantities on day 3, increasing in intensity during the next day of

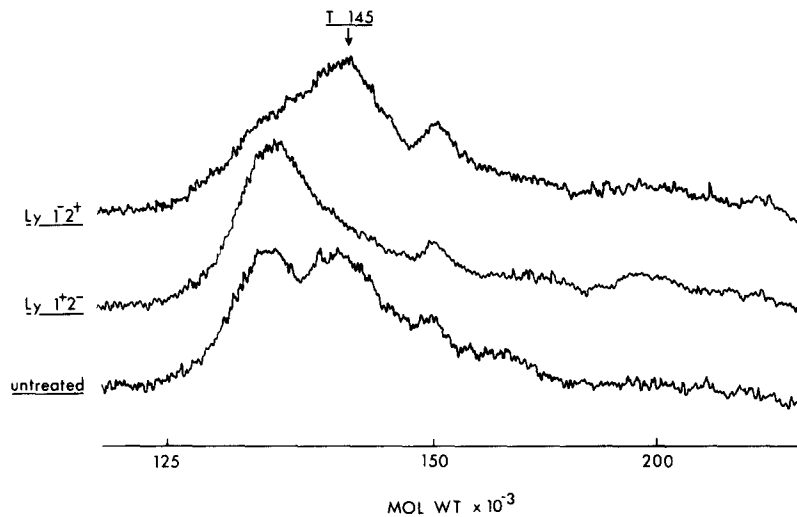


FIG. 4. Densitometric tracings of the cell surface glycoproteins from NaB^3H_4 -labeled MLC T blasts before and after enrichment for $\text{Ly } 1^{2-}$ and $\text{Ly } 1^{2+}$ blasts. Purified C57BL/6 anti-DBA/2 MLC T blasts were dissected into $\text{Ly } 1^{2-}$ and $\text{Ly } 1^{2+}$ blasts by treatment with the respective anti-Ly antisera plus complement. The individual groups of Ly phenotype-enriched blasts were surface-labeled with NaB^3H_4 , solubilized, and prepared for electrophoresis. Samples were adjusted to a total of 12,500 cpm (5–10 μg protein) and run in a linear 7.5–10% acrylamide gradient containing 0.1% SDS.

culture. Thus, there exists a positive correlation both in the time and extent of T 145 expression with that of cytolytic activity by Con A-induced lymphoblasts.

The Selective Expression of T 145 on $\text{Ly } 1^{2+3+}$ Lymphoblasts. Murine T lymphoblasts can be subdivided according to Ly 1,2,3 phenotype into three well-defined groups: $\text{Ly } 1^{2+3+}$, $\text{Ly } 1^{2-3-}$, and $\text{Ly } 1^{2+3+}$ blasts. As mentioned previously, this phenotypic variation is at least in part correlated with functional diversity. To further investigate the expression of T 145 with respect to Ly phenotype, C57BL/6 anti-DBA/2 blasts were generated in MLC so that the resulting blasts would represent activations against the entire *H-2* complex and Mls. At the peak of the proliferative response, blasts were isolated by 1 g velocity sedimentation, divided, and incubated with PBS or an excess of anti-Ly 1 or 2 specific antisera for 45 min on ice. At the end of this incubation, the cells were washed once in culture media, resuspended with appropriately diluted, absorbed rabbit complement (21), and incubated for an additional period of 30 min at 37°C. The cells were then diluted to 1 ml with ice-cold media, and cell debris was removed by centrifugation through FCS (22). The various groups of cells were then washed twice with PBS before surface labeling with NaB^3H_4 . After labeling and solubilization, the surface glycoprotein patterns from the original T lymphoblasts, as well as the populations enriched for $\text{Ly } 1^{2-}$ or $\text{Ly } 1^{2+}$ blasts, were then analyzed by SDS-polyacrylamide gel electrophoresis. As seen in Fig. 4, analysis of the densitometric tracings of the surface glycoproteins from the unfractionated and respective Ly-enriched blasts revealed a complete absence of T 145 on $\text{Ly } 1^{2-}$ blasts, which could be seen to be an exclusive surface marker by $\text{Ly } 1^{2+}$ blasts.

Whether or not such a specific enrichment for $\text{Ly } 1^{2+}$ T cells before activation

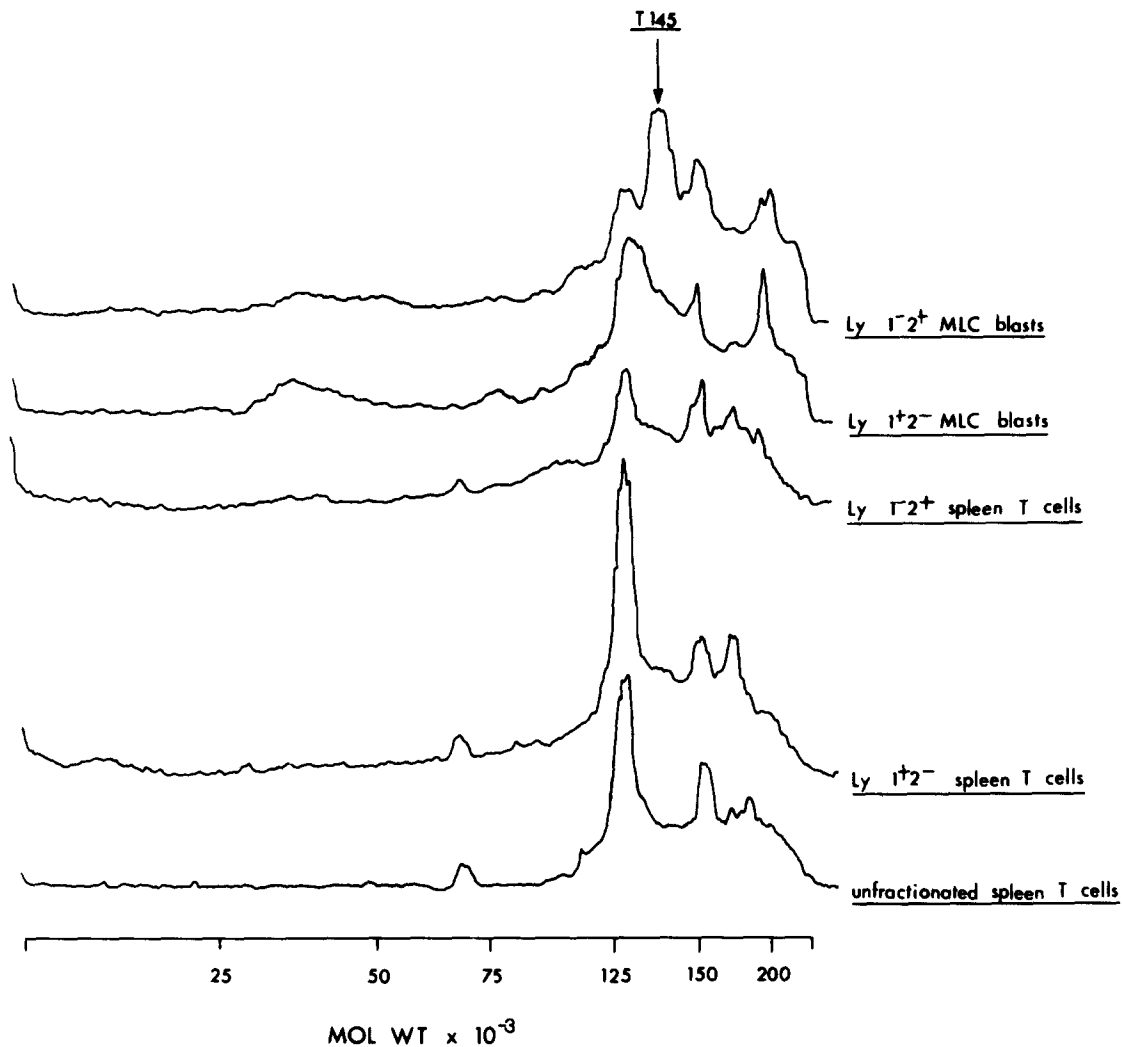


FIG. 5. Densitometric tracings of the cell surface glycoproteins of nonimmune spleen T cells before and after enrichment for Ly 1⁺2⁻ and Ly 1⁻2⁺ subpopulations of T cells. Ig-anti-Ig column-purified spleen T cells from C57BL/6 mice were treated twice with the respective anti-Ly antisera plus complement before surface labeling with NaB³H₄. Samples were solubilized and adjusted to a total of 15,000 cpm (5-10 μ g protein) and compared with the corresponding Ly-enriched populations of MLC blasts by electrophoresis through a linear 7.5-10% gradient of acrylamide containing 0.1% SDS.

would also reveal T 145 was then examined. C57BL/6 spleen cells were first purified by passage through Ig-anti-Ig-coated columns and divided into three groups as above. To ensure maximum enrichment of the respective Ly subclasses of T cells, each group was treated twice with antiserum and complement. After removal of the dead cells, the cells were labeled with NaB³H₄, solubilized, and prepared for electrophoresis. As seen in Fig. 5, a 10-fold enriched population of Ly 1⁻2⁺ cells failed to show any significant expression of T 145. These results further support the contention that T 145 arises through a maturation process

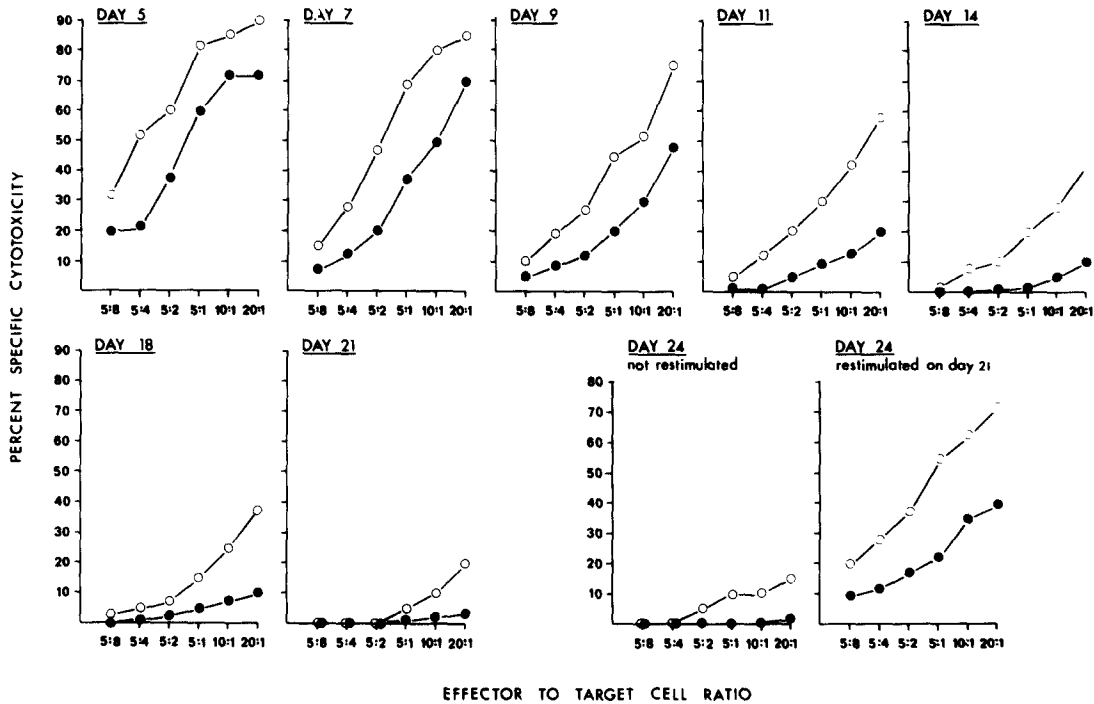


Fig. 6. Cytolytic titration curves of CBA/H anti-DBA/2 MLC T blasts during their morphological reversion to small lymphocytes. MLC blasts were harvested from culture on day 5, purified by 1 g velocity sedimentation through a linear 15-30% FCS gradient, and returned to culture at a concentration of 5×10^5 cell/ml on syngeneic macrophage feeder layers. At the indicated times, cells were tested for their cytolytic activity against ^{51}Cr -labeled target cells at a variety of effector to target cell ratios in both a 2-h (●) and 4-h (○) assay.

of Ly 1-2⁺ T cells at a time concomitant with the expression of cytolytic activity of these cells.

The T 145 Glycoprotein is a Differentiation Molecule Typical of a Restricted, Primed Subset of T Lymphocytes. The stability of T 145 in the absence of antigenic or mitogenic stimulation could be expected to further distinguish it as either a blast-stage-restricted marker or a more permanent type differentiation antigen, unrelated to the blast state or functional activity of the cell per se. To examine this question, a large number of MLC-activated T lymphoblasts (CBA/H anti-DBA/2) were isolated at the peak of the primary cytotoxic response and allowed to undergo morphological and functional reversion on syngeneic macrophage feeder layers (23). At various times during the reversion process, samples of cells were analyzed for cytolytic ability, percentage of blasts, and the presence of the T 145 glycoprotein on their surface.

The results relating to the functional activity of these cells are shown in Fig. 6. As seen, the peak of cytolytic activity occurs on day 5, followed by a relatively steady but slow decrease during the next 16 days in culture, reaching low yet significant levels on day 21. In contrast to this relatively slow decline in cytolytic activity, morphological reversion to small lymphocytes is essentially complete by day 11-12 (97% small lymphocytes). That quiescent cytotoxic

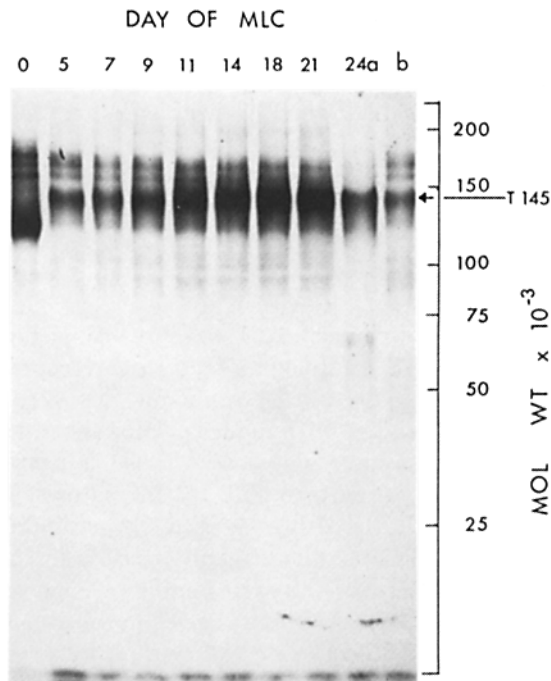


FIG. 7. Cell surface glycoprotein patterns of MLC T blasts during their morphological reversion to small lymphocytes. Aliquots of cells tested for cytolytic activity in Fig. 6 were taken on the indicated days of culture and surface-labeled with NaB^3H_4 . 24a represents the glycoprotein banding patterns of cells harvested on the 24th day of culture and 24b represents the glycoprotein pattern of a day 21 culture restimulated with irradiated DBA/2 spleen cells and harvested 3 days later. Samples were adjusted to a total of 15,000 cpm (5–10 μg . protein) and separated by electrophoresis through a linear 7.5–10% gradient of acrylamide containing 0.1% SDS.

memory cells were indeed represented in the day 21 population is shown by the positive control in which a portion of these cells could be shown to display a rapid, strong increase in cytotoxic activity upon restimulation with the relevant irradiated stimulator cells (23).

The cell surface glycoprotein patterns of cells taken at various times during this reversion process and labeled with NaB^3H_4 are seen in Fig. 7. From this figure it is clear that once induced, T 145 is expressed with seemingly equal intensity on blasts and small reverted T lymphocytes. We would thus conclude that within the limited time span studied, T 145 behaves as a permanent type differentiation structure whose expression is not restricted to the blast stage of activation. The question of the involvement of T 145 as a possible "killing-relevant" structure could not be answered by these experiments.

Discussion

T lymphocytes are known to display a group of unique surface molecules which have been determined in part by serology (1, 24, 25), and by biochemical approaches (11, 17, 26, 27). Some of these molecules are only found on the T

lymphocytes at a certain stage of differentiation (25), whereas others would seem to be maintained throughout differentiation (1, 24). In the present study, we could confirm and extend earlier findings on the characterization of T-cell surface glycoproteins and their distinction from those expressed on B lymphocytes (11, 17). Our main interest, however, has been focused upon the characterization of a unique membrane glycoprotein, absent on resting T lymphocytes and simultaneously expressed on T blasts with the development of effector cell function. We have called this glycoprotein T 145 because of its exclusive expression on T cells and its apparent molecular weight in SDS gels of 145,000 daltons.

Studies on the cellular distribution of T 145 have consistently shown that B lymphocytes, B blasts, and B lymphomas lack this glycoprotein. In the same way, resting T lymphocytes and all 15 T lymphomas analyzed thus far have also been negative. Additional studies with mouse peritoneal exudate macrophages, erythrocytes, and fibroblasts have also shown these cell types to be negative. Thus, the cellular distribution pattern of T 145 indicates that this protein is a unique surface marker of T cells with restricted expression on T lymphoblasts.

Through an analysis of various alloantigenic barriers in the mouse known to induce a primary MLC *in vitro*, we have been able to assign a ranking order of efficiency for the induction of T 145⁺ blasts, based on the genetic incompatibility between the responding and stimulating cell type. Here we could demonstrate that blasts activated against SD determinants of the *H-2D* or *K* regions expressed the highest amount of radioactivity associated with T 145 (Table I). T-cell activations involving genetic differences across the entire *H-2* complex ranked second in efficiency for the generation of T 145⁺ blasts, followed by activations against *I* region only. Blasts generated in several syngeneic, Mls-incompatible combinations have been the poorest in T 145 expression of the genetic activations studied.

In addition, we have used Con A as an antigen-independent system of activation to induce T blasts of a wider range of immunological activities (5). Through the use of previously described techniques to measure the total cytotoxic potential of mitogen-activated T cells (10) we have been able to directly correlate both the appearance in time and the extent of T 145 expression with the generation of cytolytic activity by these cells. Additional studies have now shown that PHA-induced blasts generate lower but significant levels of cytotoxicity, and that the time and extent of T 145 expression on these blasts correlate in a highly significant way with cytolytic activity. In both systems of mitogen activation, the expression of T 145 did not parallel the initial state of blast transformation, but was instead concomitant with the appearance of cytotoxic function by these cells.

As the anti-SD reactive T cells are known to constitute better killer cells than the anti-Ia and anti-Mls cells (1, 18, 19), the relative intensity of T 145 expression seemed to correlate well with the levels of cytolytic activity induced by the various genetic systems (Table I), and to account for the higher expression on Con A vs. PHA blasts (28). Aside from being functionally distinct, anti-SD reactive T cells are normally characterized by the serological phenotype Ly 1⁻2⁺, whereas the anti-Ia cells are mostly Ly 1⁺2⁻ (1). Thus, in striking accordance with the expectation that T 145 may represent a marker for killer T

cells, were the findings that Ly 1⁺2⁻ blasts were T 145⁻, and that Ly 1⁻2⁺ blasts were strongly T 145⁺. Suppressor T cells may also express an Ly phenotype similar to that of killer T cells, but treatment of MLC blasts with antisera raised against I-J (29) in the presence of complement does not eliminate T 145-positive cells in any detectable degree (unpublished observations). These findings do not exclude the possible expression of T 145 on suppressor T cells, but they would argue strongly against T 145 as constituting a marker for suppressor cells only.

Both the tissue distribution (1) and current molecular weight estimations of 35,000 daltons (30) make it highly unlikely that T 145 is an Ly 2 or 3 antigen. Furthermore, work in progress involving sequential immune precipitations of lactoperoxidase and NaB³H₄ surface-labeled T blasts with a variety of alloantisera against known antigenic systems distinguish T 145 as a unique surface molecule.

The stability of T 145 on killer T lymphoblasts undergoing morphological reversion to small, poorly cytotoxic T cells clearly indicates that the expression of this glycoprotein is not restricted to the blast state. On the contrary, the present evidence would indicate that once induced, T 145 expression persists and is no longer dependent upon the presence of stimulating antigen or the blast state.

It is known from the work of others as well as that of our own that killer T cells in the mouse (31, 32), rat (K. Welsh, personal communication), and human³ express new "killer cell-unique" surface antigens. Although the exact relationship between these findings and T 145 remains to be established, the evidence presented here strongly supports the contention that T 145 is such a unique killer T cell marker. We would at present take the view that T 145 represents a surface glycoprotein appearing on T lymphocytes undergoing immune activation by either a relevant polyclonal T-cell activator like Con A, or by alloantigens in such a way as to yield cytolytic T cells in the resulting cell population.

From internal labeling experiments on highly purified T lymphoblasts we know that the T 145 glycoprotein indeed represents an actual product of the T cell, and that it can serve as an immunogen for antibody synthesis across species barriers.⁴ The function of T 145⁺ cells may be in toto of cytolytic nature, and the actual presence of T 145 on the surface of such T cells may be a necessary requirement for directed lysis to occur, but could require additional functional activities of the cell to allow the lytic function to become expressed. If this is correct, anti-T 145 antibodies should be unusually efficient inhibitors of cytotoxic T lymphocytes, and preliminary experiments would suggest this to be the case.

In conclusion, we have presented evidence on the appearance of a new surface glycoprotein on immunocompetent T cells appearing at a given stage of differentiation. This protein could be shown to be selectively expressed on the surface of T-cell blasts endowed with cytolytic ability, reacting against SD determinants, and with the phenotype Ly 1⁻2⁺. Further analysis of this

³ L. Andersson, C. Gahmberg, A. Kimura, and H. Wigzell. Manuscript in preparation.

⁴ A. Kimura and H. Wigzell. Manuscript in preparation.

glycoprotein should now allow deeper insight as to how these specialized T lymphocytes exert their biologically relevant functions.

Summary

T lymphocytes at various stages of maturation and differentiation have been isolated by cellular fractionation procedures and characterized by cell surface markers and functional assays. The cell surface glycoproteins of the various T-cell preparations have been selectively radiolabeled by the galactose oxidase-tritiated sodium borohydride technique and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography.

Details are presented on the appearance of a new cell surface glycoprotein (T 145), present on immunocompetent T lymphocytes after activation by either major histocompatibility complex alloantigens or by concanavalin A. The intensity of T 145 expression on T lymphoblasts is shown to be directly correlated in time and extent to the levels of cytotoxicity generated in a variety of T-cell activations.

Specific enrichment procedures of purified populations of mixed leukocyte culture blasts have shown Ly 1⁺2⁻ blasts to be T 145⁻ and Ly 1⁻2⁺ blasts to be strongly T 145⁺. Similar enrichment procedures on normal peripheral T cells have failed to reveal any significant expression of T 145 on a highly enriched population of Ly 1⁻2⁺ T cells. Further studies on the stability of T 145 expression after induction have shown it to be a more permanent-type differentiation structure whose expression is clearly not linked to the blast stage of activation.

T 145 would thus appear to represent a membrane glycoprotein whose exclusive expression on T lymphoblasts is further restricted to a defined group of cells endowed with cytolytic activity and bearing the Ly phenotype Ly 1⁻2⁺.

We wish to thank Dr. J. Andersson for the generous supply of purified IgG₁ myeloma proteins and mitogens used in these studies, and Dr. E. A. Boyse for supplying us with anti-Ly sera. The skillful secretarial assistance of Birgitta Ehrsson is gratefully acknowledged.

Received for publication 18 October 1977.

References

1. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* 141:1376.
2. Huber, B., O. Devinsky, R. K. Gershon, and H. Cantor. 1976. Cell-mediated immunity: delayed-type hypersensitivity and cytotoxic responses are mediated by different T-cell subclasses. *J. Exp. Med.* 143:1534.
3. Gershon, R. K. 1974. T cell suppression. *Contemp. Top. Immunobiol.* 3:1.
4. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen reactive cells. *Transplant. Rev.* 1:3.
5. Jandinski, J., H. Cantor, T. Tadakuma, D. L. Peavy, and C. W. Pierce. 1976. Separation of helper T cells from suppressor T cells expressing different Ly components. I. Polyclonal activation: suppressor and helper activities are inherent properties of distinct T-cell subclasses. *J. Exp. Med.* 143:1382.
6. Kimura, A. K., and H. Wigzell. 1977. Cytotoxic T lymphocyte membrane components: an analysis of structures related to function. *Contemp. Top. Mol. Immunol.* 6:209.

7. Wigzell, H. 1976. Specific affinity fractionation of lymphocytes using glass or plastic bead columns. *Scand. J. Immunol. Suppl.* 5:23.
8. Miller, R. G., and R. A. Phillips. 1969. Separation of cells by velocity sedimentation. *J. Cell. Physiol.* 73:191.
9. Brunner, K. T., J. Mauel, J. Cerottini, and B. Chapuis. 1968. Quantitative assay of the lytic action of immune lymphoid cells on ⁵¹Cr-labelled antigenic target cells in vitro. Inhibition by isoantibody and drugs. *Immunology* 14:181.
10. Bevan, M. J., and M. Cohn. 1975. Cytotoxic effects of antigen- and mitogen-induced T cells on various targets. *J. Immunol.* 114:559.
11. Gahmberg, C. G., P. Häyry, and L. C. Andersson. 1976. Characterization of surface glycoproteins on mouse lymphoid cells. *J. Cell Biol.* 58:642.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680.
13. Melchers, F., and J. Andersson. 1973. Synthesis, surface deposition and secretion of immunoglobulin M in bone-marrow derived lymphocytes before and after mitogenic stimulation. *Transplant Rev.* 14:76.
14. Hunter, W. M. 1973. Radioimmunoassay. In *Handbook of Experimental Immunology*. D. M. Weir, editor. Blackwell Scientific Publications Ltd., Oxford, England. 17-3.
15. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83.
16. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335.
17. Trowbridge, I. S., P. Ralph, and M. J. Bevan. 1975. Differences in the surface proteins of mouse B and T cells. *Proc. Natl. Acad. Sci. U. S. A.* 72:157.
18. Nabholz, M., H. Young, A. Rynbeek, R. Baccardo, C. S. David, T. Meo, V. Miggiano, and D. Shreffler. 1975. I-region-associated determinants: expression on mitogen-stimulated lymphocytes and detection by cytotoxic T cells. *Eur. J. Immunol.* 5:594.
19. Wagner, H., D. Götze, L. Ptschelinzew, and M. Rölinghoff. 1975. Induction of cytotoxic T lymphocytes against I-region-coded determinants: in vitro evidence for a third histocompatibility locus in the mouse. *J. Exp. Med.* 142:1477.
20. Watanabe, T., C. G. Fathman, and A. Coutinho. 1977. Clonal growth of T cells in vitro: preliminary attempts to a quantitative approach. *Transplant. Rev.* 35:3.
21. Boyse, E. A., L. Hubbard, E. Stockert, and M. E. Lamm. 1970. Improved complementation in the cytotoxic test. *Transplantation (Baltimore)* 10:446.
22. Shortman, K., N. Williams, and P. Adams. 1972. The separation of different cell classes from lymphoid organs. V. Simple procedures for the removal of cell debris, damaged cells and erythroid cells from lymphoid cell suspensions. *J. Immunol. Methods.* 1:273.
23. Andersson, L. C., and P. Häyry. 1975. Clonal isolation of alloantigen-reactive T-cells and characterization of their memory function. *Transplant. Rev.* 25:121.
24. Reif, A. E., and J. M. V. Allen. 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissue. *J. Exp. Med.* 120:413.
25. Boyse, E. A., L. J. Old, and E. Stockert. 1966. The TL (thymus leukemia) antigen: a review. In *Immunopathology, Fourth International Symposium*. P. Grabar and P. A. Miescher, editors. Schwabe & Co., Basel, Switzerland.
26. Morris, R. J., and A. F. Williams. 1977. Serological and preliminary biochemical characteristics of a T lymphocyte differentiation antigen detected by rabbit antiserum to rat thymocyte membranes. *Eur. J. Immunol.* 118:1600.
27. Trowbridge, I. S., and C. Mazauskas. 1976. Immunological properties of murine thymus-dependent lymphocyte surface glycoproteins. *Eur. J. Immunol.* 6:557.
28. Falkoff, R. M., and R. W. Dutton. 1977. Evidence that Con A induces cytotoxicity in

- the same subclass as does alloimmunization. *J. Immunol.* 118:1600.
29. Murphy, D. B., L. A. Herzenberg, K. Okumura, L. A. Herzenberg, and H. O. McDevitt. 1976. A new *I* subregion (*I-J*) marked by a locus (*Ia-4*) controlling surface determinants on suppressor T lymphocytes. *J. Exp. Med.* 144:699.
 30. Durda, P. J., and P. D. Gottlieb. 1976. The Ly-3 antigens on mouse thymocytes: immune precipitation and molecular weight characterization. *J. Exp. Med.* 144:476.
 31. Sullivan, K. A., G. Berke, and D. B. Amos. 1973. An antigenic determinant of cytotoxic lymphocytes. *Transplantation (Baltimore)*. 16:388.
 32. Kimura, A. K., K. I. Welsh, and H. Wigzell. 1975. Specific gel precipitation of a T cell membrane component involved in T cell-mediated killing. *In Leukocyte Membrane Determinants Regulating Immune Reactivity*. V. Eijsvogel, D. Roos, and W. P. Zijlemaker, editors. Academic Press, Inc., New York. 611.