

# INVOLVEMENT OF THE T CELL ANTIGEN RECEPTOR AND OF LYT-2 IN THE CYTOTOXIC FUNCTION OF AGED KILLER (AK) T CELLS

BY DRAGANA LJ. JANKOVIĆ, DAVID WOODLAND, INGA MELCHERS,  
HANS-ULRICH WELTZIEN, RALPH T. KUBO, AND KLAUS EICHMANN

*From the Max-Planck-Institut für Immunbiologie, 78 Freiburg, Federal Republic of Germany;  
and the National Jewish Center for Immunology and Respiratory Medicine,  
Denver, Colorado 80206*

Tissue culture-adapted cytotoxic T cell lines and clones often do not maintain their original recognition specificity and function. For example, many T cell lines that acquired broad specificity spectra, in some cases resembling NK activity have been described (1-3). In contrast, our group has observed that long-term cytotoxic clones, after having lost their original antigen specificity, frequently acquire a new but equally specific cytotoxic activity for the DBA/2 mastocytoma tumor cell line, P815 (4). This activity is distinct from the NK or "NK-like" activity of other long term T cell lines by its exclusive specificity for P815 targets and by the sensitivity of the cytotoxicity to inhibition with anti-Lyt-2 antibody. We have therefore termed this function aged killer (AK)<sup>1</sup> activity (4). Because of their stability, independence of restimulation with antigen, and generally good adaptation to tissue culture, AK cells have obvious advantages over bona fide cytotoxic T cell lines for experimental purposes. In addition, several properties of their cytotoxic function resemble that of bona fide cytotoxic T cells (4). We therefore evaluated the possibility of using AK cells for the study of the cytotoxic function of T lymphocytes. In particular, we investigated whether or not the T cell receptor and/or Lyt-2 are involved in AK cytotoxicity. To this end, we generated a series of functionally deficient variants from the prototype AK cell clone 96 (Cl 96). A characterization of this collection of functional variants for the expression of the T cell antigen receptor and of Lyt-2 reveals a clear correlation of both with AK activity: Loss or low levels of Lyt-2 results in loss of specific cytotoxicity for P815 but is compatible with maintenance of lectin-mediated cytotoxic function; loss or low levels of the T cell receptor results in loss of all cytotoxic activity. The data are compatible with the notion that the antigen receptor of AK cells has a low affinity for P815, which needs stabilization either by Lyt-2 or by a lectin. We conclude that the recognition process of AK T cells is similar to that of bona fide cytotoxic T cells.

D. Lj. Janković is a recipient of an Alexander von Humboldt Fellowship. She is currently on leave from the Immunology Research Center, 11221 Belgrade, Yugoslavia. Address correspondence to Dr. K. Eichmann, Max-Planck-Institut für Immunbiologie, Stübeweg, D-7800 Freiburg i.Br. FDR.

<sup>1</sup> *Abbreviations used in this paper:* AK, aged killer;  $\alpha$ MM,  $\alpha$ -methyl-mannoside; ConASN, Con A-induced rat spleen cell supernatant.

### Materials and Methods

*Cell Lines and Culture Conditions.* Cl 96 cells, originally obtained from Dr. P. Krammer, Deutsches Krebsforschungszentrum, Heidelberg, and all other cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS,  $10^{-5}$  M 2-ME, 2.5 mM Hepes, 2 mM glutamine, kanamycin (100  $\mu$ g/ml), and tyrosine (10  $\mu$ g/ml) in 5% CO<sub>2</sub>/air. IL-2-dependent cells were grown in the presence of 10% rat spleen cell Con A supernatant (ConASN) as a source of IL-2 and 10 mg/ml  $\alpha$ -methyl-mannoside ( $\alpha$ MM). IL-2-independent cell lines were grown in the absence of IL-2 and  $\alpha$ MM. In some experiments recombinant human IL-2 (rec. IL-2; Sandoz Forschungsinstitut, Wien, Austria) was used, as indicated, adjusted to 100 U/ml (1 U = 100 pg rIL-2).

*Antibodies and Antisera.* Rabbit anti-mouse T cell antigen receptor antiserum was produced by immunizing rabbits with immunoprecipitated T cell receptor material using a clonotypic antibody and the OVA-specific T cell hybridoma DO11.10 (R. Kubo, Manuscript in preparation). It was shown to react with most normal T cells and T cell tumors and to immunoprecipitate T cell receptor. Also used were anti-Thy-1.2 (Code 585, Olac), anti-L3T4(GK 1.5) anti-Lyt-2 (56-6.7) (5). Anti-H-2 class I antibody 9C5 was obtained by fusion of spleen cells of AKR mice repeatedly immunized with Cl 96 cells, with X63.Ag8.653 cells. Antibody 9C5 precipitates class I MHC molecules from Cl 96 and from other cells (unpublished observation). Strain distribution is similar to public specificity H27.

*Cytotoxic Assay.* Cells were assayed in a 4 h <sup>51</sup>Cr release assay at various E/T cell ratios. Effector cells in 0.1 ml of culture medium (see above) were added to  $2 \times 10^3$  <sup>51</sup>Cr-labeled P815 cells. For lectin facilitated cytotoxicity, PHA (Gibco Laboratories, Grand Island, NY; 670/0576, 1% of stock solution) was added to the test mixture. After incubation for 4 h at 37°C the plates were centrifuged at 1,000 rpm for 10 min and 0.1 ml supernatant was removed for counting. Percent specific lysis was calculated as  $100 \times (\text{experimental} - \text{low control}) / (\text{acid lysis} - \text{low control})$ . Each effector cell concentration was tested in triplicate.

*Cytofluorometric Analysis.* Cytofluorometric analyses were made using a 50 H cell sorting device (Ortho Diagnostic Systems, Inc., Raritan, NJ). FITC-labeled F(ab)<sub>2</sub> anti-MIg and F(ab)<sub>2</sub> anti-rat Ig were purchased from Medac, Hamburg, FRG. Incubations with first and second antibodies were for 30 min on ice, and were followed by 3 washes.

*Radiolabeling of Cells.* Cell surface proteins were labeled by lactoperoxidase-catalysed iodination using a modification of the method described by Hubbard and Cohn (6). Briefly,  $50 \times 10^6$  cells were suspended in 1 ml PBS and 250  $\mu$ l of Enzymobead™ reagent (Bio-Rad Laboratories, München, FRG) and 1.5 mCi of <sup>125</sup>I (Amersham Corp., London) were added. The iodination was carried out by adding, eight times, 50  $\mu$ l of  $\beta$ -D-glucose (Sigma Chemical Co., Taufkirchen, FRG) in two minute intervals. After the last addition of  $\beta$ -D-glucose, the reaction was allowed to proceed for an additional 30 min and stopped by adding a large volume of ice-cold PBS containing 5 mM KI. Labeled cells were further washed five times with PBS containing 10 mM iodoacetamide and then treated for 45 min on ice with lysis buffer (50 mM Tris-HCl, pH 8.0; 0.15 M NaCl; 1% NP-40; 1 mM EDTA, 2 mM PMSF; 10 mM iodoacetamide; and 0.02% NaN<sub>3</sub>). Cell lysates were centrifuged for 15 min in an airfuge (Beckman Instruments Inc., Fullerton, CA) at 10,000 g. Supernatants were stored at -70°C.

*Two-dimensional Gel Electrophoresis.* Two-dimensional (nonreduced/reduced) polyacrylamide gel electrophoresis employed the protocol described by Goding and Harris (7). The first dimension consisted of a 7.5% acrylamide tube gel. Sample volumes were 50  $\mu$ l and contained 10  $\mu$ l of labeled cell supernatant (corresponding to  $\sim 1.5 \times 10^6$  cells). Gels were run at 1 mA per tube constant current for 3 h. When the bromphenol blue marker reached the bottom, the gel was removed and equilibrated for 1 h at 25°C in sample buffer with 5% 2-ME and 50 mM DTT. The second dimension was performed using a 10% acrylamide slab gel. Gels were fixed, stained, and destained. Molecular weights were determined by reference to the mobility of low molecular weight standard proteins (Pharmacia Fine Chemicals, Freiburg, FRG) applied on the same gel. Autoradiographs of

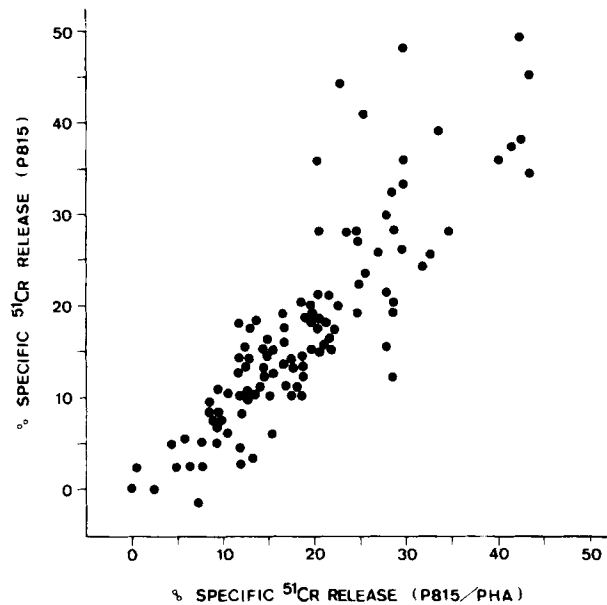


FIGURE 1. Limiting dilution cloning of Cl 96 cells at 0.3 cells/microwell in 15% ConASN. Wells with growing cells (108/480, cloning efficiency = 0.86) were tested in  $^{51}\text{Cr}$  release assay for cytotoxicity against P815 cells in the absence and presence of 2% PHA. Each dot represents the result on one culture well.

the dried gels were visualized using intensifying screens (Dr. Goos, Heidelberg, FRG) and X-R5 films (Eastman Kodak Co., Stuttgart, FRG) at  $-70^{\circ}\text{C}$ .

**Immunoprecipitation.** Aliquots of cell lysate supernatant, precleared with packed protein A-Sepharose beads (Pharmacia Fine Chemicals), were mixed with either  $50\ \mu\text{l}$  of rabbit anti-mouse T cell antigen receptor serum or with  $50\ \mu\text{l}$  of normal nonimmune rabbit serum. Two h later,  $50\ \mu\text{l}$  of protein A beads were added and after an overnight incubation at  $4^{\circ}\text{C}$  they were thoroughly washed with lysis buffer. The specifically bound material was removed by boiling in sample buffer and subjected to two-dimensional gel electrophoresis.

## Results

**General Characteristics of Cl 96.** Most functional properties of Cl 96 have been described before (4). Generated in 1980, the cells were grown in IL-2-containing media for several years before their cytotoxic activity for P815 targets was discovered fortuitously. Assays for P815 cytotoxicity performed early in its development had been negative (8), suggesting that the cell line had switched from its original, unknown specificity to AK activity. Cytotoxicity for P815 can be shown in a specific 4 h,  $^{51}\text{Cr}$  release assay, as well as in the presence of a lectin such as PHA and results in target cell death (unpublished observations). Not only the specific P815 kill, but also the lectin-facilitated cytotoxic activity is largely restricted to P815 targets (Woodland et al., manuscript in preparation). Specific cytotoxic activity, but not lectin-facilitated cytotoxicity, is readily inhibited by anti-Lyt-2 antibody (4).

**Selection of Noncytotoxic Variants.** A first set of functional variants of Cl 96 was selected in cloning experiments for loss of all cytotoxic activity. Fig. 1 shows

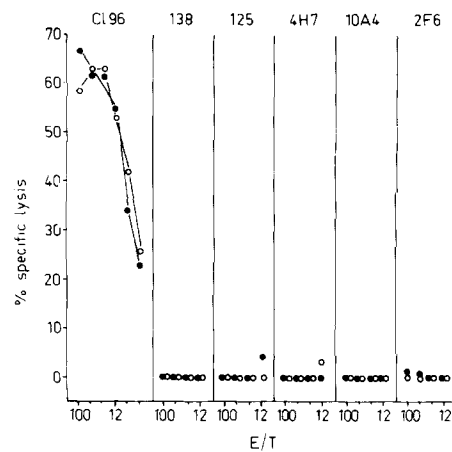


FIGURE 2.  $^{51}\text{Cr}$  release assay of Cl 96 cells and of five noncytotoxic variants in the absence (●) and presence (○) of 2% PHA against P815 target cells. Variants 138 and 125 were obtained from the first, and variants 4H7, 10A4, and 2F6 were obtained from the second cloning experiment. E/T ratios are twofold dilution steps.

results from assaying 108 subclones growing in microwells originally seeded with 0.3 Cl 96 cells/well (88% clonality). Cytotoxic activities for P815 with and without PHA were measured at a single unknown cell concentration, and were found to be highly correlated. Clones with <10% specific  $^{51}\text{Cr}$  release in both assays were grown up and retested in a similar experiment (data not shown). 3 out of 14 clones from this experiment, and 4 out of 10 clones from another experiment, remained essentially negative for all cytotoxic activity upon repeated assays over a period of more than 1 yr. Five of these seven clones were studied in this paper, and representative  $^{51}\text{Cr}$  release assays are given in Fig. 2.

*Selection of IL-2-independent Variants.* In a routine assay for IL-2 dependence of the growth of various T cell lines, we found that the noncytotoxic variants described above grew essentially independent of exogenously added IL-2 (see Fig. 4), even though these cells had been derived and subsequently cultured in the presence of ConASN. We suspected a correlation between IL-2-independent growth and loss of cytotoxic function and performed experiments in which Cl 96 cells were selected for growth in the absence of exogenous IL-2. Various dilutions of cells were cultured in medium without added IL-2 in 1 ml cultures, 24 per cell concentration. Analysis of the growing cultures according to the Poisson distribution (9) suggested that about 1/400 Cl 96 cells grew under these conditions (Fig. 3), whereas the plating efficiency of Cl 96 cells in the presence of IL-2 is close to 1/1 (see Fig. 1). Among a series of IL-2-independent cell lines thus obtained, we present here data on 2 lines, and on 3 clones derived from a third line. The lines (96 MedA and 96 MedC) were grown from 300 Cl 96 cells/well. The clones (96 E7, 96 G12, and 96 E12) were derived from a line originally grown from  $2 \times 10^4$  Cl 96 cells/well. This line was cloned at 0.3 cells/well in the presence of ConASN, because its plating efficiency in the absence of IL-2 was unsatisfactory for effective cloning. Culture of the clones thus obtained was continued in medium without IL-2.

*IL-2-independent Growth of Variants of Cl 96.* As mentioned above, variants

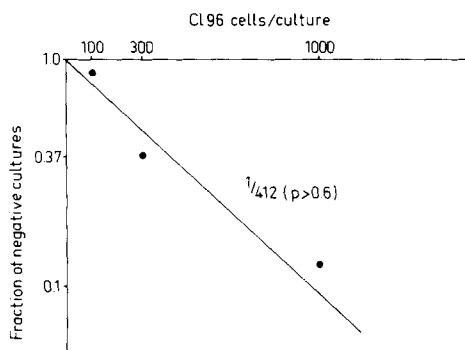


FIGURE 3. Limiting dilution analysis of CI 96 cells that grow in the absence of ConASN. 24 2-ml culture wells were set up for each cell concentration. Growth was recorded by visual examination twice weekly and the results given are from day 14. Frequencies were determined according to Taswell (9) and varied between 1/300 and 1/4000 in several experiments. Most but not all cultures that initially grew could be developed into continuous lines.

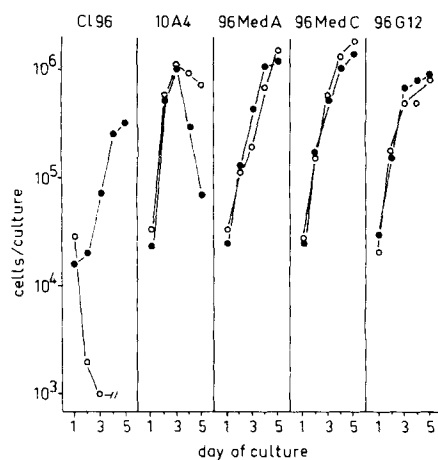


FIGURE 4. Growth curves of CI 96 cells and of several variants in the presence (●) and absence (○) of 100 U recombinant human IL-2. Cells were seeded at  $10^4$ /ml in 2 ml cultures on day 0, and viable cells were counted by trypan blue exclusion daily. 10A4 is a noncytotoxic variant and its growth behaviour is representative for all other noncytotoxic variants studied (see Fig. 2). 96MedA, 96MedC, and 96G12 are IL-2-independent variants and their growth behavior is representative for that of the other IL-2-independent variants (see Fig. 5). Generation times varied between ~7.5 h (10A4) and 14 h (CI 96).

selected for loss of cytotoxic function turned out to be IL-2-independent, with respect to growth. Since all additional variants were selected for IL-2 independence we should expect all variants described above to grow in the absence of exogenously added IL-2. Fig. 4 shows that growth curves of representative variants are essentially identical in the absence and presence of 100 units of recombinant human IL-2 (rIL-2). All other variants described in this paper have growth properties similar to those shown in Fig. 4. Thus, IL-2 does not seem to have any influence on the growth of these variant cells, whereas CI 96 cells are essentially dependent on IL-2. No difference was observed between cells grown in rIL-2 and in ConASN (data not shown). None of the IL-2-independent cell

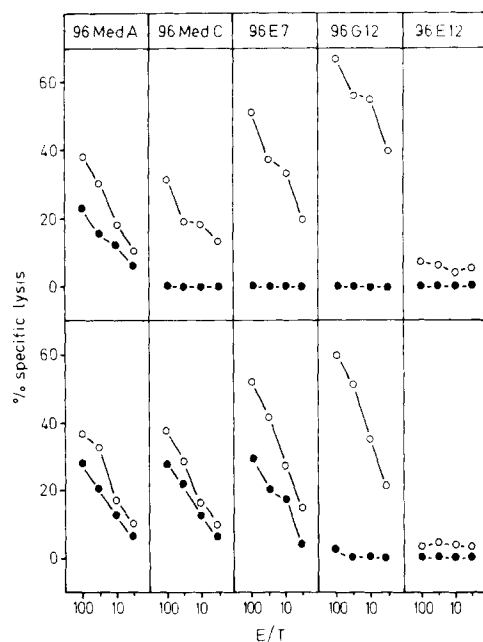


FIGURE 5. Cytotoxic activities of IL-2-independent variants that have been grown in the absence (*top*) and for 2 d in the presence (*bottom*) of 100 U of recombinant human IL-2.  $^{51}\text{Cr}$  release assay was performed in the absence (●) and presence (○) of 2% PHA on P815 target cells. E/T ratios are threefold dilution steps.

lines constitutively secretes detectable concentrations of IL-2 or IFN- $\gamma$  into its supernatant, as determined in standard assays (data not shown).

*Cytotoxic Function of IL-2-independent Variants of Cl 96.* Functionally, Cl 96 variants selected for IL-2-independent growth fall into four types. Type I is represented by 96 MedA, and the results of its analysis (Fig. 5) show that all functional properties of Cl 96 are maintained, i.e., cytotoxic activity for P815 in the absence and presence of PHA. The reduced cytotoxic activity of 96MedA as compared with Cl 96 (compare Fig. 2) is the result of a diminished activity of each individual cell and not of contamination with noncytotoxic cells, as shown by limiting dilution analysis (data not shown). Culture of 96 MedA cells for 2 d in the presence of rIL-2 or ConASN induces only a small enhancement of their cytotoxicity.

Type II is represented by two cell lines, 96MedC and 96E7. These cells, when cultured without IL-2, retain PHA-facilitated cytotoxicity for P815 but are inactive in a specific assay (Fig. 5). When cultured for 2 d or more in the presence of rIL-2, or ConASN, specific cytotoxicity for P815 returns. This IL-2-inducible cytotoxic activity is clearly weaker than in Cl 96 and was unstable in all type II variants obtained to date. After culture periods of several weeks to several months, type II variants gradually lose inducibility by IL-2 and turn into type III variants, which are represented by 96G12 in Fig. 5. These cells are capable of lectin-facilitated, but not of specific P815 kill, independent of whether they are cultured in the presence or absence of IL-2. Type III represents a stable phenotype over many months of culture.

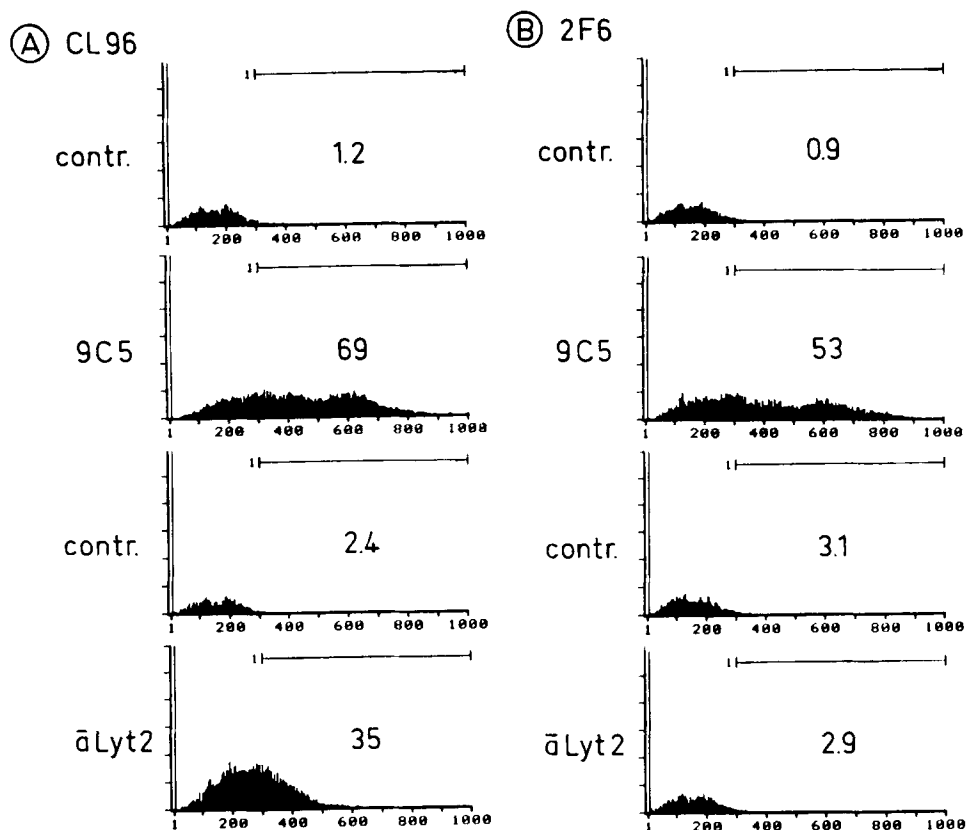


FIGURE 6. Cytofluorograms of Cl 96 cells (A) and of non-cytotoxic variant 2F6 (B), both cultured in presence of ConASN, using monoclonal rat anti-Lyt-2 (56-6.7) and monoclonal mouse anti-H-2 class I (9C5) antibodies. Panels marked control are FITC labeled goat anti-mouse Ig(Fab<sub>2</sub>) and FITC labeled goat anti-rat Ig (Fab<sub>2</sub>) used as second antibodies. Numbers in each panel give the percent of cells that appear within region I. The fluorescence intensity is plotted in logarithmic scale. All other noncytotoxic variants (see Fig. 2) have the same Lyt-2<sup>-</sup> phenotype as 2F6.

Variant 96E12 had negligible cytotoxic activity in both assays (Fig. 5). These cells are therefore referred to as type IV, together with the cells originally selected as noncytotoxic variants.

*Lyt-2 Expression of Cl 96 Variants.* Routine typing of cell lines by flow cytofluorometry showed that Cl 96, as well as all variants, express Thy-1.2 and are negative for L3T4 (data not shown). Typing for Lyt-2, however, revealed significant differences between different cell lines. Fig. 6 and 7 show cytofluorograms of the staining of various cell lines with monoclonal rat anti-Lyt-2

FIGURE 7. Cytofluorograms of IL-2-independent variants cultured without IL-2 (-IL-2) and cultured for 2 d in the presence of 100 U of recombinant human IL-2 (+IL-2). Antibodies are the same as in Fig. 6. 96MedC (A) and 96E7 (B) are type II variants whose specific cytotoxic activity and Lyt-2 expression is induced by culture in IL-2. 96MedA (C) has specific cytotoxic activity, and 96G12 (D) lacks specific cytotoxic activity both in the presence and absence of IL-2.

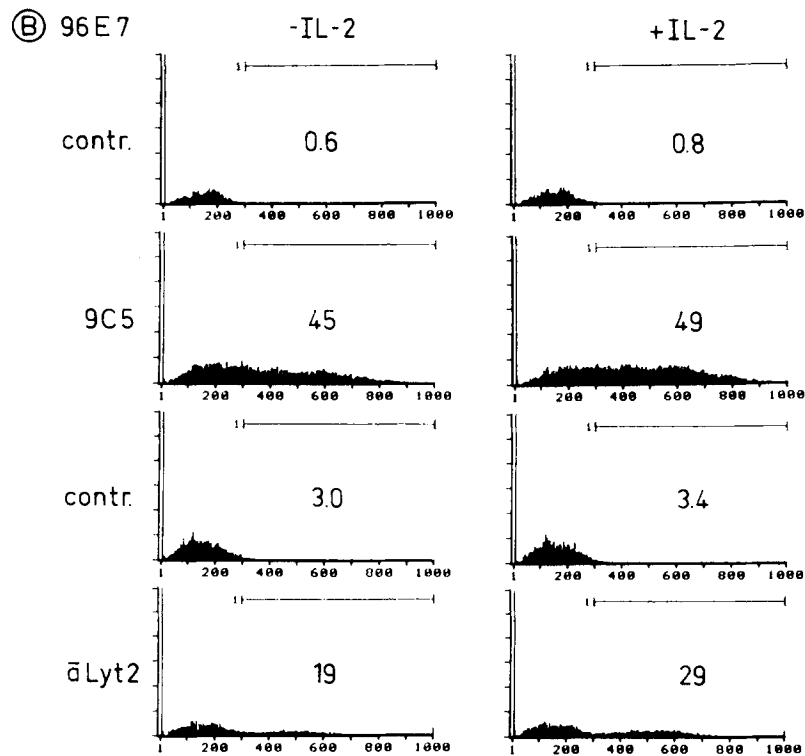
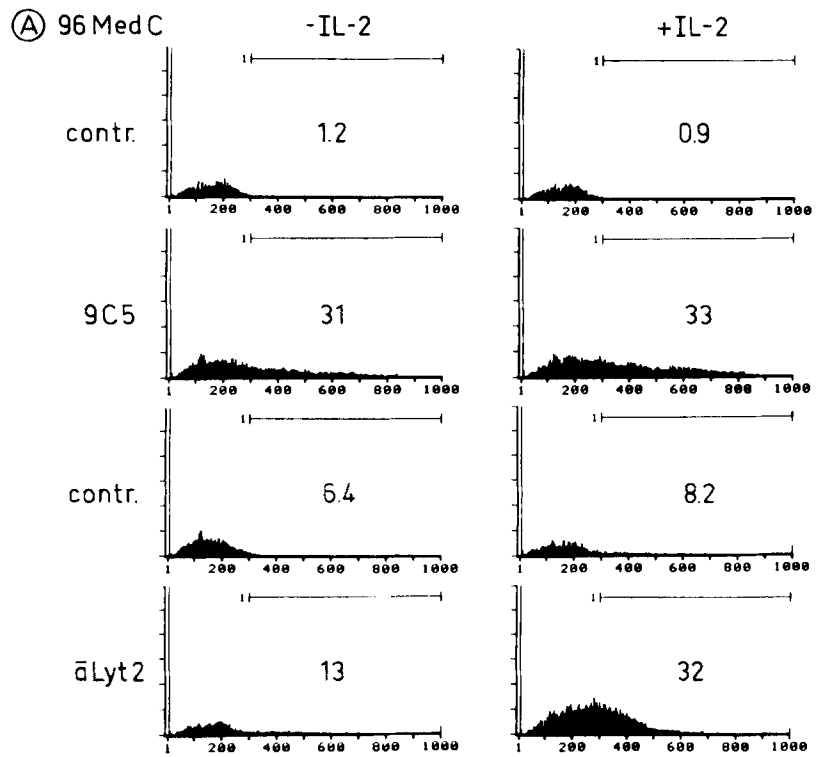


FIGURE 7



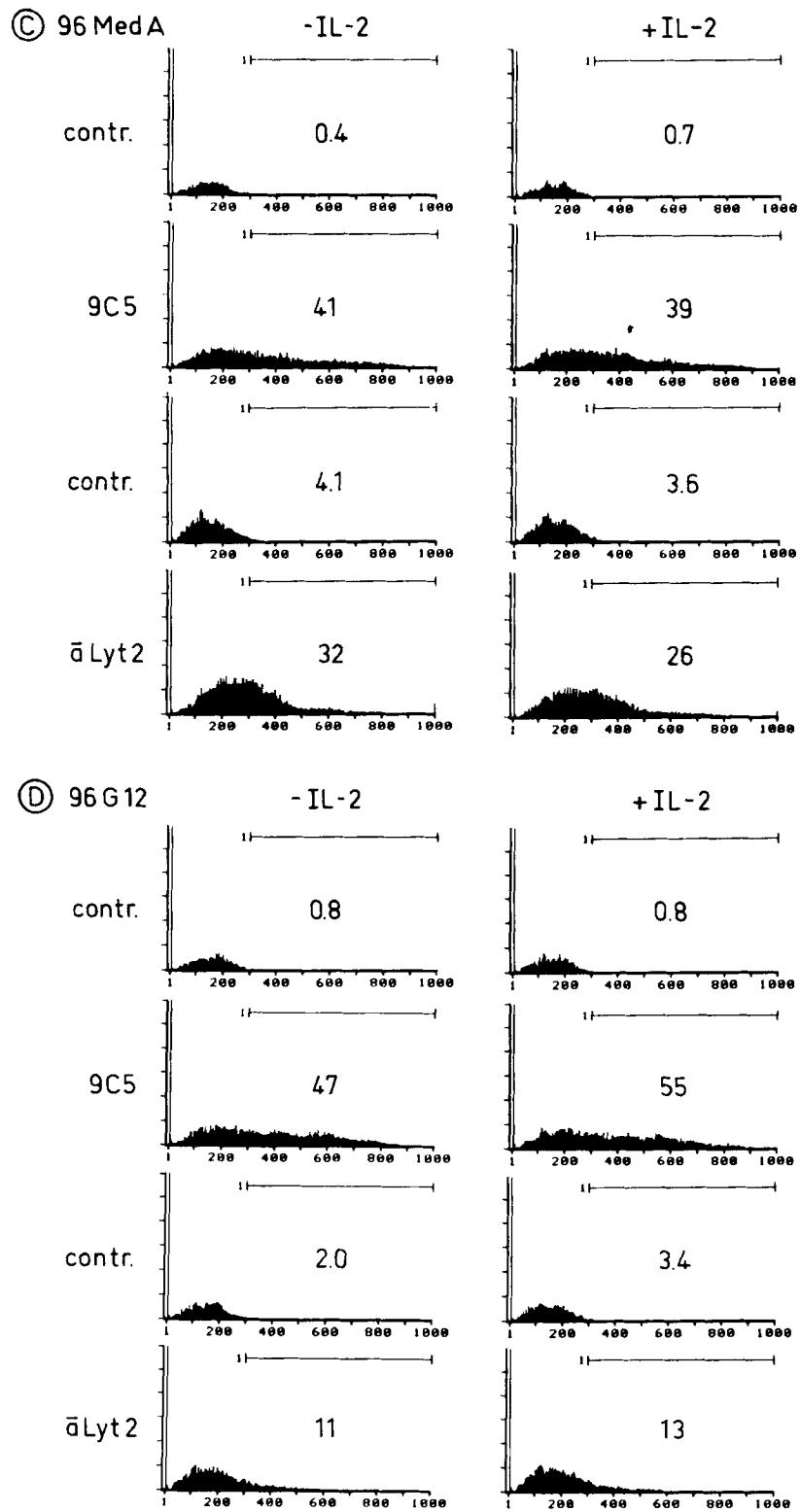


FIGURE 7

antibody (56-6.7) and with monoclonal mouse anti-MHC class I antibody (9C5) for control. All cell lines stain with similar intensity with this control antibody, which appears to detect class I epitopes similar in distribution to public specificity H27 (unpublished observations). In contrast, Lyt-2 expression varies between different cell lines and clearly correlates with cytotoxic activity: Fig. 6 compares Cl 96 (Lyt-2<sup>+</sup>) with the noncytotoxic variant 2F6, which is, like all other noncytotoxic variants, Lyt-2 negative. Fig. 7A and B show the striking behaviour of type II variants that are low or negative for Lyt-2 when cultured without IL-2, and increase their Lyt-2 expression when cultured with IL-2. This induction of Lyt-2 by IL-2 is more clearly seen with 96MedC but also apparent in 96E7. The increase in Lyt-2 expression is selective because MHC class I expression is not significantly enhanced by culture in IL-2. Fig. 7C and D show that Lyt-2 expression of type I and type III variants is not altered by culture in IL-2; 96MedA is high, whereas 96G12 is low, under both culture conditions.

These results strongly suggest that Lyt-2 is involved in the specific cytotoxic activity of Cl 96 cells; lack or low expression of Lyt-2 is accompanied by the inability to kill P815 in a specific assay but does not seem to affect lectin-facilitated cytotoxicity. Reinduction of Lyt-2 by IL-2 is accompanied by return of specific cytotoxicity but leaves lectin-facilitated activity largely unaffected.

*Expression of the T cell Antigen Receptor on Cl 96 Variants.* The mouse T cell antigen receptor is a disulfide-linked heterodimer of two polypeptide chains,  $\alpha$  and  $\beta$ , each of an approximate molecular mass of 40–45 kD (10–13). Due to the fact that very few cell surface proteins of mouse T cells are disulfide-linked heterodimers, the T cell receptor can be identified by running a total lysate of <sup>125</sup>I surface-labeled T cells by 2-dimensional SDS-gel electrophoresis in which the first dimension is run under nonreducing conditions and the second dimension is run under reducing conditions (7). The T cell receptor appears as a spot in a characteristic position (first dimension is ~90 kD, second dimension is ~45 kD) below the diagonal that contains the bulk of the nonreducible cell surface components. Among the few other reducible proteins, Lyt-2 as well as the transferrin receptor can be usually identified (7).

Fig. 8 contains examples of the analyses of Cl 96 cells and some of its functional variants by this procedure, and Table 1 gives a synopsis of all results. All cells that possess lectin-dependent cytotoxic activity (Cl 96, type I, II, and III variants) express the T cell antigen receptor, regardless of their specific P815 killing activity. On the contrary, all but one of the noncytotoxic (type IV) variants are negative for the T cell receptor. In some cases of negative variants a faint spot in this region appears (Fig. 8e), which may or may not represent low amounts of T cell receptor.

The T cell antigen receptor of Cl 96 appears as a short diagonal itself, suggesting a certain degree of size heterogeneity which we attribute to different degrees of glycosylation (Fig. 8a and 9a). For its direct identification, immunoprecipitation was performed using rabbit anti-mouse T cell antigen receptor serum. Compared with the negative control, in which normal nonimmune rabbit serum was used, the anti-mouse T cell receptor serum precipitated a single spot below the diagonal with an apparent molecular mass of 45 kD under reducing conditions and 84 kD under nonreducing conditions (Fig. 9, b and c). According

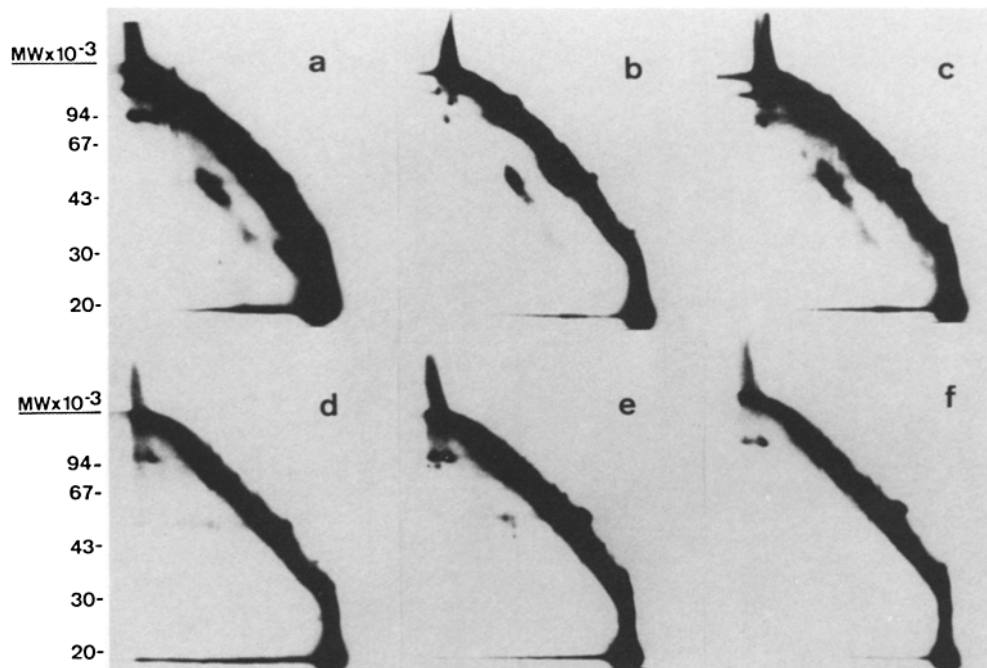


FIGURE 8. Two-dimensional nonreduced/reduced SDS-PAGE of  $^{125}\text{I}$ -labeled membrane proteins of (a) Cl 96; (b) 96MedA, without ConASN; (c) 96 MedC cultured with ConASN; (d) 2F6; (e) 4H7; (f) 138, all cultured with ConASN. The spot corresponding to T cell antigen receptor runs at  $\sim 45$  kD in the second dimension. The spot corresponding to Lyt-2 runs at  $\sim 34$  kD in the second dimension.

to these estimates, the spot precipitated by the anti-T cell receptor antiserum corresponds to the spot at the same position seen in the total lysate (Fig. 9, a). The same type of autoradiograms were obtained in immunoprecipitation experiments with several other cytotoxic variants, and, as expected, rabbit anti-mouse T cell receptor serum did not precipitate any reducible molecules from noncytotoxic variants (data not shown).

Culture of IL-2-independent cells in IL-2 has no detectable influence on T cell receptor expression. This was shown for both type II variants and is shown for 96E7 in Fig. 10. The results of this analysis also confirm serological typing for Lyt-2; the spot corresponding to Lyt-2 increases in intensity when the cells are cultured in IL-2. Thus, the IL-2-inducible, specific P815 cytotoxicity is accompanied by an increase in Lyt-2 expression, whereas the expression of T cell receptor remains, within the sensitivity of the method, unaltered.

Table I summarizes the results of all assays performed. The results strongly suggest that Lyt-2 and the T cell receptor together are necessary for specific recognition of P815 targets by AK cells. Loss of Lyt-2 leads to loss of specific recognition. Loss of the T cell receptor leads to loss of all cytotoxic activity. The one exception to this rule is the type IV variant, 96E12, which possesses negligible cytotoxicity in spite of its expression of Lyt-2 and of the T cell receptor. We conclude that these molecules are necessary but not sufficient for cytotoxic activity of AK cells.

TABLE I  
*Expression of Lyt-2 and of T Cell Antigen Receptor on Cl 96 and Variants Grown in Presence and Absence of rIL-2*

Type of variants	IL-2 in culture	Lysis of:		Lyt-2	T receptors
		P815	P815/PHA		
Cl 96	+	+	+	+	+
I: 96 MedA	-	+	+	+	+
	+	+	+	+	+
II: 96 MedC	-	-	+	Low	+
	+	+	+	+	+
96E7	-	-	+	Low	+
	+	+	+	+	+
III: 96G12	-	-	+	Low	+
	+	-	+	Low	+
IV: 96E12	-	-	-	+	+
	+	-	-	+	ND
138	-	-	-	ND	ND
	+	-	-	-	-
125	-	-	-	ND	ND
	+	-	-	-	-
4H7	-	-	-	ND	ND
	+	-	-	-	-
10A4	-	-	-	ND	ND
	+	-	-	-	-
2F6	-	-	-	ND	ND
	+	-	-	-	-

### Discussion

Although the T cell antigen receptor has been identified and molecularly characterized to some degree (reviewed in Reference 14) it is far from being understood how this molecule exerts its function: recognition of antigen and of MHC products on the surfaces of other cells (15). In the present work we evaluated whether AK T cells would lend themselves to a study of T cell receptor structure-function relationships, since these cells are particularly well adapted to growth in culture, and their function is stable and easily analyzed. However, since AK T cells express a somatically acquired specificity that differs from the specificity of their clonal precursors (4), it was essential to decide whether the T cell antigen receptor or some other surface structure is responsible for the newly acquired specificity.

Acquisition of lectin-like receptors leading to polyspecific cytotoxicity has been postulated for long term and short term cultured T cells (3, 16). If the T cell antigen receptor were responsible for the new recognition specificity, one would have to invoke mechanisms for somatic alteration of its structure and specificity.

Our results lend strong support to the view that the recognition apparatus of AK cells closely resembles that of bona fide cytotoxic T cells. The expression of T cell antigen-receptor molecules on AK cells is clearly shown. Moreover, we

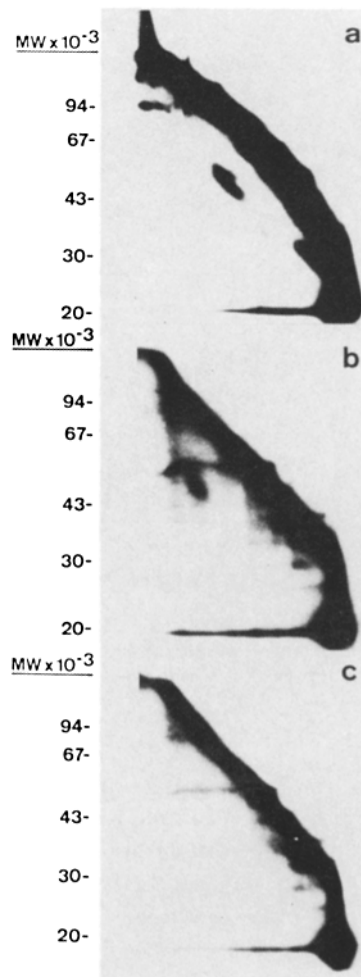


FIGURE 9. The T cell antigen receptor of Cl 96 analyzed by two-dimensional nonreduced/reduced SDS-PAGE of  $^{125}\text{I}$ -labeled membrane proteins (a) whole cell lysate, (b) immunoprecipitate with rabbit anti-mouse T cell receptor serum, and (c) immunoprecipitate with rabbit nonimmune serum. In addition to precipitating the T cell receptor, the rabbit anti-mouse T cell receptor serum, as well as rabbit nonimmune serum, forms a background precipitate which appears in the diagonal. The photographs of the autoradiographs do not bring out the fact that the background precipitates represent only a small proportion (total cpm in *b*, 67,805; total cpm in *c*, 69,220) of the total counts in the lysate (total cpm in *a*,  $10^6$ ). The horizontal configuration across the position of the T cell receptor spot in *b* and *c* is an artifact often seen in our gels.

found that Cl 96 and three out of three cytotoxic variants are receptor<sup>+</sup> and that five out of six noncytotoxic variants are receptor<sup>-</sup>. Formally, it is possible that other molecules, which are obligatorily coexpressed with the T cell receptor, are responsible for P815 recognition by AK cells. The T cell antigen receptor is part of a macromolecular complex that besides diverse  $\alpha$  and  $\beta$  subunits, includes the invariant polypeptides of the T3 antigen. It is unlikely, though, that T3 is responsible for the specificity of AK cells because in this case one should expect

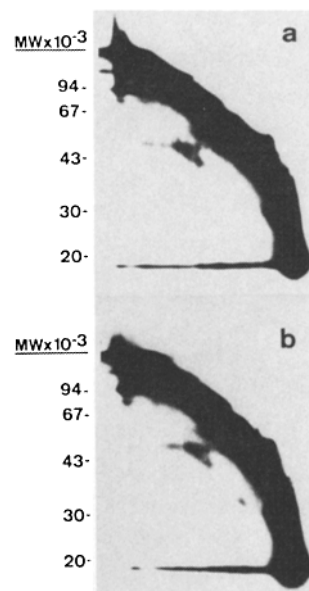


FIGURE 10. Two-dimensional nonreduced/reduced SDS-PAGE of  $^{125}\text{I}$ -labeled membrane proteins of Type II variant 96E7, cultured in the absence (a) and in the presence (b) of 15% ConASN. The spot corresponding to the T cell receptor runs at  $\sim 45$  kD in the second dimension. The spot corresponding to Lyt-2 runs at  $\sim 34$  kD in the second dimension and is seen only in b.

that all  $\text{T3}^+$  cytotoxic cells lyse P815 targets. The loss of the T3 molecule, as well as the loss of any of the  $\alpha$  or  $\beta$  subunits, however, may be the explanation for the absence of the complete T cell antigen receptor complex from the surface of noncytotoxic variants. Alternatively, on the basis of our data it is possible that the T cell receptor is required only for the cytotoxicity of AK cells but not for their target recognitions. In this case, Lyt-2 would be the prime candidate for target recognition, which is again unlikely because all  $\text{Lyt-2}^+$  cytotoxic cells should then lyse P815 target cells. Thus we conclude that the T cell antigen receptor of AK cells is involved in their target cell recognition.

From this it follows that long-term cytotoxic T cell clones may change their receptors from one specificity to another during tissue culture. This is a further example of somatic changes in T cell receptor specificity and complements previous observations from our laboratory on changes in restriction crossreactivity of TNP-specific CTL clones (17), observations by Augustin and Sim (18) on specificity changes in MHC class II-reactive hybridomas, and observations by Reimann and Miller (19) on specificity changes of CTL clones in limiting dilution cultures. Considering the apparent lack of somatic hypermutation in both T cell antigen receptor genes, the molecular mechanism that leads to such specificity changes needs to be determined. It is interesting in this context that a specific  $V\beta$  gene with an unusual degree of homology with a human leukemic  $V\beta$  sequence (20) is expressed in more than one independently arisen murine AK line (21), including Cl 96 (Eppelen, J. T., personal communication). *In vivo* systems need to be established to investigate the intriguing possibility that similar

intraclonal specificity changes occur during T cell development and represent mechanisms for the somatic diversification of the T cell repertoire.

Our data extend previous models of cytotoxicity by showing that the T cell receptor, in addition to its recognition function, is necessary for the activation of the cytotoxic machinery. Cells lacking the T cell receptor are also noncytotoxic in a lectin-facilitated assay. So far, it has been unclear whether or not lectin-dependent cytotoxicity is antigen receptor-dependent (22, 23). For AK cells it seems clear that activation of the cytotoxic machinery occurs via the T cell antigen receptor.

Recently, evidence that the T cell antigen receptor is a lectin-binding molecule has been obtained by several laboratories (24, 25). Inhibition experiments have suggested that the target for PHA-binding on human T cells is not the T3 antigen, but rather the antigen-specific part of the T cell receptor (26). We suggest that the role for the lectin in AK cytotoxicity may be the stabilization of a low-affinity receptor antigen interaction. Since the lectin-facilitated AK cytotoxicity is largely specific for P815, whereas that of bona fide CTL applies to many different target cells, AK cells may differ somewhat from bona fide CTL. It remains to be studied, by experiments similar to those described here, whether the T cell receptor of bona fide CTL is also necessary for lectin-facilitated cytotoxicity.

The role of Lyt-2 in specific target recognition of AK cells seems to be similar to the role of the lectin in lectin facilitated lysis. As suggested by previous experiments (27, 28), Lyt-2 participates in target recognition of CTL by enhancing the affinity of the interaction of the T cell receptor with its antigenic structure. Accordingly, specific cytotoxicity of AK cells for P815 needs both Lyt-2 and the T cell receptor. This observation is in full agreement with the model proposed by McDonald et al. (29) for the function of Lyt-2 in the recognition of low-affinity cytotoxic T lymphocytes.

In addition to the role of the T cell receptor and of Lyt-2 in AK killing, we find the inducibility by IL-2 of specific cytotoxicity in type II variants especially interesting. This inducibility correlates with an increase in the expression of Lyt-2, and leads to the conclusion that Lyt-2 is essential for specific target cell recognition. IL-2 has recently been shown to induce specific cytotoxicity in a cytotoxic T cell hybridoma whose growth was independent of IL-2 (30). Moreover, IL-2 was shown to induce its own receptor on helper T cell lines (31, 32). In none of these reports was evidence obtained that Lyt-2 (or L3T4) was subject to regulation by IL-2. Whether or not the present observation reflects a physiological effect of IL-2 or is unique to the AK system needs to be established.

The fact that specificity variants of AK type are generated frequently in a number of CTL clones of independent specificity suggests to us that the expression of AK activity is intimately connected with an unknown selective advantage in tissue culture, either through recognition of an unknown antigen in culture or through concomitant activation of a growth promoting gene. Therefore, the switch to AK activity may not represent an important event *in vivo*. However, irrespective of how this activity arises, our data show that the lytic activity of AK cells uses a classical recognition mechanism, and we therefore suggest that these cells can be readily used as a model for studying the lytic process of bona fide

CTL. Moreover, AK cells are interesting because they can give rise to IL-2-independent T cell lines with complete or partial maintenance of cytotoxic function. Such IL-2-independent variants may represent preleukemic cells. Fully tumorigenic variants may arise from AK cells that lose cytotoxic function (Simon et al., manuscript submitted for publication) or remain active as cytotoxic cells (unpublished observation). Taken together, we feel that AK cells may be useful tools, not only for the study of recognition and cytotoxic effector function of T lymphocytes, but also for general questions in cell biology such as induction of function and growth control.

### Summary

Aged killer (AK) T cells are antigen-independent, IL-2-requiring variants of antigen-dependent CTL clones that have lost their original antigen specificity and have acquired, instead, specific cytotoxicity for P815 target cells. In this report we study whether AK cells use a similar or a different target cell recognition system than that of bona fide CTL. To this end, we selected from a cloned AK line variants that are partially or completely deficient in specific target recognition and/or in cytotoxic function, and analyzed these variants for expression of the T cell antigen receptor and of Lyt-2. Variants were selected from the prototype AK line (Cl 96) with specific, as well as lectin-facilitated, cytotoxicity for P815 tumor cells. Variants could be grouped into four types with increasing degrees of functional deficiency, which correlated with loss of T cell receptor and/or loss of Lyt-2. In short, loss of Lyt-2 was reflected in loss of specific target recognition, and loss of the T cell antigen receptor was reflected in loss of all cytotoxic activity. We conclude from these results that (a) both Lyt-2 and the T cell antigen receptor are required for specific target cell recognition and (b) the T cell antigen receptor is, in addition, required for cytotoxic function. Moreover, since AK cells express a somatically acquired specificity that differs from that of their clonal precursors, it appears that cytotoxic T cells may change their antigen receptor from one specificity to another during tissue culture.

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