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Cytotoxic Activity of CD4$^+$ T Cells against Autologous Tumor Cells

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The ^{51}Cr-release assay is mostly applied to detecting the cytotoxic activity of CD8$^+$ T cells, and little is known about the activity of CD4$^+$ T cells. Therefore, the correlation between the cytotoxic activity of CD4$^+$ or CD8$^+$ T cells and the incubation period with autologous tumor cells was analyzed by two methods. The incubation periods were 4 and 20 h (4 h and 20 h assay) for the ^{51}Cr-release assay. Eight pairs of tumor cells and T cells were assayed. T cells were fractionated into CD4$^+$ and CD8$^+$ T cells by using magnetic beads and panning methods, and those cells were activated by culture with recombinant interleukin-2 and immobilized anti-CD3 monoclonal antibody. In 6 out of 8 cases, no cytotoxic activity of CD4$^+$ T cells was detected by the 4 h assay, whereas cytotoxic activity was detected in all cases in the 20 h assay. The cytotoxic activities in 20 h assay of CD4$^+$ T cells were increased 67-fold in comparison with the activities in 4 h assay (range: 5–197). In the case of CD8$^+$ T cells, cytotoxic activities were detected in 6 out of 8 cases in the 4 h assay. The lytic unit ratio of CD4$^+$ and CD8$^+$ T cells was calculated as 1.5 in the 20 h assay (range: 0.2–>7.2) versus 0.4 in the 4 h assay (range: <0.1–1.3). Cytotoxic activities in colorimetric assay using Crystal Violet with a 24 h incubation were similar to those in the 20 h ^{51}Cr-release assay in all eight cases. These results indicate that CD4$^+$ T cells have cytotoxic activity as strong as that of CD8$^+$ T cells towards autologous tumor cells.

Key words: Autologous cytotoxicity — CD4$^+$ T cell — T cell separation — Incubation time — Colorimetric assay

CD8$^+$ T cells,[1–3] lymphokine activated killer (LAK) cells,[4,5] large granular lymphocytes (LGL)[6,7] and macrophages[8] have come to be recognized as major effector cells in tumor immunity. The ^{51}Cr-release assay with a short incubation time such as 4–6 h has often been used to detect cytotoxic activity,[1,6,9,10] because cytotoxic activities of the above cells, except for macrophages, can be detected within a short time. It has also been reported that a longer incubation time is needed to detect cytotoxic activity,[11–14] but little analysis has been done on the correlation between the incubation period and cytotoxic activity, since the high spontaneous release of the ^{51}Cr-release assay leaves only a small margin between total incorporation and the unreleased materials in a long-term assay.[12] Improvement of the ^{51}Cr-labeling method of the target cells, however, allows the spontaneous release to be held at a low level even in a long-term assay.[11]

Recent studies have dealt with the adoptive transfer of tumor-infiltrating lymphocytes (TIL),[3,14–16] or cytotoxic T lymphocytes.[17–21] In some cases, predominantly CD4$^+$ T cells were effective for tumor regression.[16,18,19] In particular, Rosenberg *et al.* obtained some partial responses based on objective evidence of cancer regression after infusion of TIL which contained mostly CD4$^+$ T cells.[16] Itoh *et al.* reported cytotoxic activities of cloned CD4$^+$ T cells against autologous tumor cells.[18] LeMay *et al.* found that detection of the cytotoxic activity of cloned CD4$^+$ T cells required long-term incubation in the ^{51}Cr-release assay.[13]

In this study, bulk cultures of T cells were fractionated into CD4$^+$ and CD8$^+$ T cells by using two magnetic beads methods[22–24] and a panning method[25,26] from peripheral blood lymphocytes (PBL) or TIL, in order to analyze whether CD4$^+$ T cells have greater cytotoxic activity towards autologous tumor cells than do CD8$^+$ T cells in PBL and TIL, by using both shorter- and longer-term ^{51}Cr-release assays. Furthermore, a colorimetric assay using Crystal Violet (colorimetric assay) was compared with the long-term ^{51}Cr-release assay.

MATERIALS AND METHODS

Tumor cells Eight samples consisting of renal cell carcinoma (case No. 1), three melanomas (cases No. 2–4), two hepatocellular carcinomas (cases No. 5 and 6), breast cancer (case No. 7), and hemangiosarcoma (case No. 8) were cultured by the following method and subjected to cytotoxicity assay (Table I). Tumor tissues or metastatic lymphnodes were minced and treated with 40 ml of

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Table I. Patients and Original Sources of Lymphocytes

Case No.	Diagnosis	Age/Sex	Lymphocytes		
			Origin	Vol.	Cell No.[a]
1	Renal cell ca.	63/M	TIL[b]	2.5 g	4.4×10^6
2	Melanoma-1	46/M	TIL[c]	6.3 g	7.5×10^6
3	Melanoma-2	51/F	PBL	20 ml	4.2×10^7
4	Melanoma-3	28/F	TIL[d]	0.8 g	1.6×10^7
5	Hepatocellular ca.-1	56/F	PBL	20 ml	3.3×10^7
6	Hepatocellular ca.-2	56/M	TIL[e]	2.5 g	2.2×10^7
7	Breast ca.	47/F	PBL	20 ml	2.0×10^7
8	Hemangiosarcoma	89/F	PBL	20 ml	1.1×10^7

a) Total number of pre-culture mononuclear cells.
b) TIL from metastatic left axillar lymphnode.
c) TIL from original tumor (skin).
d) TIL from metastatic right axillar lymphnode.
e) TIL from original tumor (liver).

enzyme mixture (40 ml of saline containing 4 mg of deoxyribonuclease, 40 mg of collagenase, and 100 units of hyaluronidase (Sigma Chemical Co., Mo., USA)) for 40 min at room temperature. The mixture was filtered through 100 and then 200 mesh stainless steel grids. The clusters of tumor cells on the 200 mesh grid were seeded in 24-well culture plates (Primaria, Falcon, Becton Dickinson, Calif., USA) as a tumor cell culture with a culture medium (RPMI-1640; Gibco Laboratories, N.Y., USA) containing 10% heat-inactivated fetal bovine serum (FBS, Cell Culture Laboratories, Ohio, USA) (10% FBS) and recombinant epithelial growth factor (10 ng/ml rEGF; Sigma Chemical Co.). The culture medium was supplemented with sodium pyruvate (1 m*M*), oxalacetic acid (1 m*M*), L-glutamate (2 m*M*), insulin (0.2 U/ml), kanamycin sulfate (60 μg/ml), and streptomycin sulfate (20 μg/ml). Fibroblasts were not observed in any cultures.[27] K562 and Daudi cell lines were used as allogeneic target cells.

T lymphocytes TILs (cases No. 1, 2, 4 and 6) were separated from the pass-through fraction on the 200 mesh grid mentioned above by density sedimentation with Lymphosepar I (density 1.077; Immuno-Biological Laboratories, Gunma). TILs were suspended in 10% FBS containing 10% heat-inactivated human serum (type compatible)(10% serum) and cultured in an anti-CD3 monoclonal antibody (anti-CD3MoAb; Janssen-Kyowa Co., Ltd., Tokyo)-coated 96-well microculture plate (flat-bottomed plate; Nunc Intermed, Roskilde, Denmark). About 1 week later, the TILs were transferred to a culture flask (Costar 225 cm^2; Costar, Mass., USA). PBLs (cases No. 3, 5, 7 and 8) were separated by the use of Lymphosepar I from heparinized peripheral blood. TILs and PBLs were stimulated and propagated *in vitro* with recombinant interleukin-2 (700 U/ml rIL-2; Shionogi Pharmaceutical Co., Ltd., Tokyo) and anti-CD3MoAb in a culture flask (SUMILON 225 cm^2; Sumitomo Bakelite Co., Ltd., Tokyo) after being fractionated into CD4$^+$ and CD8$^+$ T cells by using two kinds of magnetic cell separation beads methods (MACS; Becton Dickinson and Dynabeads; Dynal A.S., Norway) and the panning method (AIS microcellector; Applied Immune Science, Inc., Calif., USA). The T cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 5 to 7 days.[27] A surface phenotypic analysis of the cultured and expanded lymphocytes was performed on a FACScan (Becton Dickinson FACS system) after staining with CD4/CD8, CD3/HLA-DR, CD16/CD56, TCR-α/β (Becton Dickinson Immunocytometry Systems) and TCR-γ/δ (Coulter Immunology, Fla., USA).

Cytotoxicity assay ^{51}Cr-Release assays with incubation for 4 and 20 h (4 h and 20 h assays) were performed to detect the cytotoxic activities of T cells towards autologous tumor cells, K562 and Daudi cells.[11, 12] Target cells (1.0–1.5$\times10^6$ cells) in pellet form after centrifugation were radiolabeled with 7–10 MBq of sodium [^{51}Cr]chromate (ICN Biochemicals, Inc., Calif., USA) in 10 μl of phosphate-buffered saline (PBS) and then incubated for 20 min at 37°C. The radiolabeled cells were washed with 3% FBS and suspended in 40 ml of 10% FBS for 2 h at 37°C. After having been washed in 3% FBS, target cells were suspended in 10% FBS at 5×10^4 cells/ml. Viable target cells were counted using the trypan blue exclusion test. The target cells were seeded in 100 μl aliquots (5×10^3 cells/well) for each microculture plate (96-well round-bottomed plate; Sumitomo Bakelite Co., Ltd.). The effector cells were added to each well at the effector:target (E:T) ratio of 30:1, 10:1, 3:1 or 1:1. These plates were incubated for 4 and 20 h. After incubation, plates were centrifuged and the supernatant fluid of each well was collected for determination of ^{51}Cr released. Spontaneous release of the target cells was

detected by incubation with the culture medium alone, and maximal lysis was detected by incubation with 1% NP-40 detergent. Spontaneous release in the 4 h assay was a mean of 10% (±3.9% SD) and that in the 20 h assay was a mean of 20% (±3.6% SD).

The percentage lysis (% lysis) on ^{51}Cr-release assay was calculated as follows: % lysis = 100 × {soluble ^{51}Cr (cpm) in the experimental sample − spontaneous cpm}/{maximal ^{51}Cr (cpm) − spontaneous cpm}.

Relative cytotoxic activity was presented in lytic units (LU).[27] One LU was defined as the number of effector cells required to cause 30% of 5×10^3 target cells to lyse during the incubation period. LU was calculated as follows: LU = 10^6 effector cells/the number of effector cells required to produce 1 LU.

A colorimetric assay using Crystal Violet solution (colorimetric assay) was employed to detect cytotoxic activities during a longer incubation period. In brief, target cells in 10% FBS were seeded in wells of 96-well microculture plates (flat-bottomed plate; Sumitomo Bakelite Co., Ltd.) at 5×10^3 cells per well. After a one-day culture, the effector cells were added to the wells at an E:T ratio of 30:1, 10:1, 3:1 or 1:1. After incubation for 1, 2 or 3 days, the plates were washed once gently with PBS, then 50 μl/well of 0.1% Crystal Violet solution (1% in 0.1 *M* $NaHPO_4$) (Aldrich Chemical Co., Inc., Wis., USA) was added and the cells were allowed to settle for 30 min at room temperature. They were then washed carefully with PBS and the color was extracted with 100 μl/well of elution buffer (0.33 *M* disodium citrate and 0.02 *N* HCl in 50% ethanol). The absorbance of the extracts was measured at 577 nm by an automatic microprocessor-controlled analyzer (ELISA analyzer; Toyo Sokki Co., Ltd., Tokyo). Then the plate was washed and residual cells on the plate were stained with Giemsa's solution (Merck, Darmstadt, Germany). Cytotoxic activities in colorimetric assay were calculated as follows: cytotoxic activity (%) = 100 × {absorbance of control well without effector cells − absorbance of experimental well/absorbance of control well}.

Calculated results were confirmed by the microscopic observation of stained plates.

RESULTS

Fractionation of T cells The fractionated T cells were more than 90% pure in terms of phenotype, as shown in Table II, and they showed no phenotypic change following culture. In $CD4^+$ T fractionation, phenotypes of CD3/HLA-DR and TCR-α/β were present, but CD16/CD56 and TCR-γ/δ were not detected.

Detection of cytotoxic activity towards autologous tumor cells by ^{51}Cr-release assay Spontaneous release was reduced by a shorter incubation time with the ^{51}Cr compound and a longer incubation time with 10% FBS after radiolabeling. The viability of the ^{51}Cr-labeled tumor cells was more than 95% by the trypan blue exclusion test. As shown in Table III, the cytotoxic activities of the $CD4^+$ and $CD8^+$ T cells in the 20 h assay were greater than those in the 4 h assay in all eight cases. In cases No. 2, 6 and 8, the activities of $CD4^+$ T cells exceeded those of $CD8^+$ T cells in the 20 h assay. In cases No. 2 and 4, $CD4^+$ T cells showed higher activity even in the 4 h assay. Enhancement of lytic activity with longer incubation time was indicated by the LU of the 20 h assay/LU of the 4 h assay (4 h/20 h ratio), as shown in Table III. The 4 h/20 h ratios of $CD4^+$ T cells were higher than those of the $CD8^+$ T cells in all eight cases (Table III).

Table II. Phenotypes of Cultured PBL and TIL

Fractionated T cells	%[a]
$CD4^+$ T: $CD4^+/CD8^-$	93±3.6
$CD8^+$ T: $CD4^-/CD8^+$	97±4.5
$CD4^+$ T fraction	
$CD3^+/HLA\text{-}DR^+$	92±7.0
$CD16^-/CD56^-$	97±5.0
TCR-$\alpha\beta^+$	84±7.8
TCR-$\gamma\delta^-$	96±1.1

a) Values shown as mean±SD.

Table III. Effect of Incubation Period on ^{51}Cr-release Assay

Case No.	CD4, CD8	LU[a]		LU ratio[b]
		4 h[c]	20 h[c]	
1	CD4	<0.1	9.3	>130
	CD8	4.4	22	5
2	CD4	15	>200	>13
	CD8	12	28	2
3	CD4	2.8	15	6
	CD8	8.7	27	3
4	CD4	14	154	11
	CD8	31	ND[d]	ND[d]
5	CD4	2.9	13	5
	CD8	9.3	21	2
6	CD4	0.7	24	36
	CD8	4.2	12	3
7	CD4	<0.1	14	>200
	CD8	1.5	83	56
8	CD4	2.0	20	10
	CD8	2.4	17	7

a) LU is defined as the number of effector cells ($\times10^6$) required to lyse 30% of 5×10^3 target cells during the incubation period.
b) LU ratio is defined as the ratio of the values in 4 h and 20 h assay.
c) Incubation period.
d) Not done.

This was not due to the contamination of $CD8^+$ T cells. LU in cases No. 1 and 7 could be detected in the 20 h assay but not the 4 h assay (less than 0.1). In contrast, the 4 h/20 h ratio in cases No. 3, 5 and 8 increased a little.

The 4 h/20 h ratios of $CD8^+$ T cells were less than 7 (range: 2–7), but in case No. 7 it was 56. The ratio of cytotoxic activities of $CD4^+$ T and $CD8^+$ T cells ($CD4^+/CD8^+$ ratio) is shown in Table IV. $CD4^+$ T cells showed low activities in the 4 h assay. After 20 h, $CD4^+$ T cells showed similar activities to $CD8^+$ T cells. The $CD4^+/CD8^+$ ratio in cases No. 1, 3, 5–7 was less than 0.3, but changed to 0.2–0.6 or more in the 20 h assay. The LU of the $CD4^+$ T cells in case No. 2 already exceeded that of the $CD8^+$ T cells in the 4 h assay. In the 20 h assay, $CD4^+$ T cells of cases No. 2, 6 and 8 showed superior activity to the $CD8^+$ T cells. The $CD4^+/CD8^+$ ratio in case No. 2 was more than 7.

Comparison of cytotoxic activity of activated and non-activated T cells towards autologous tumor cells by the ^{51}Cr-release assay Effector and target cells were obtained from case No. 7. As shown in Table V, T cells activated with IL-2 and anti-CD3MoAb lysed the autologous target cells even in the 4 h assay, but non-activated peripheral blood mononuclear cells did not lyse the target even in the 20 h assay.

Detection of cytotoxic activity towards autologous tumor cells by the colorimetric assay Cytotoxic activities detected by the colorimetric assay on day 1 were similar to those in the ^{51}Cr-release 20 h assay, as shown in Table V. Microscopic observations revealed that the target cells in control wells grew and adhered on the bottom of the plate. The target cells reduced their cytoplasmic volume and then separated from the bottom of the plates. Some lymphocytes adhered to the target cells strongly. The adhering cells could not be separated from each other even by washing with PBS several times. Marked cytotoxic activity was not detected in cases No. 7 and 8, since many lymphocytes adhered to the target cells. The cytotoxic activities in the other cases increased as time passed (Table VI).

Cytotoxic activity towards allogenic tumor cells The T cells of case No. 7 were examined in the ^{51}Cr-release assay. As shown in Fig. 1, the $CD8^+$ T cells were active, especially in the 20 h assay, but $CD4^+$ T cells did not lyse the target cells even in the 20 h assay.

DISCUSSION

It has recently been reported that $CD4^+$ T cells have effector activity in allograft rejection.[28–30] Unlike $CD8^+$ T cells, little is known about the killing mechanism or the

Table IV. Ratio of Lytic Unit (LU) Value between $CD4^+$ T vs. $CD8^+$ T Cells in ^{51}Cr-release Assay

Case No.	4 h[a]	20 h[a]
1	<0.1[b]	0.4[b]
2	1.3	>7
3	0.3	0.6
5	0.3	0.6
6	0.2	2.0
7	<0.1	0.2
8	0.8	1.2

a) Incubation period.
b) Ratio.

Table V. Comparison of Cytotoxic Activity between ^{51}Cr-release Assay and Colorimetric Assay

Case No.	T cells	^{51}Cr-release assay (20 h)	Colorimetric assay (day 1)
1[b]	$CD4^+$ T	37[a]	25[a]
	$CD8^+$ T	82	89
5[c]	$CD4^+$ T	45	53
	$CD8^+$ T	59	82
7[b]	$CD4^+$ T	38	0[e]
	$CD8^+$ T	69	63
7[b]	Activated T[d]	12	46
	No activated T[d]	0[f]	0[f]

a) Percentage lysis of target cells after 20 h assay in ^{51}Cr-release assay and day 1 in colorimetric assay.
b) Patients 1 and 7: E:T ratio of 30:1.
c) Patient 5: E:T ratio of 10:1.
d) T cells were not fractionated.
e) Cytotoxic activity was not detected because of tight adhesion of many lymphocytes to the tumor cells.
f) Cytotoxic activity was not detected.

Table VI. Colorimetric Assay against Autologous Tumor Cells (E:T ratio=30:1)

Case No.	CD4, CD8	Day 1	Day 2	Day 3
1	CD4	0[a]	54	86
	CD8	89	90	91
2	CD4	73	85	84
	CD8	86	96	93
3	CD4	69	86	95
	CD8	29	75	76
5	CD4	53	63	ND[b]
	CD8	82	89	ND
7	CD4	0[a]	0[a]	ND
	CD8	63	90	ND
8	CD4	0[a]	0[a]	ND
	CD8	0[a]	0[a]	ND

a) Cytotoxic activity was not detected because of tight adhesion of many lymphocytes to the tumor cells.
b) ND, not done.

role of CD4$^+$ T cells in antitumor immunity. Accordingly, T cells should be separated into CD4$^+$ and CD8$^+$ T cells to examine the cytotoxic activity towards autologous tumor cells[31, 32] and the correlation between activity and incubation period.

CD4$^+$ and CD8$^+$ T cells were easily fractionated from heparinized blood by one positive selection, but it was difficult to separate them from cultured T cells. Two magnetic beads methods were mainly used for the fractionation of T cells on account of their convenience. The purity of CD4$^+$ or CD8$^+$ T cells from peripheral blood was not changed following a long-term culture (data not shown). It is not clear whether or not the beads affect the cytotoxic activities of T cells.

In all eight cases, the cytotoxic activities of CD4$^+$ T cells to autologous tumor cells were much greater in the 20 h assay than in the 4 h assay, whereas the cytotoxic activities of the CD8$^+$ T cells increased little in the 20 h assay (Table III). Thus, the activities of the CD8$^+$ T cells were detectable in the short-term assay. CD4$^+$ T cells did not lyse K562 and Daudi cells even in the 20 h assay, as compared with the CD8$^+$ T cells, which killed the target cells even in the 4 h assay (Fig. 1). These results suggest that CD4$^+$ T cells have cytotoxic activity towards autologous tumor cells, like CD8$^+$ T cells, but it takes a longer time to detect that activity. Furthermore, the activity was seen after activation with IL-2 and CD3MoAb (Table V). The activity of the CD4$^+$ T cells as detected by the 20 h assay exceeded that of the CD8$^+$ T cells in 3 out of 8 cases (cases No. 2, 6 and 8). No cytotoxic activity was seen with the non-activated T cells in preliminary experiments, but non-cytotoxic non-activated cells such as PBL acquired cytotoxicity after activation. This is important in analyzing the killing mechanism of T cells, since the cytotoxic activities were not inhibited with anti-CD3MoAb or with class I and II MoAb,[3, 33] even in the 4 h and 20 h assays (data not shown).

The ^{51}Cr-release assay has been used to detect cytotoxic activity in a short period. Bulk cultures of T cells have often been used as effector cells,[1, 10, 14, 15, 19] but the activities depend on CD8$^+$ T cells and NK cells, whose activities can be detected by a short-term assay. In our experiments, the 4 h/20 h ratio with CD8$^+$ T cells was less than 7-fold except for case No. 7. These results suggest that the cytotoxic activity of CD8$^+$ T cells can be detected within a 4 h incubation period. However, it is unclear whether or not there is a correlation between the activity and the incubation period, though some reports have suggested that cytotoxic activity could be detected through an assay in which the incubation period was over 16 h.[11–14] High spontaneous release in the ^{51}Cr-release assay is restricted to the long-term assay. However, improvement of the ^{51}Cr labeling method allows a lower spontaneous release to be obtained, and a 20 h incubation is feasible in this assay.

The colorimetric assay is easy and reproducible, because no radioisotopes are required and the values correlate well with the results of the ^{51}Cr-release assay (Table V). This assay has the following characteristics: (1) it is suitable for the detection of cytotoxic activities when a longer incubation period is needed; (2) it can be run with an adherent target cell culture; (3) it is superior in reproducibility without technical difficulty; (4) the plate can be stained with Giemsa's solution and the cells can be counted with a microscope after the assay. The colorimetric assay also has some disadvantages, in that: (1) it is difficult to maintain the fixed E:T ratio during the

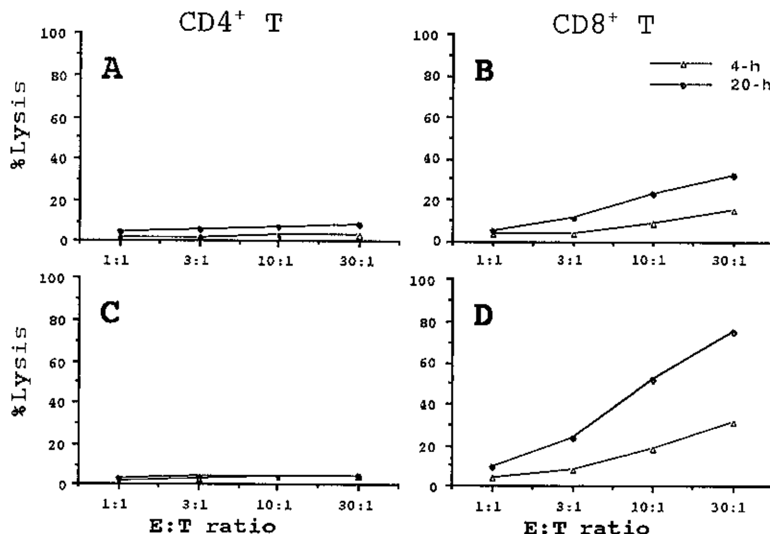

Fig. 1. Cytotoxic activity against allogeneic tumor cells. Target cells in A and B were K562 cells, and those in C and D were Daudi cells. Effector cells were obtained from case No. 7. Activities of CD4$^+$ T cells are shown in A and C, and those of CD8$^+$ T cells are shown in B and D as measured by 4 h (△) and 20 h (◆) ^{51}Cr-release assay.

incubation period; (2) in some cases, effector cells which adhere tightly to the target cells also incorporate Crystal Violet, and it reduces the cytotoxic activity. In this assay, no cytotoxic activity was detected in cases No. 7 and 8, even when the cells were incubated for 2 days (Table VI). Examination with a microscope showed that the tumor cells were dead and T cells had proliferated. These results indicate that colorimetric assay is valid for detecting cytotoxic activities with a longer incubation period and the cytostatic activities of T cells.

In summary, the present study indicates that $CD4^+$ T cells cannot kill allogeneic tumor cells, but can kill autologous tumor cells to much the same degree as $CD8^+$ T cells. Therefore, it is surmised that $CD4^+$ T cells may be involved in tumor regression, possibly through a cytostatic effect. Further studies are needed to establish the mechanism.

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REFERENCES

1) Itoh, K., Tilden, A. B. and Balch, C. M. Interleukin 2 activation of cytotoxic T-lymphocytes infiltrating into human metastatic melanomas. *Cancer Res.*, **46**, 3011–3017 (1986).
2) Kan-Mitchell, J., Huang, X. Q., Steinman, L., Oksenberg, J. R., Harel, W., Parker, J. W., Goedegebuure, P. S., Darrow, T. L. and Mitchell, M. S. Clonal analysis of *in vivo* activated $CD8^+$ cytotoxic T lymphocytes from a melanoma patient responsive to active specific immunotherapy. *Cancer Immunol. Immunother.*, **37**, 15–25 (1993).
3) Topalian, S. L., Solomon, D. and Rossenberg, S. A. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. *J. Immunol.*, **142**, 3714–3725 (1989).
4) Mittelman, A., Savona, S., Gafney, E., Penichet, K. O., Lin, B., Levitt, D., Ahmed, T., Arlin, Z. A., Baskind, P., Needleman, D., Levere, R. D. and Podak, E. R. Treatment of patients with advanced cancer using multiple long-term cultured lymphokine-activated killer (LAK) cell infusions and recombinant human interleukin-2. *J. Biol. Response Modif.*, **8**, 468–478 (1989).
5) Rosenberg, S. A., Lotze, M. T., Muul, L. M., Chang, A. E., Avis, F. P., Leitman, S., Lineham, W. M., Robertson, C. N., Lee, R. E., Rubin, J. T., Seipp, C. A., Simpson, C. G. and White, D. E. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N. Engl. J. Med.*, **316**, 889–897 (1987).
6) Timonen, T., Ortaldo, J. R. and Herberman, R. B. Characteristics of human large granular lymphocytes and relationship to natural killer cells. *J. Exp. Med.*, **153**, 569–582 (1981).
7) Uchida, A. and Micksche, M. Lysis of fresh human tumor cells by autologous large granular lymphocytes from peripheral blood and pleural effusions. *Int. J. Cancer*, **32**, 37–44 (1983).
8) Mantovani, A., Jerrells, T. R., Dean, J. H. and Herberman, R. B. Cytolytic and cytostatic activity on tumor cells of circulating human monocytes. *Int. J. Cancer*, **23**, 18–24 (1979).
9) Main, E. K., Lampson, L. A., Hart, M. K., Kornbluth, J. and Wilson, D. B. Human neuroblastoma cell lines are susceptible to lysis by natural killer cells but not by cytotoxic T lymphocytes. *J. Immunol.*, **135**, 242–246 (1985).
10) Topalian, S. L., Solomon, D., Avis, F. P., Chang, A. E., Freerksen, D. L., Linehan, W. M., Lotze, M. T., Robertson, C. N., Seipp, C. A., Simon, P., Simpson, C. G. and Rosenberg, S. A. Immunotherapy of patients with advanced cancer using tumor-infiltrating lymphocytes and recombinant interleukin-2; a pilot study. *J. Clin. Oncol.*, **6**, 839–853 (1988).
11) Ayres, R. C. S., Shaw, J., Mills, C. O., Coleman, R. and Neuberger, J. M. A ^{51}Cr release cytotoxicity assay for use with human intrahepatic biliary epithelial cells. *J. Immunol. Methods*, **141**, 117–122 (1991).
12) Katz, A., Feldman, M. and Eisenbach, L. The use of [^{35}S]methionine as a target cell label in long term cytotoxic assays. *J. Immunol. Methods*, **149**, 255–260 (1992).
13) LeMay, L. G., Kan-Mitchell, J., Goedegebuure, P. S., Harel, W. and Mitchell, M. S. Detection of melanoma-reactive $CD4^+$ HLA-class I-restricted cytotoxic T cell clone with long-term assay and pretreatment of targets with interferon-γ. *Cancer Immunol. Immunother.*, **37**, 187–194 (1993).
14) Sato, T., Sato, N., Takahashi, S., Koshiba, H. and Kikuchi, K. Specific cytotoxicity of a long-term cultured T-cell clone on human autologous mammary cancer cells. *Cancer Res.*, **46**, 4384–4389 (1986).
15) Kradin, R. L., Kurnick, J. T., Lazarus, D. S., Preffer, F. I., Dubinett, S. M., Pinto, C. E., Gifford, J., Davidson, E., Grove, B., Callahan, R. J. and Strauss, H. W. Tumor-infiltrating lymphocytes and interleukin-2 in treatment of advanced cancer. *Lancet*, **ii**, 577–580 (1989).
16) Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P., Lotze, M. T., Yang, J. C., Seipp, C. A., Simpson, C., Carter, C., Bock, S., Schwartzentruber, D., Wei, J. P. and White,

D. E. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma; a preliminary report. *N. Engl. J. Med.*, **319**, 1676–1680 (1988).
17) Arinaga, S., Karimine, N., Adachi, M., Inoue, H., Nanbara, S., Asoh, T., Ueo, H. and Akiyoshi, T. Cytotoxic cell function and phenotypic analysis of peripheral blood mononuclear cells in cancer patients treated with low-dose interleukin-2 and mitomycin C. *Cancer Immunol. Immunother.*, **37**, 220–226 (1993).
18) Itoh, K., Platsoucas, C. D. and Balch, C. M. Autologous tumor-specific cytotoxic T lymphocytes in the infiltrate of human metastatic melanomas; activation by interleukin 2 and autologous tumor cells, and involvement of the T cell receptor. *J. Exp. Med.*, **168**, 1419–1441 (1988).
19) Osband, M. E., Lavin, P. T., Babayan, R. K., Graham, S., Lamm, D. L., Parker, B., Sawczuk, I., Ross, S. and Krane, R. J. Effect of autolymphocyte therapy on survival and quality of life in patients with metastatic renal-cell carcinoma. *Lancet*, **335**, 994–998 (1990).
20) Sporn, J. R., Ergin, M. T., Robbins, G. R., Cable, R. G., Silver, H. and Mukherji, B. Adoptive immunotherapy with peripheral blood lymphocytes cocultured *in vitro* with autologous tumor cells and interleukin-2. *Cancer Immunol. Immunother.*, **37**, 175–180 (1993).
21) Mukherji, B. and MacAlister, T. J. Clonal analysis of cytotoxic T cell response against human melanoma. *J. Exp. Med.*, **158**, 240–262 (1983).
22) Rasmussen, A.-M., Smeland, E. B., Erikstein, B. K., Caignault, L. and Funderud, S. A new method for detachment of Dynabeads from positively selected lymphocytes. *J. Immunol. Methods*, **146**, 195–202 (1992).
23) Semple, J. W., Allen, D., Chang, W., Castaldi, P. and Freedman, J. Rapid separation of CD4$^+$ and CD19$^+$ lymphocyte populations from human peripheral blood by a Magnetic Activated Cell Sorter (MACS). *Cytometry*, **14**, 955–960 (1993).
24) Smeland, E. B., Funderud, S., Kvalheim, G., Gaudernack, G., Rasmussen, A.-M., Rusten, L., Wang, M. Y., Tindle, R. W., Blomhoff, H. K. and Egeland, T. Isolation and characterization of human hematopoietic progenitor cells; an effective method for positive selection of CD34$^+$ cells. *Leukemia*, **6**, 845–850 (1992).
25) Letessier, E. M., Heo, D. S., Okarma, T., Johnson, J. T., Herberman, R. B. and Whiteside, T. L. Enrichment in tumor-reactive CD8$^+$ T-lymphocytes by positive selection from the blood and lymph nodes of patients with head and neck cancer. *Cancer Res.*, **51**, 3891–3899 (1991).
26) Morecki, S., Topalian, S. L., Myers, W. W., Okrongly, D., Okarma, T. B. and Rosenberg, S. A. Separation and growth of human CD4$^+$ and CD8$^+$ tumor-infiltrating lymphocytes and peripheral blood mononuclear cells by direct positive panning on covalently attached monoclonal antibody-coated flasks. *J. Biol. Response Modif.*, **9**, 463–474 (1990).
27) Sekine, T., Shiraiwa, H., Yamazaki, T., Tobisu, K. and Kakizoe, T. A feasible method for expansion of peripheral blood lymphocytes by culture with immobilized anti-CD3 monoclonal antibody and interleukin-2 for use in adoptive immunotherapy of cancer patients. *Biomed. Pharmacother.*, **47**, 73–78 (1993).
28) Loveland, B. E., Hogarth, P. M., Ceredig, R. and McKenzie, I. F. C. Cells mediating graft rejection in the mouse; 1. Lyt-1 cells mediate skin graft rejection. *J. Exp. Med.*, **153**, 1044–1057 (1981).
29) Rosenberg, A. S., Mizuochi, T., Sharrow, S. O. and Singer, A. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. *J. Exp. Med.*, **165**, 1296–1315 (1987).
30) Tyler, J. D., Galli, S. J., Snider, M. E., Dvorak, A. M. and Steinmuller, D. Cloned LYT-2$^+$ cytolytic T lymphocytes destroy allogeneic tissue *in vivo*. *J. Exp. Med.*, **159**, 234–243 (1984).
31) Wong, J. T., Pinto, C. E., Gifford, J. D., Kurnick, J. T. and Kradin, R. L. Characterization of the CD4$^+$ and CD8$^+$ tumor infiltrating lymphocytes propagated with bispecific monoclonal antibodies. *J. Immunol.*, **143**, 3404–3411 (1989).
32) Muul, L. M., Spiess, P. J., Director, E. P. and Rosenberg, S. A. Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. *J. Immunol.*, **138**, 989–995 (1987).
33) Hoskin, D. W., Reynolds, T. and Blay, J. Colon adenocarcinoma cells inhibit anti-CD3-activated killer cell induction. *Cancer Immunol. Immunother.*, **38**, 201–207 (1994).