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Specific Studies on *In Vitro* Generated Cytotoxic T Lymphocytes Directed Against Human Target Cells Grown in Monolayer Culture*)

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ABSTRACT

Specific cytotoxic T lymphocytes (CTL) against HLA antigens of skin fibroblasts were generated with mixed leukocyte culture (MLC).

Nonspecific cytotoxicity (CTX) in ^{3}H-proline microcytotoxicity test was detected as early as the 4th day by primary in vitro sensitization (IVS), and the activity continued until around the 8th day. The specific CTX by primary IVS was detectable from the 7th day to 12th day, and by secondary IVS, from around the 2nd to 4th day. However, obtainment of specific CTX only was rarely the case, and the probability of obtaining them did not increase until the 9th to 10th day by primary IVS and until the 3rd to 4th day by by secondary IVS. This is related to reduction of blastogenic response in MLC and suggests that inactivation of blast cells generated by MLC is of importance in obtaining specific CTX.

A CTX assay was conducted using cryopreserved effector cells, which revealed that in most cases nonspecific CTX either decreases or disappears altogether. On the other hand, specific CTX activity (more than 30% reduction of only test target cells) remained and the specific CTX pattern was good and evident in many cases.

Specific CTL was generated against fibroblast antigens by adding allogenic lymphocytes as third party stimulators. Cryopreservation technique was effective in increasing the specificity of CTX.

INTRODUCTION

MLC and cell-mediated cytotoxicity (CMC) studies have shown that lymphocytes cultured with allogeneic lymphocytes respond to allogeneic HLA antigens (HLA-D) by undergoing blast transformation, and subsequently CTL can be differentiated and directed against the target cell antigens present on the stimulating cells[1,6)]. Whereas target cells autologous to or scngenic with responding lymphocytes are not usually destroyed in CMC assay, in which the target cells used were grown in suspension culture[21,23)].

In practice, however, most human solid tumors are grown as monolayer culture cells *in vitro*, and when the CMC tests are performed by using effector cells generated *in vitro* against monolayer tissue culture target cells, nonspecific CTX often develops. These monolayer culture target cells appear to be very sensitive to nonspecific cytotoxic effects of blast cells[3,5,9)].

Therefore, the present study was undertaken

*) 秋山實利，山木戸道郎，小武家暁子，藤原佐枝子，Micheal A. Bean，西本幸男：ヒト単層培養細胞に対する細胞障害性Tリンパ球の *in vitro* 誘導

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to establish selectively specific CTL generation by MLC against surface antigens expressed by human skin fibroblast target cells, and also to study methods of cryopreservation of effector cells in order to check whether their function can be maintained. Cryopreservation has proven to be an effective way for reducing nonspecific killing cells and retaining function of specific CTX of effector cells.

For the purpose of generating specific CTL to autologous tumor cells, the conditions necessary to generate CTL against monolayer cultured tumor target cells were also studied by using allogeneic fibroblasts as stimulator cells, since they are known to bear serologically defined (SD) HLA surface alloantigens just as tumor cells bear SD antigens.

MATERIALS AND METHODS

Details of HLA locus antigens of the study subjects from whom lymphocytes and skin fibroblasts were obtained are shown in Table 1.

Isolation of human lymphocytes from peripheral bllod. It was performed as described elsewwhere[10]. Briefly, human peripheral blood mononuclear cells were isolated from normal volunteers, who were also donors of skin fibroblast cell lines, by defibrination and use of Ficoll-Hypaque solution (specific density 1.077 ±0.001) followed by three washes before suspension in medium for IVS. The medium consisted of Eagle's minimal essential medium (MEM) containing 10% heat-inactivated fresh normal human serum which is usually obtained from stimulator donors in IVS, and 100 IU of penicillin and 100 μg streptomycin/ml (1% P/S), 1% nonessential aminoacid (NEAA) and 2 mM fresh L-glutamine (1% L-glutamine). These

Table 1. HLA locus antigens on lymphocytes and skin fibroblasts of donors

Normal Donors	Sex	HLA Locus Antigens			
		A	B	Cw	Dw
M.B.	M	2	7, 12	5	3
C.S.	M	2, 29	12		2
Y.K.	M	2, 9	5	1	
J.E.	F	2, 10	12, 27	1	
T.P.	F	11, w24	12, 40		
J.M.	M	1, w24	8, 15		2, 3
M.C.	F	2, 29	12		1

mononuclear cells were used as responder and stimulator cells for IVS.

In vitro sensitization. One-way MLC was used to sensitize responder normal lymphocytes under various conditions against alloantigens on irradiated stimulating lymphocytes or fibroblasts (irradiated with ^{60}Co to 2,250 rad and 4,500 rad, respectively). MLC was performed in tissue culture flasks at several ratios of responder and stimulator cells and keeping the conditions similar to those used for MLC in microtest plate (Falcon # 3040) in order to maintain comparable culture conditions between the systems. The responder cell density and amount per medium surface area of culture flasks were kept constant (i. e., 125×10^3 responding cells in 0.2 ml of test medium per 0.282 cm^2 of surface area of culture flasks). Unless otherwise noted, half volume of culture medium in the flasks was usually replaced every three days with fresh test medium.

Harvesting MLC cells from flasks and checking mixed leukocyte response by ^{3}H-thymidine uptake. Eighteen hours before terminating sensitization, 0.2 ml of cell suspencion was tranferred from each culture flask into wells of the microtest plate. Triplicate samples were made and labelled with ^{3}H-thymidine (^{3}H-TdR, 0.5 μCi/well, specific activity 5 Ci/mmole) for 18 hours, and the cells were harvested. The remaining MLC cells in the flasks were washed with Eagle's balanced salt solution and resuspended in CMC medium (consisting of MEM+ 10% prescreened heat-inactivated fetal calf serum (FCS), 1% P/S, 1% NEAA, and 1% L-glutamine) for CMC assay.

Freezing and thawing MLC cells. The cryopreservation method used was a slight modification of Miller et al.[17]. The cells were centrifuged and resuspended in the CMC medium, to which, then, were added an equal amount of cold-freezing medium containing 50% heat-inactivated FCS, 20% dimethyl sulfoxide (DMSO) in MEM to secure a final concentration of 30% FCS, 10% DMSO, and $10\text{-}20\times10^6$ cells/ml. One ml of this in a vial was kept in ice water for 5-10 minutes and placed in a −80°C freezer overnight prior to transferring into liquid nitrogen. The rate of freezing in this system is four times as fast as that in a rate-controlled freezer.

For recovery of frozen cells, the vials were

thawed in 37°C water bath with gentle agitation until the last ice crystals disappeared. Thawed vials were kept in ice water and within five minutes the contents were slowly diluted with an equal volume of cold medium containing 15% FCS and thoroughly mixed. This was repeated two more times to secure a final dilution of 1 : 8–1 : 12. This suspension was washed twice, and the pellets were resuspended in CMC medium for determination of viability, recoveıy, and cytotoxic activity. The persent viability of frozen-thawed cells was 86.5±8.1% in unsensitized cells and 84.6±6.7% in sensitized cells, and the percent recovery was 53.6±14.7% in unsensitized cells and 62.4±14.1% in sensitized cells.

Cell culture. Culture of normal skin fibroblasts was initiated with finely miced explants from seven healthy donors. The culture was maintained in a complete medium consisting of 1% NEAA, 2 mM fresh L-glutamine, 1% P/S, and 15% prescreened FCS in MEM. These fibroblasts were used as monolayer culture target cells in microcytotoxicity assay and as a source of HLA-SD antigens in IVS between the 2nd to 17th passages *in vitro*.

^{3}H-Proline cell-mediated cytotoxicity microassay. Cultured skin fibroblasts were labelled overnight with 50 μCi/ml of ^{3}H-proline (specific activity 21.9–24.6 Ci/mmole), trypsinized, resuspended in test medium, and distributed into microtest plates at 1,000 viable labelled target cells per well, as described elsewhere[2,3]. Unless otherwise noted, a ratio of 250 effector cells to one target cell in used for 3H-prolioe CMC assay. After 40 hours of incuaation in 5% CO_2 and humidified air at 37°C, unattached dead target cells and effector cells were washed away with 37°C phosphate-buffered saline containing 5% FCS. Then, the residual radioactivity of cells attached to each well was measured to indicate the number of viable target cells remaining.

Analysis. Percent CMC (% reduction of target cells) is calculated as $[1-(b)/(a)] \times 100$, where (a) = mean count per minute (com) of target monolayer incubated with effector cells from unsensitized culture, and (b) = mean cpm of target monolayer incubated with effector cells from sensitized culture. Here, for positive CTX of effector cells, more than 30% reduction of target cells and significant difference (at $p < 0.05$ by t-test) from mean cpm of five replicates of target monolayer incubated with medium alone were used.

For the determination of specific CTX by effector cells from sensitization culture, negative CTX against control target and positive CTX against test target were considered.

RESULTS

Kinetic study of primary and secondary mixed leukocyte culture. These studies were performed in microtest plates. All cultures were set up in triplicate containing 125×10^3 responding cells and 125×10^3 irradiated (2,250 rad) stimulating cells in 0.2 ml of test medium, cultured for 1 to 15 days, and labelled with ^{3}H-TdR.

Primary MLC response reached a peak on the 7th day and subsequently diclined and became zero, or weak, around the 12th day. When primary MLC cells were restimulated by cells autologus to primary stimulating cells, secondary proliferative response was induced and reached a peak within 2–3 days.

Detection of cell-mediated cytotoxicity in effector cells sensitized in vitro. Fig. 1 A shows, that as compared with destruction of target cells by unsensitized cells, effector cells sensitized to allogeneic cells for 3–6 days strongly destroyed both target cells autologous to responder cells and target cells autologous to stimulator cells. But in the following days, CTX to target cells autologous to responder cells (nonspecific CTX) decreased. In contrast to this nospecific CTX, specific CTX came to be detected after 7 days of sensitization (6 of 18 CMC tests showed killing only test targets). After this, detection of the specific CTX became more clear by effector cells sentitized for 8 or 9–11 days, and subsequently in 12–14 days generation, CTX against both target cells became weak or negative.

At the ratio of two responder cells to one stimulator cell, less blastogenesis was usually seen (data not shown), but rather strongly reactive specific cytotoxic lymphocytes were seen seven days after primary stimulation, with decreasing changes of nonspecific CTX, i.e., decreasing from 12 of 18 nonspecific CTX tests at 1 : 1 ratio (Fig. 1A) to 5 of 19 tests at 1 : 0.5 ratio (Fig. 1B).

Fig. 1C shows CTX test by effector cells

generated in secondary stimulation of 10-day primed MLC. There was a rapid increase of specific CTX without much nonspecific CTX at 2-3 days after restimulation.

Fig. 2 summarizes the CTX of cryopreserved effector cells generated under several conditions of MLC. Effector cells sensitized for 5-6 cays still showed high CTX against target cells auto-

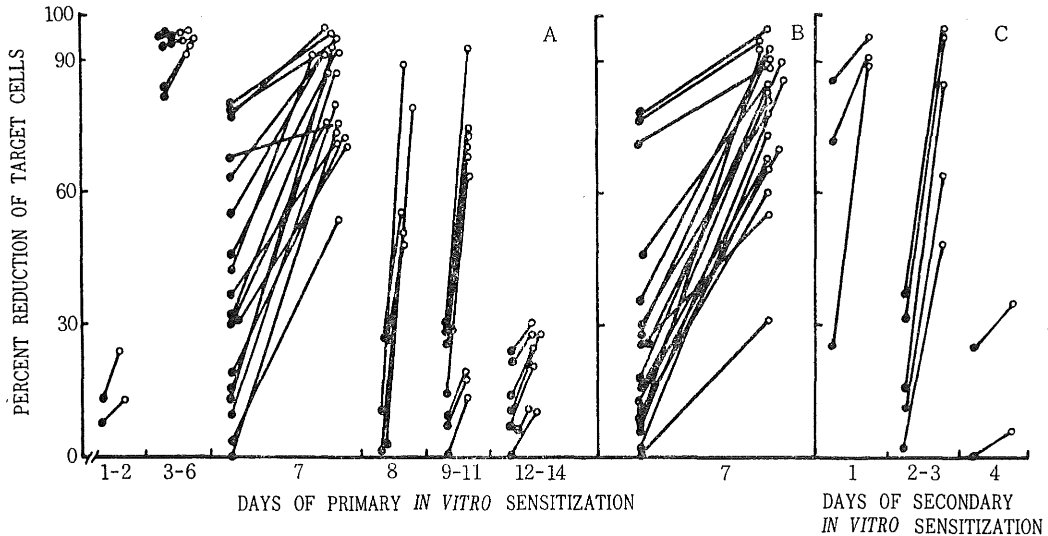


Fig. 1. Cytotoxicity of effector cells generated for various times of in vitro sensitization. Effector cells were prepared at various periods of MLC. 250,000 effector cells (per well) were incubatedwith 1,000 ^{3}H-proline prelabelled fibroblast target cells for 40 hours. The data show percent reduction of target cells by sensitized effector cells compared to usensitized cells against target cells autologous to responder cells (●), and to stimulator cells (○). Primary in vitro sensitization was done with 1 : 1 ratio of responder and stimulator cells (A), and 1 : 05 ratio (B). Secondary in vitro sensitization was done with 1 : 1 ratio of 10 days-primary IVS cells and stimulator cells (C).

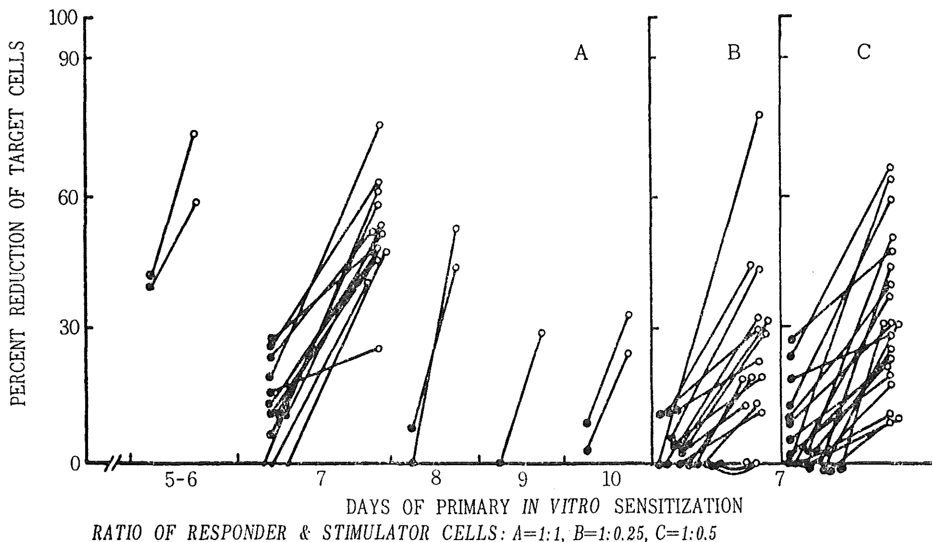
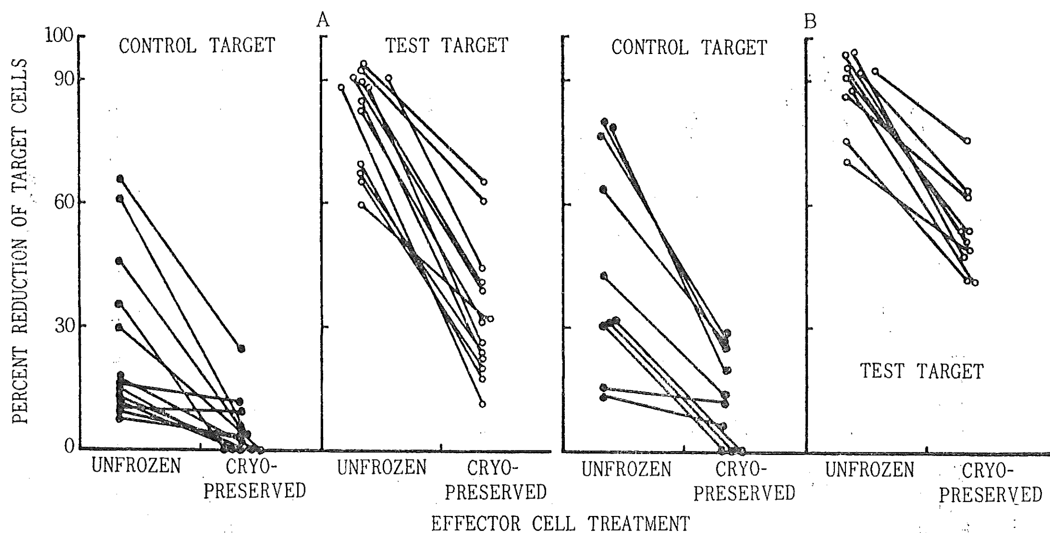


Fig. 2. Cytoxic activity of cryopreserved effector cells generated for various times of in vitro sensitization. Cytotoxicity tests were performed as described in the text. 250,000 cryopreserved effector cells were incubated with 1,000 ^{3}H-proline prelabelled fibroblast target cells for 40 hours. The data showed percent reduction of target cells by sensitized cells compared to unsensitized cells against target cells autologous to responder cells (○), and to stimulator cells (●).

Table 2. Effect of cryopreservation oncytotoxic activity of effector cells

Experiment No.	Effector cells[1] (250×10³ cells/well)	3H-Tdk Uptake[2] (c. p. m.)	Target Cell (1000 cells/well) (c. p. m.)[3]					
			M. C. Fibroblast (A 2, A 29, B 12, -)[5]		T. P. Fibroblast (A11, Aw 24, B 12,B 40)		J. E. Fibroblast (A 2, A 10, B 12, B 27, CW 1)	
			Unfrozen Effector Cells	Cryo-Preserved Effector Cells	Unfrozen Effector Cells	Cryo-Preserved Effector Cells	Unfrozen Effector Cells	Cryo-Preserved Effector Cells
1	M. C.+M. Cx	675± 25	3280±122	2873±152	2030± 93	1952± 96	1010± 27	989± 43
	M. C.+T. Px	53168±2004	2275± 71*	3467± 81	492± 43*	1152± 43*	578± 19*	957± 35
	M. C. + J. Ex	28030± 474	1892± 96*	2483± 55	1201± 37*	1877± 70	86± 4*	462± 23*
	M. A.+PHA[4]		867±46		684±48		99± 4	
	MEDIUM ALONE		3526±77		2302±90		1169±37	
2	T. P.ALONE	2129± 51	3801±110	4304±157	2467± 27	2140±154	1168± 19	1018± 58
	T. P.+M. Cx	56594±1097	1122± 89*	2207± 82*	1706± 28*	2240± 63	229± 9*	568± 72*
	T. P.+J. Ex	57031± 562	1724±142*	3004±127*	1680± 83*	2193± 62	153± 14*	391± 33*
	M. A.+PHA		1150±67		943±126		132±18	
	MEDIUM ALONE		4138±93		2615± 70		1144±18	

1) Effector cells were prepared from 7 days primary in vitro sensitization with 1 : 1 ratio of responder and stimulator cells. X after stimulator cells means irradiation at 2,250 rad. Cryopreserved effector cells were divided into unfrozen and cryopreserved cells from the same culture system. Cytotoxicity test was performed at the same time.
2) 3 H-thymidine incorporation was measured before freezing effector cells, mean cpm±standard error or triplicate samples from culture flasks.
3) 1000 target cells prelabeled with 3 H-proline were seeded in wells of Falcon 3040 microtest plates. Mean cpm±standard error of five replicates of remaining target cells after 40 hours incubation with effector cells.
4) Phytohemagglutinin-P (1 : 800) was added to induce cytotoxicity to freshly prepared lymphocytes.
5) HLA locus antigens. * Signifcant cytotoxicity ($\geq 30\%$ reduction compared with unsensitized control cells and $p<0.05$ by student's "t" test).



CONTROL TARGET=TARGET: AUTOLOGOUS FIBROBLASTS TO RESPONDER CELLS
TEST TARGET=TARGET: AUTOLOGOUS FIBROBLASTS TO STIMULATOR CELLS
RATIO OF RESPONDER & STIMULATOR CELLS: A=1.0.5, B=1.1

Fig. 3. Comparative studies on cytotoxic activity of unfrozen and cryopreserved effector cells generated in 7 days-primary MLC. Cytotoxicity tests were performed as described in Materials and Methods, with 250,000 effector cells and 1,000 target cells prelabelled with ^{3}H-proline in microtest plates. Effector cells generated in 7 days-primary MLC were divided into unfrozen and cryopreserved cells from same culture systems. These effector cells were used at the same time or at different times against the same donor's fibroblast target cels.

logous to both responder and stimulator cells. In contrast, 7–8 days sensitized cells killed target cells autologous to responder cells less (0 of 14 tests showed$\geq$30% reduction) but killed target cells autologus to stimulator cells strongly (13 of 14 tests showed$\geq$30% reduction, mean 51.3%). Effector cells from 1 : 0.25 and 1:0.5 ratios of responder and stimulator cells also killed only target cells autologus to stimulator cells, but the frequency and magnitude of the CTX were low and weak. (Six of 16 tests showed$\geq$30% reduction, mean of 26.2%, and 11 of 20 tests, mean of 34.3%).

Comparison of activity of unfrozen and cryopreserved effector cells in cell-mediated cytotoxicity. Table 2 shows the effect of cryopreservation on CTX of sensitized cells against cells autologous to responder cells (nonspecific CTX). Unfrozen effector cells of M. C. + T. Px and M. C. + J. Ex in experiment 1 and T. P. + M. Cx and T. P. + J. Ex in experiment 2 showed high nonspecific pattern of CTX against all of the target cells, but when these effector cells were frozen and thawed, they came to kill selective target cells autologous to stimulator cells in each MLC; they do not destroy target cells which are autologous to responder cells.

Fig. 3 presents a summary of comparative studies made on CTX of unfrozen and cryopreserved effector cells. Some unfrozen effector cells obtained from 1 : 0.5 ratio of responder and stimulator cells killed more than 30% of control target cells (4 of 13 tests), but after freezing and thawing of these cells, reduction of target cells never exceeded 30%. Although all of the unfrozen effector cells killed test target cells at a high rate, 6 of 13 cryopreserved effector cells did not kill more than 30% (Fig. 3 A). In Figure 3 B, unfrozen effector cells generated with 1 : 1 ratio of responder and stimulator cells showed strong nonselctive patterns of CTX, but, when cryopreserved, the same effector cells presented highly selective killing of test target cells. The frequency of 30% reduction of target cells and magnitude of CTX of cryopreserved cells against test target cells was 7 of 13 tests and with a mean of 31.4$\pm$16.5% reduction by effector cells from 1 : 0.5 ratio of MLC, and 10 of 10 tests with a mean of 53.5$\pm$10.7% reduction by effector

Table 3. Representative experiment on generating cytotoxic lymphocytes against alloantigens on the surface of fibroblast

Effector cells (250×10^3 cells/well)	3H-TdR Uptake	Target Cell (1000 cells/well)		
		M. F. Fibroblast (A 2,—, B 7, B 12, CW 5)	C. S. Fibroblast (A 2, A 29, B 12, —)	J. E. Fibroblast (A 2, A 10, B 12, B 27, CW 1)
C. S. ALONE	233± 26	2306±57	1412±59	1228±28
C. S.+M. Bx	28860±1139	1081±62*	1068±20	867±23
C. S.+J. Ex	43677±1512	1704±49	1042±82	455±13*
C. S. ON M. B. FIBROx	998± 68	2610±57	1411±31	1325±41
C. S.+J. Ex ON M. B. FIBROx	21625±1132	1330±68	1154±31	560±29*
NORMAL LYM + PHA	—	131± 5	87± 4	65± 2
MEDIUM ALONE	—	2795±97	1524±31	1250±23

Effector cells were prepared from 7 days primary *in vitro* sensitization. Effector cells were frozen in Revco overnight and then transferred into liquid nitrogen bank until use.
Ratio of responder and atimulator lymphocytes in vitro sensitization was 1 : 1.
1.68×10^6 fibroblast in the culture of in vitro sensitization were irradiated at 4,500 rad. The ratio of responder cells and fibroblast was 1 : 0.05.
* See Table 2.

cells from 1 : 1 ratio of MLC.

Generation of cytotoxic lymphocytes against monolayer cultured fibroblasts used as stimulator cells. The conditions necessary to generate cytotoxic cells against monolayer cultured cells used as stimulator cells were studied. For this model, allogeneic fibroblasts were used as the stimulator cells as they are known to bear HLA-SD surface antigens. Thus, they serve as a model for tumor cells bearing SD antigens including tumor antigens. As shown in Table 3, when responder cells were cultured with only fibroblasts there was little or no blastogenesis or development of cytotoxic cells. Based on the findings of Eijsvoogel et al.[7] and Zarling et al.[24] on a two signal stimulus necessary for proliferation and generation of cytotoxic cells, third party experiments were performed.

Donors M. B., C. S., and J. E. were not HLA identical. After seven days *in vitro* MLC, cryopreserved effector cells were used for the testing of CMC on fibroblast targets from donors M. B., C· S., and J. E. The CTX of these effector cells could be easily and specifically generated to M. B. fibroblasts as well as J. E. fibroblast targets.

DISCUSSION

The MLC and CMC assay systems are extensively used for the study of alloantigens or tumor antigens and for typing transplant recipients and donors[1,6,8]. However, sometimes complicated interpretations are made of these assays due to unexpected destruction of autologous or third party targets used as controls[4,5,14].

The secific studies we have attempted here were made to answer: 1) why MLC cells nonspecifically kill autologous monolayer targets, as it is unusual for target cells grown in suspension culture to be so killed, 2) whether we can differentiate specifically cytotoxic lymphocytes to monolayer target cells and under what condition of MLC we can do this, 3) whether specific cytotoxic effector cells generated in vitro can be preserved without loss of much of their reactivity, and 4) whether cytotoxic lymphocytes can be generated to HLA-SD antigens present on fibroblasts as a model for SD antigen-bearing tumor cells.

The results of the present study demonstrated that lymphocytes sensitized with allogeneic lymphocytes in vitro have two distinct cytotoxic activities, which are quite distinguishable by the kinetics of generation of effector cells and by the different type of destroyed target cells. There was a strong nonspecific CTX of effector cells cultured for 3-6 days while mixed leuko-

cyte responses were increasing and reaching a peak. However, specific patterns appear with decreasing response of MLC.

Although all of the tissue culture cells were carried in prescreened FCS, this nonspecific CTX was not the result of recognition of FCS antigens by responder cells because IVS was always performed in normal human serum.

Another possibility is that this nonselective and wide spectrum CTX may be attributable to *in vitro* augmentation of natural killer (NK) CMC[19,20,25]. It is well known that NK cells are induced or activated by interferon[11] which may be produced in MLC. And a relation has been observed between NK cell activity and graft vs disease[16]. Thus, *in vitro* augmented or generated NK cells may react against some normal cells[12].

It is more likely that nonspecific CTX against monolayer targets is caused by lymphoblasts attacking directly or releasing lymphocyte effector molecules, which are cytotoxic to nonlymphoid cells *in vitro*[9].

Several means were tried to knock out these blast cells in the effector cell suspension, but the procedures for this (adsorption of effector cells on monolayer cultured cells, irradiation, treatment of effector cells with drugs blocking cell metabolism) were not always perfect (data not shown). Subsequently, the method of cryopreservation was studied in order to determine whether known specific cytotoxic effector cells are preservable after once tested with unfrozen effector cells. It was found that such specific CTX could be cryopreserved. In addition, interestingly enough, another experiment revealed cryopreservation to be the most effective way for reducing nonspecific CTX and enchancing specific patterns.

Our freezing rate is four times as fast as that with a rate-controlled freezer. According to the study of Knight et al.[13], rate-controlled slowfreezing is better to preserve blast cells and cells actively synthesizing DNA. The lymphoblast cells may be easily inactivated or destroyed by rapid rate freezing.

Effect of DMSO on diminishing activity of nonspecific CTX may be present. But the toxicity, if any, may be little because the cells are kept at lower temperatures during the exposure period and CMC assay is usually performed for 40 hours, which is long enough to kill target cells.

Cryopreserved effector cells, however, show reduced activity against specific target cells as compared with unfrozen cells, the degrees of diminishment of percent reduction of target cells against control and specific target cells were nearly equal (against control target cells: from $46.3 \pm 26.1\%$ to $13.5 \pm 11.0\%$, against specific target cells: from $88.1 \pm 8.6\%$ to $53.5 \pm 10.7\%$). So, when the MLC are performed under appropriate and sufficient doses of alloantigens (suitable ratio of responder and stimulator cells seems 1 : 1 when effector cells are cryopreserved), dimminishment of effector cell activity against specific target cells seems due to diminishment of the nonspecific activity, or due to both maximal diminishment of the nonspecific activity and minimal diminishment of specific activity.

To perform studies on the conditions necessary to generate cytotoxic cells against monolayer cultured cells, we used allogeneic fibroblasts as the ttimulator cells for generation of cytotoxic cells because they are known to bear SD HLA surface alloantigens[22]. Thus they serve as a model for SD antigen-bearing tumor cells. When we set up person A responding to fibroblast Bx, we found little or no blastogenesis or development of cytotoxic cells.

On the basis of the evidence of Eijsvoogel et al.[6,13] and Schendel and Bach[18], who showed that generation of CTL was possible even though HLA-D lymphocyte-activating determinants were not present on the same cells as HLA-A, B, or C SD antigens. Zarling et al.[24] and Lee and Oliver[15] made these three-cell experiments in which remission lymphocytes of leukemia patients cultured with autologous leukemic blast cells and allogeneic lymphocytes differentiate to CTL which were cytotoxic for the autologous leukemic blast cells.

We added unrelated allogeneic lymphocytes Cx to supply HLA-D antigens to responder lymphocytes A and fibroblast Bx culture. We could quite easily generate CTL to fibroblast B under this condition.

More studies are necessary on these points, however, all of the observations presented here are important from the point of view of generating cytotoxic lymphocytes to human tumor antigens.

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