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T Cell Response to Embryonal Carcinoma F9 Cells: Induction and Characterization of T Cell Receptor $\alpha\beta^+$ Double-negative Cytotoxic T Lymphocytes

Mie Imada and Shigeyoshi Fujimoto

Department of Immunology, Kochi Medical School, Kohasu, Okoh-cho, Nankoku-shi, Kochi 783

We investigated the mechanism of T cell response to murine embryonal carcinoma F9 cells. Thy-1$^+$, CD4$^-$, CD8$^-$ (double-negative) cytotoxic effector cells were induced in spleen cells obtained from immune A.BY mice to F9 cells, and the cytotoxic activity was major histocompatibility complex (MHC)-unrestricted. Furthermore, CD4$^+$ T cells were essential for the induction of double-negative cytotoxic T lymphocytes directed to F9 cells. Most of the double-negative cytotoxic T lymphocyte lines obtained by long-term culture of the effector cells had CD3 molecule and T-cell receptor β chain on their cell surface, and the CD3 molecule was found to be involved in target cell recognition. The T cell receptor $\alpha\beta^+$ double-negative cytotoxic T lymphocyte line (2A5) also lysed various tumor cells in a non-MHC-restricted manner, but did not lyse concanavalin A-stimulated blasts of 129 strain, from which F9 cells had originated. These results indicate that T cell receptor $\alpha\beta^+$ double-negative cytotoxic T lymphocytes induced by F9 cells recognize a common antigen(s) expressed on F9 cells and other tumor cells but not minor histocompatibility antigens.

Key words: Embryonal carcinoma F9 cell — Double-negative cytotoxic T lymphocyte — CD4$^+$ T cell

It was reported that embryonal carcinoma cells possess common antigens shared with early embryo,[1] and various other tumor lines are also considered to have such antigens.[2-4] However, effector cells in the host immune response to the antigens have not been fully identified. In order to elucidate this problem, we investigated T cell response against F9 cells, which are representative embryonal carcinoma cells[5] lacking major histocompatibility complex (MHC) molecules.[6] Some investigators reported that the host response to embryonal carcinoma cells is mediated by natural killer cells,[7] while recently Sponaas *et al.*[8] reported that Thy-1$^+$, CD4$^-$, CD8$^-$ double-negative cytotoxic T lymphocytes (DN CTL) can be induced from F9-primed spleen cells. According to the latter, the DN CTL lysed various tumor cells in a non-MHC-restricted manner. The effector cells were not specific for either MHC molecules or minor histocompatibility antigens but recognized common tumor antigens or differentiation antigens on F9 cells shared with various tumor cells. In this study, we analyzed effector cells which can be generated in splenocytes of A.BY after immunization with attenuated F9 cells, since A.BY was found to be resistant and to reject viable F9 cell inoculation after the immunization. DN CTL to F9 cells were also induced even in A.BY and several DN CTL lines were established. We describe here the generation mechanisms of DN CTL directed to F9 cells and characterization of the DN CTL.

MATERIALS AND METHODS

Mice Breeders of A.BY strain were kindly donated by Dr. K. Moriwaki from the National Institute of Genetics (Mishima) and propagated and maintained in the animal facilities of Kochi Medical School as specific pathogen-free mice. Six- to twelve-week-old mice were used for the experiments.

Cell lines F9 cells were supplied by the Japanese Cell Research Bank (Tokyo) and maintained in stationary culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Flow Laboratories Inc., McLean, VA) and 100 μg/ml of kanamycin (Meiji Pharmaceutical Co. Ltd., Tokyo). EL4, P815, BW5147, and YAC-1 were maintained in stationary culture in RPMI 1640 medium supplemented with 4% heat-inactivated FCS and 100 μg/ml of kanamycin. N24.3 cells are T cell receptor (TCR) $\gamma\delta^+$ T cell clones derived from BALB/c *nu/nu*[9] and were kindly donated by Dr. H. Tamura in the Division of Immunology, National Institute of Neurosciences (Tokyo).

Immunizations F9 cells were harvested from plastic culture flasks using phosphate-buffered saline (PBS), pH 7.4, containing 1 mM EDTA, washed with PBS, and resuspended in the culture medium. The tumor cells were then attenuated with 100 μg/ml of mitomycin C (Kyowa Hakko Industrial Co. Ltd., Tokyo) for 45 min. After washing 3 times with PBS the cells were resuspended in PBS. Each mouse was intraperitoneally injected with two

million F9 cells. This immunization procedure was performed 3 times at 10-day intervals.

Lymphocyte preparation and induction of cytotoxic effector cells Two weeks after the last immunization, spleens were removed from immunized mice. A spleen cell suspension was prepared by gentle grinding in a glass homogenizer in DMEM supplemented with 5% heat-inactivated FCS, 100 μg/ml of kanamycin, 5×10^{-5} *M* 2-mercaptoethanol, and 20 m*M* HEPES, followed by filtration through nylon mesh to remove fibrous tissue.

The lymphoid cells were cultured at a cell density of 6×10^6/ml in supplemented DMEM in an atmosphere of 7% CO_2/93% air at 37°C. Stimulator F9 cells were attenuated with 100 μg/ml of mitomycin C for 45 min at 37°C followed by washing with PBS 3 times. The *in vitro* stimulation of the primed lymphoid cells with attenuated F9 cells was performed at a responder/stimulator ratio of 100/1. The cultured lymphoid cells were harvested at day 4 and used as cytotoxic effector cells for cell-mediated cytotoxicity tests.

Cell-mediated cytotoxicity test Cytotoxic activity was measured by 16 h ^{51}Cr release assay according to the method described previously.[10] Ten million target cells/ml in Hanks' solution containing 10% heat-inactivated FCS were incubated with 50 to 100 μCi of ^{51}Cr in the form of sodium chromate (Japan Atomic Energy Research Institute, Tokai) for 30 min at 37°C. The target cells were then washed four times with Hanks' solution and finally resuspended in supplemented DMEM. Serial dilutions of activated culture lymphoid cells or T cell lines were plated into the wells of round-bottomed microtiter plates (Nunc, Roskilde, Denmark) in quadruplicate, and 1.5×10^4 ^{51}Cr-labeled target cells were added to each well. The total volume of each well was adjusted to 0.2 ml with supplemented DMEM. The plates were centrifuged at 200*g* for 1 min, and then were incubated for 16 h at 37°C to allow completion of the cytotoxic reaction. An 0.1 ml aliquot of the supernatant was taken from each well, and the radioactivity was measured in a gamma counter. Target cell lysis was expressed as percentage specific ^{51}Cr release calculated by means of the following formula:

$$\text{Percent specific } ^{51}\text{Cr release} = \frac{\text{cpm experimental} - \text{cpm control}}{\text{cpm maximum release} - \text{cpm control}} \times 100.$$

The maximum releasable counts, which amounted to 85 to 90% of the total radioactivity incorporated into target cells, were determined by lysis of the labeled target cells with 1.25% Saponin (Nacalai Tesque Inc., Kyoto). The spontaneous release from the target cells in the wells averaged less than 30% of the maximum release.

Antibodies Anti-Thy-1.2 (HO-13-4) hybridoma was kindly supplied by Dr. H. Ishikawa of the Department of Microbiology, Keio University (Tokyo). Anti-CD4 (GK1.5), anti-CD8 (3.155),[11] anti-CD3 (145-2C11),[12] and anti-LFA-1 (FD441.8),[11] anti-H-2K^{b}D^b (20-8-4),[13] anti-IL-4 (11B11)[14] and anti-rat kappa light chain (MAR18.5) hybridomas were kindly donated by Dr. N. Shinohara of the Cellular Immunology Division, Mitsubishi Life Science Institute (Tokyo). Anti-TCR β chain (H57-597)[15] and anti-IL-2 (S4B6)[16] were obtained from American Type Culture Collection (ATCC). These monoclonal antibodies were obtained from culture supernatant and purified by Protein G Sepharose 4 Fast Flow chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden). Monoclonal antibody anti-TCR $\gamma\delta$ (3A10)[17] was kindly donated by Dr. H. Yagita of the Department of Immunology, Juntendo University (Tokyo).

Treatment with monoclonal antibodies In order to determine the phenotype of cells involved in the generation of cytotoxic effector cells, 1×10^7/ml of lymphoid cells obtained from A.BY mice immune to F9 cells were treated with appropriate antibodies for 60 min at room temperature, and after washing once with supplemented DMEM, they were further incubated for 60 min at 37°C with rabbit complement diluted to 1:25. The cells were washed once, resuspended in supplemented DMEM and cultured with attenuated F9 cells for 4 days.

To determine the phenotype of cytotoxic effector cells, cultured lymphoid cells were treated with appropriate antibodies for 60 min at room temperature. They were then washed once and further incubated with rabbit complement diluted to 1:25, for 60 min at 37°C. Their cytotoxic activity was determined by cell-mediated cytotoxicity testing.

Inhibition test for cytotoxic activity was performed by treating effector cells with an appropriate dose of monoclonal antibodies for 60 min at room temperature just before cell-mediated cytotoxicity testing.

Establishment of T cell lines CD4$^+$ and CD8$^+$ T cell-depleted effector cells (1×10^5/ml) were plated into the wells of flat-bottomed microtiter plates (Nunc) with 1×10^6/ml irradiated syngeneic spleen cells as filler cells. Total volume of each well was adjusted to 0.2 ml. The medium was changed every 3–4 days. Growing cells were restimulated with attenuated F9 cells on day 7. After two months, established cell lines were checked for cytotoxic activity against F9 cells. At first, we failed to raise T cells in RPMI 1640 medium supplemented with 20% culture supernatant of concanavalin A-activated rat spleen cells (Con A-sup) and 10% heat-inactivated FCS. When the culture period was over three weeks, the cells hardly proliferated any longer. The cells could not be maintained even in medium supplemented with 40% Con A-sup. But this problem was solved by the addition of culture supernatant of a CD4$^+$ T cell clone. T cell lines grew well in

RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 20% Con A-sup and 50% culture supernatant of the $CD4^+$ T cell clone, but they did not proliferate in the culture supernatant of the $CD4^+$ T cell clone alone. The $CD4^+$ T cell clone was established beforehand from effector cells directed to F9 cells according to the method of Haas and von Boehmer.[18] This clone was grown in RPMI 1640 medium containing 20% Con A-sup and the culture supernatant was taken from this culture.

Flow cytometric analysis T cell lines were incubated in appropriate dilutions of the first antibody for 30 min on ice and washed once with PBS containing 1% FCS. The cells were further incubated with FITC-conjugated second antibody for 30 min on ice. The cells were washed once, and flow cytometric analysis was performed in an EPICS 752 (Coulter Electronics, Hialeah, FL).

RESULTS

Induction of cytotoxic effector cells Cytotoxic activity to F9 cells was induced in splenocytes obtained from immune A.BY mice to F9 cells within 4 days of culture, whereas it was not induced in normal splenocytes (Table I). Cytotoxic activity was augmented by *in vitro* restimulation with attenuated F9 cells. The effector cells lysed various tumor target cells in a non-MHC-restricted manner (Table II), but EL4 cells were not lysed as much as the other target cells.

Table I. Cytotoxicity against F9 Cells Generated in A.BY Immune Spleen Cells

In vivo priming	*In vitro* restimulation	% Specific ^{51}Cr release[a]	
		E/T=40[b]	E/T=20[b]
−	−	0.2±2.35	2.0±2.52
−	+	−4.1±2.40	1.3±2.72
+	−	38.6±1.99	19.1±1.51
+	+	62.1±2.09	34.5±1.64

a) Values are %lysis±SE.
b) Effector:target ratio in microcytotoxicity tests.

Table II. Cytotoxicity of the Effector Cells against Various Tumor Cells

Target cell line (origin and H-2 haplotype)	% Specific ^{51}Cr release[a]	
	E/T=10[b]	E/T=5[b]
F9 (129, b)	69.0±1.62	52.3±1.77
EL4 (C57BL/6, b)	25.7±1.23	18.3±0.61
P815 (DBA/2, d)	56.1±1.63	41.0±1.66
BW5147 (AKR, k)	80.7±1.31	65.5±0.89

a) Values are %lysis±SE.
b) Effector:target ratio in microcytotoxicity tests.

Phenotype of effector cells exhibiting cytotoxic activity against F9 cells The *in vitro*-activated immune lymphoid cells were treated with monoclonal antibodies against several surface markers of T cell in the presence of complement. As shown in Table III, cytotoxic activity was clearly abrogated by the treatment with anti-Thy-1.2 antibody and complement but not with either anti-CD4 or anti-CD8 plus complement, thus indicating that the effector cells exhibiting cytotoxic activity were DN T cells.

Cells involved in the generation of cytotoxic activity Next, we examined T cell subsets involved in the induction of DN CTLs by depletion of $CD4^+$ T cells or $CD8^+$ T cells at day 0. $CD4^+$ T cell-depleted responder cells could not acquire cytotoxic activity after 4 days of culture (Table IV). Therefore, $CD4^+$ T cells were essential to generate the effector cells, while $CD8^+$ T cells had no effect on the generation of the effector cells. As $CD4^+$ T cells were necessary, we expected that cytokines were required for the generation of the effector cells. Interleukin-2 (IL-2) and IL-4 are representative cytokines secreted by $CD4^+$ helper T cells, and both have the potential to augment cytotoxic activity of T cells. We added monoclonal antibodies against these cytokines to the culture medium at day 0. Cytotoxic activity was completely abrogated by anti-IL2 antibody (Table V).

Table III. Phenotypic Analysis of Cytotoxic Effector Cells Induced by F9 Cells[a]

Effector cells treated with	% Specific ^{51}Cr release[b]	
	E/T=20[c]	E/T=10[c]
Complement (C) alone	43.3±1.00	25.1±0.69
Anti-Thy-1.2+C	6.4±1.68	4.5±0.58
Anti-CD4+C	32.8±1.00	20.5±1.06
Anti-CD8+C	44.4±1.76	27.4±1.94

a) F9 cells were used as target cells.
b) Values are %lysis±SE.
c) Effector:target ratio in microcytotoxicity tests.

Table IV. T-Cell Subsets Involved in the Induction of Cytotoxic Effector Cells[a]

Responder cells treated with	% Specific ^{51}Cr release[b]	
	E/T=20[c]	E/T=10[c]
Complement (C) alone	61.3±1.55	36.8±0.79
Anti-Thy1.2+C	−1.9±1.08	NT
Anti-CD4+C	11.2±1.05	4.9±0.80
Anti-CD8+C	58.8±1.05	32.0±0.55

a) F9 cells were used as target cells.
b) Values are %lysis±SE.
c) Effector:target ratio in microcytotoxicity tests.
NT=not tested.

Table V. Anti-IL2 and Anti-IL4 Effects on the Induction of Effector Cells[a)]

Antibodies[b)]		% Specific ^{51}Cr release[c)]	
anti-IL2	anti-IL4	E/T=20[d)]	E/T=10[d)]
−	−	34.3±1.86	16.5±0.36
−	+	46.9±2.12	23.4±0.98
+	−	−0.7±0.61	1.0±1.98
+	+	0.6±0.54	0.4±1.04

a) F9 cells were used as target cells.
b) The antibodies (25 μg/ml) were added to the culture medium of immunized spleen cells at day 0 and cytotoxic activity was measured at day 4.
c) Values are %lysis±SE.
d) Effector:target ratio in microcytotoxicity tests.

Phenotype of established cytotoxic T cell lines According to the method described in "Materials and Methods," we obtained 10 CTL lines. They were all Thy-1.2$^+$, CD4$^-$, CD8$^-$, CD3$^+$, TCR β^+, and TCR $\gamma\delta^-$ as determined by flow cytometric analysis. Fig. 1 shows the results of analysis of one of them, 2A5. Although the T cell lines were double-negative, they were not stained by monoclonal antibody anti-TCR $\gamma\delta$, while TCR $\gamma\delta^+$ N24.3 cells were clearly stained by the antibody (data not shown). We failed to obtain T cell lines with a similar phenotype from unprimed splenocytes under the same conditions.

Cytotoxicity of DN T cell line 2A5 One of the DN TCR $\alpha\beta^+$ T cell lines, 2A5, lysed F9, P815, BW5147, and YAC-1 cells in a non-MHC-restricted manner (Table VI). As 2A5 failed to lyse 129 blasts, this cell line was thought not to recognize minor histocompatibility antigens of 129. EL4 cells were also not lysed by this T cell line.

We tried to block the cytotoxicity with monoclonal anti-CD3 antibody in order to clarify whether CD3/TCR complex of 2A5 is functional for target cell lysis. As shown in Fig. 2, cytotoxicity of 2A5 against F9 cells was inhibited by anti-CD3 antibody. This inhibition was reproducible and not a non-specific effect of the antibody, since the antibody did not inhibit the cytotoxic activity of lymphokine-activated killer (LAK) cells at the same concentration (data not shown). These findings indicate that CD3 molecule of 2A5 acts to recognize the target cells. LFA-1 molecule was also involved in target cell lysis, as anti-LFA-1 antibody inhibited the cytotoxicity. As 2A5 cells have no CD4 and CD8 molecules and F9 cells have no MHC molecules, either monoclonal anti-CD4 or anti-CD8 or anti-H-2K^{b}D^b antibodies did not inhibit the cytotoxicity of 2A5 against F9 cells. They had no effect even at high concentrations of antibodies (data not shown).

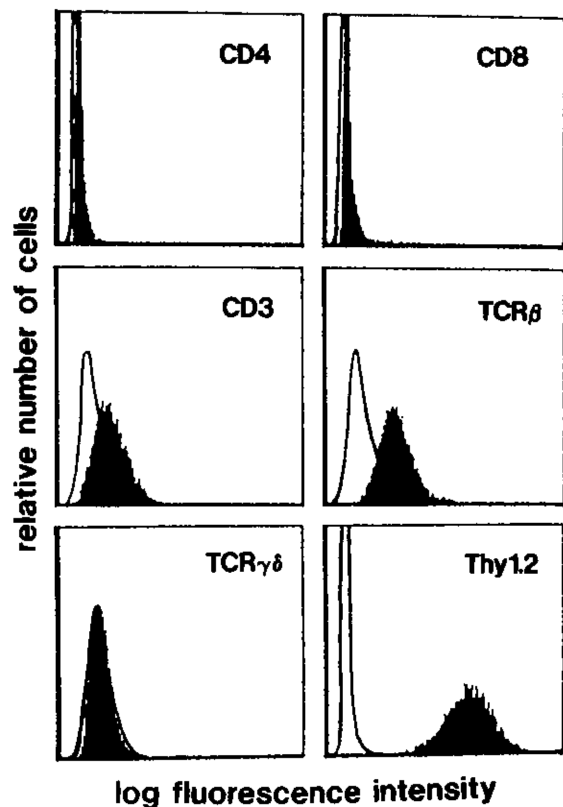

Fig. 1. Expression of T cell surface markers on a DN T cell line, 2A5. Cells were treated with each monoclonal antibody (mAb) at 10 μg/ml for 30 min and stained with corresponding second antibodies on ice (solid phase). Cells stained with second antibodies alone were used as a negative control (open phase). FITC-conjugated goat anti-hamster immunoglobulin (Ig) (Cappel, West Chester, PA) was used as the second antibody to mAb anti-CD3 (145-2C11), anti-TCR β (H57-597) and anti-TCR γδ (3A10). FITC-conjugated mouse anti-rat kappa light chain was used as the second antibody to mAb anti-CD4 (GK1.5) and anti-CD8 (3.155). The second antibody to mAb anti-Thy-1.2 (HO-13-14) was FITC-conjugated rabbit anti-mouse Ig. A total of 10^4 cells were analyzed for each histogram.

DISCUSSION

We could induce DN cytotoxic T cells from primed spleen cells of A.BY. CD4$^+$ T cells and IL-2 played an important role in the generation of cytotoxicity. The stimulation of CD4$^+$ T cells by the antigens might be long-acting because cytotoxic activity was induced sufficiently without restimulation *in vitro* (Table I).

We should consider the presence of at least two kinds of immunogenic molecule on F9 cells. One of them stimulates CD4$^+$ helper T cells and another is recognized by TCR $\alpha\beta^+$ DN T cells. As Ono *et al.*[19)] reported that the induction of double-negative anti-melanoma CTLs

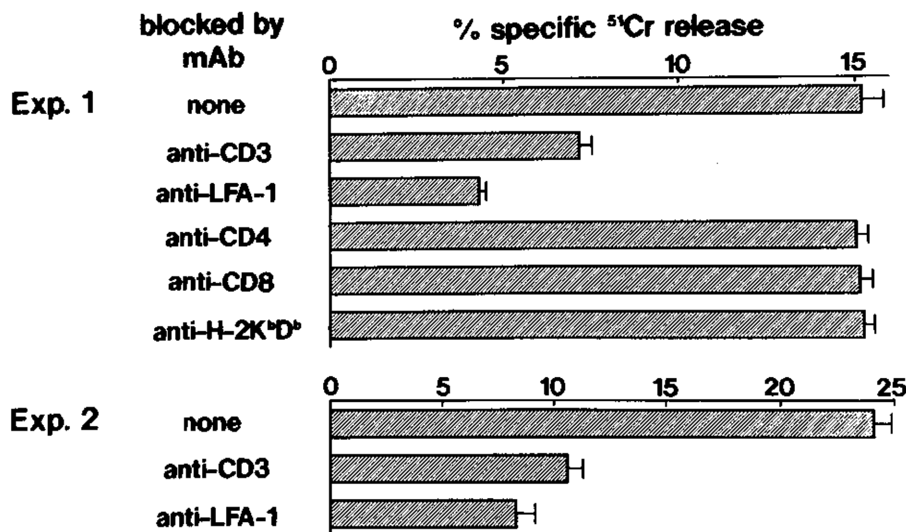

Fig. 2. Inhibition of cytotoxic activity of 2A5 by monoclonal antibodies. Antibodies (6 μg/ml) were added to the wells plated with effector cells and incubated at room temperature for 1 h before the effector cells were mixed with target cells. Effector:target ratio was 10:1.

Table VI. Specificity of Cytotoxicity of TCR $\alpha\beta^+$ DN T Cell Line, 2A5

Target cells	% Specific ^{51}Cr release[a]	
	E/T=10[b]	E/T=5[b]
F9	19.2±1.13	13.1±0.23
P815	19.2±1.04	10.7±0.32
BW5147	18.2±1.41	16.6±1.73
YAC-1	26.4±1.02	20.9±0.98
EL4	2.8±0.24	1.4±0.64
129 blast[c]	1.9±0.77	−0.7±0.62
A.BY blast[c]	0.0±1.07	−1.2±0.55

a) Values are %lysis±SE.
b) Effector:target ratio in microcytotoxicity tests.
c) Blasts were induced in medium supplemented with 1 μg/ml concanavalin A. Microcytotoxicity testing was performed for 6 h to keep the spontaneous release of blasts at less than 30% of the maximum release.

which recognize GM3-protein complex on melanoma cells was dependent on CD4$^+$ T cells, there may be a common mechanism in the immune response to these tumor cells. Although many epitopes regulated developmentally have been found in embryonal carcinoma cells,[20] antigens to which T cells respond are still undefined in F9 cells.

As TCR $\alpha\beta^+$ DN T cells in our experiments lysed various tumor cells but did not lyse Con A-blasts, the molecule recognized by TCR $\alpha\beta^+$ DN CTLs seemed to be common to tumor cells. However, it still remains to be elucidated whether our DN T cell lines were only stimulated by endogenous IL-2 secreted from CD4$^+$ T cells or whether they were stimulated directly by antigens expressed on F9 cells. Actually, most non-MHC-restricted cytotoxic TCR $\alpha\beta^+$ DN T cells reported previously were generated by large amounts of exogenous IL-2 as LAK cells.[21, 22] Effector cells generated in bulk spleen cells from F9-immune mice showed high cytotoxicity and they were expected to consist of heterogenous cell populations activated by IL-2, for example, CD3-negative NK lineage cells. But only TCR $\alpha\beta^+$ DN T cells were selected during the long-term culture. This fact might imply that some TCR $\alpha\beta^+$ DN T cells were preferentially stimulated by F9 cells and expanded.

In regard to the mechanism of target cell lysis, a previous report showed that MHC-unrestricted cytotoxicity of TCR $\alpha\beta^+$ DN T cells was not mediated via CD3/TCR complex because anti-CD3 antibody failed to inhibit the cytotoxic activity.[22] The authors assumed that some unknown molecule mediated the cytotoxicity. However, our T cell line 2A5 had a functional pathway via CD3/TCR complex, since anti-CD3 antibody inhibited the cytotoxicity. But, as the inhibition was about a half of the cytotoxicity in antibody-free medium, the presence of an alternate mechanism for the recognition via some unknown molecule was also suggested. In other reports, cytotoxic activity of TCR $\alpha\beta^+$ DN T cells was inhibited by anti-CD3 antibody.[23, 24] These reports claimed that the ligand of the TCR $\alpha\beta$ was MHC class I molecule on the target cells, since the cytotoxicity was

inhibited by anti-class I antibodies. However, as F9 cells lack MHC molecules and anti-MHC class I antibodies did not inhibit the cytotoxicity in our experiments, the ligand of TCR $\alpha\beta$ of 2A5 remains to be identified. Monomorphic MHC molecules such as Qa and Tla are unlikely to be the ligand of the TCR because of the specificity of the cytotoxicity mediated by 2A5.

DN TCR $\alpha\beta^+$ T cells were raised in medium consisting of 10% Con A-sup and 50% culture supernatant of the $CD4^+$ T cell clone. This clone was also established from spleen cells primed with attenuated F9 cells as described in "Materials and Methods." This clone secreted IL-4 in Con A-sup-free medium (in preparation). However, at the induction phase of the effector cells in bulk splenocytes, anti-IL4 antibody did not inhibit the generation of cytotoxicity (Table V). At present, IL-4 is supposed to be necessary for the proliferation of TCR $\alpha\beta^+$ DN T cell lines, but not for the generation of their cytotoxicity.

As the effector cells that were induced in F9-primed spleen cells lysed various tumor cells in a non-MHC-restricted manner, the immunopotent antigens of F9 cells might have the potential to make the hosts resistant to syngeneic tumor cells. A previous report claimed that the immunization of F9 cells was effective for different syngeneic tumor reduction.[8] We did not perform a similar study, but such a result is possible if the syngeneic tumor cells have common antigens with F9 cells. In the immunotherapy of cancer, MHC-lacking embryonal carcinoma cells might have potential for a vaccine, or as a source of antigens for restimulation of lymphocytes used for adoptive immunotherapy. Embryonal carcinoma cells could augment immune responses to antigens shared with syngeneic tumor cells and could not induce another immune response to allo-MHC molecules, even if the MHC of the host is different from that of the source of the embryonal carcinoma cells.

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