CD4⁻CD8⁻ T Cell Receptor $\alpha\beta$ T Cells: Generation of an In Vitro Major Histocompatability Complex Class I Specific Cytotoxic T Lymphocyte Response and Allogeneic Tumor Rejection

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Summary

The generation of an in vitro major histocompatibility complex class I specific response of $CD4^-CD8^-$ T cell receptor (TCR) $\alpha\beta$ cytotoxic T lymphocytes (CTL) and their allogeneic tumor rejection were investigated. Inocula of BALBRLO 1 were rejected in C57BL/6 (B6) mice treated with minimum essential medium (MEM) (control), anti-L3T4 (CD4) monoclonal antibody (mAb) or anti-Lyt-2.2 (CD8) mAb and CTL against the tumor were generated in vitro. No rejection and no induction of CTL were observed in B6 mice treated with anti-L3T4 (CD4) plus anti-Lyt-2.2 (CD8) mAb. CTL with the classical $Thy\text{-}1^+CD3^+CD4^-CD8^+$ TCR $\alpha\beta$ phenotype were generated in mixed lymphocyte tumor cell culture (MLTC) spleen cells from B6 mice treated with MEM (control) or anti-L3T4 (CD4) mAb, whereas CTL with an unusual $Thy\text{-}1^+CD3^+CD4^-CD8^-$ TCR $\alpha\beta$ phenotype were generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice. Both types of CTL were reactive with both $H\text{-}2K^d$ and D^d (L^d) class I antigen. These findings suggest that when $CD4^+$ cells were blocked by anti-L3T4 (CD4) mAb, $CD8^+$ CTL mediated rejection, and when $CD8^+$ cells were blocked by anti-Lyt-2.2 (CD8) mAb, $CD4^+$ cells were capable of mediating rejection, although less efficiently than $CD8^+$ cells, by inducing $CD4^-CD8^-$ TCR $\alpha\beta$ CTL. The finding that adoptive transfer of CD4 and CD8-depleted MLTC spleen cells, obtained from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRLO 1, resulted in rejection of BALBRLO 1 inoculated into B6 *nu/nu* mice confirmed the above notion. CTL clones with the $CD4^-CD8^-$ TCR $\alpha\beta$ phenotype specific for L^d were established.

Thymocytes with the $CD4^-CD8^-$ phenotype constitute about 5% of all cells in the thymus of adult mice (1). The TCR $\alpha\beta$-bearing subset constitutes 2–30% of the $CD4^-CD8^-$ thymocytes in different mouse strains and displays a $CD3^+$, HSA^-, $CD5^{high}$, $IL\text{-}2R^-$, $Pgp\text{-}1^+$, $B220^-$, and $Qa\text{-}2^+$ phenotype (2–6). Although these cells appear to have a functional TCR (7), the origin and developmental pathway of $CD4^-CD8^-$ TCR $\alpha\beta$ cells is currently unknown. Recently, however, it was shown that tolerance-related $V\beta$ clonal deletion took place in $CD4^-CD8^-$ TCR $\alpha\beta$ thymocytes from mice with Mls^a and Mls^c alleles and mice expressing I-E (8). Clonal deletion has been shown to occur at the stage of $CD4^+CD8^+$ thymocytes and to involve the CD4 or CD8 molecule (9–11). These findings suggest that $CD4^-CD8^-$ TCR $\alpha\beta$ thymocytes are formed at a late stage of differentiation by down-regulation of CD4 and CD8 molecules (1, 8). Alternatively, these cells could represent an independent lineage of T cells that never express CD4 and CD8 molecules during their differentiation.

Although appreciable numbers of $CD4^-CD8^-$ TCR $\alpha\beta$ cells are found in the thymus, these cells are rarely detected in peripheral lymphoid tissues. However, $CD4^-CD8^-$ TCR $\alpha\beta$ cells have been shown to accumulate in the lymph nodes of autoimmune *lpr* (12–15) or *gld* (13, 16, 17) mice. The $CD4^-CD8^-$ TCR $\alpha\beta$ cells in these mice also undergo $V\beta$ clonal deletion (8, 18) and thymectomy prevents disease development (19, 20). These findings suggest that $CD4^-CD8^-$ TCR $\alpha\beta$ cells in the lymph nodes of these mice are derived from thymocytes with this phenotype. Recently, T cells with this phenotype have been shown to be present in the lymph nodes of normal mice (21).

In this paper, we report that a $CD4^-CD8^-$ TCR $\alpha\beta$

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CTL response specific for both H-$2K^d$ and D^d (L^d) class I antigen was generated in C57BL/6 (B6)[1] mice that had been treated with anti–Lyt-2.2 (CD8) mAb and had rejected the BALBRL♂ 1 tumor, and that adoptive transfer of these cells to B6 *nu/nu* mice resulted in rejection of the tumor.

Materials and Methods

Mice. A/J, BALB/c, C57BL/6 (B6), B10.A, B10.D2 and C3H/He mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Breeding pairs of C3H.OH, B10.A (5R), B10.AQR and B10.HTG mice were provided by Dr. K. Moriwaki (National Institutes of Genetics, Mishima, Japan). Breeding pairs of B6 *nu/+* mice were provided by Dr. K. Kuribayashi (Faculty of Medicine, Kyoto University, Kyoto, Japan). Breeding pairs of BALB.B mice were provided by Dr. H. Fujiwara (Osaka University Medical School, Osaka, Japan). These mice were bred in the Laboratory Animal Center for Biomedical Research (Nagasaki University School of Medicine, Nagasaki, Japan).

Tumor. BALBRL♂ 1 is a radiation-induced leukemia (22). B6LK is a nitrosomethylurea-induced thymic leukemia that expresses TCR $\alpha\beta$, CD4, and CD8 on the cell surface. K4D11 is a T cell lymphoma that developed in a C3H Tg.Tla^a-3-1 transgenic mouse and expresses TCR $\gamma\delta$, but not CD4 or CD8, on the cell surface (23). The tumors were maintained in the strain of origin.

Antibodies. Anti-L3T4 (CD4) mAb, a rat antibody of the IgG2b immunoglobulin class, produced by hybridoma GK1.5 (24), was provided by Dr. F. Fitch (University of Chicago, Chicago, IL). Anti–Lyt-2.2 (CD8) mAb, a mouse antibody of the IgG2a class, produced by hybridoma 19/178 (25) was provided by Dr. U. Hammerling (Memorial Sloan-Kettering Cancer Center, New York, NY). Anti-TCR β mAb, a hamster antibody of the IgG class, produced by hybridoma H57-597 (26), was provided by Dr. R. Kubo (National Jewish Center, Denver, Co.). Anti-TCR δ mAb, a hamster antibody of the IgG class, produced by hybridoma 3A10 (27) was provided by Dr. S. Itohara (M.I.T., Cambridge, MA). Anti-CD3 mAb, a hamster antibody of the IgG class, produced by hybridoma 145-2C11 (28), was provided by Dr. J.A. Bluestone (University of Chicago, Chicago, IL). Anti-thymus leukemia (TL) mAb's were rat antibodies of the IgG2a and IgG2b classes produced by hybridoma 168 and 177, respectively (29). Anti-Qa 2 mAb is a mouse IgM antibody produced by a hybridoma HDQa-2. Anti-Qa 3 mAb is a mouse IgG2a antibody produced by a hybridoma Qa3-59. Anti-H-$2K^d$ mAb is a mouse IgG2a antibody produced by a hybridoma HB159. Anti-H-$2D^d$ mAb is a mouse IgG2a antibody produced by a hybridoma HB102 (30). Both these mAbs were obtained from the American Type Culture Collection (Rockville, MD). Anti–H-$2L^d$ mAb is a mouse IgG2a antibody produced by a hybridoma 30-5-7 (31). H-2^d antibodies used are B6 anti-BALBRL♂ 1, B6 anti-BALB/c methylcholanthrene sarcoma Meth A (BALBMeth A) and B6 anti-BALB/c spleen cells.

Cytofluorometric Analysis. Cells (1–2 × 10^6) were incubated with mAb for 30 min at 4°C. The following mAb's were used: PE-conjugated anti-L3T4 (CD4) mAb (clone GK1.5), FITC-conjugated anti–Lyt-2 (CD8) mAb (clone 53-6.7), and FITC-conjugated anti–Thy-1.2 mAb (clone 30-H12) were obtained from Becton Dickinson & Co. (Mountain View, CA). Biotin-conjugated anti–Thy-1.2 mAb (25) and FITC-conjugated anti-CD3 mAb (145-2C11) were provided by Dr. M. Kawai (Osaka University Medical School, Osaka, Japan). Biotin-conjugated anti-$V_\beta 6$ mAb (44-22-1) (32) and anti-$V_\beta 8$ mAb (F23.1) (33) were provided by Dr. K. Kuribayashi (Kyoto University, Kyoto, Japan). For unconjugated anti-L3T4 (CD4) mAb and anti–Lyt-2.2 (CD8) mAb, FITC-conjugated goat anti-rat IgG and FITC-conjugated goat anti–mouse Ig(G+M) $F(ab')_2$ were used, respectively, as second antibody. For unconjugated anti-TCR β mAb (H57-597), FITC-conjugated goat anti–hamster IgG that had been absorbed with mouse Ig (Caltag, San Francisco, CA) was used as second antibody. PE-conjugated streptavidin (Becton Dickinson and Co.) was used for biotinylated mAb. After treatment, the cells were washed, suspended in PBS, and analyzed in a FACScan (Becton Dickinson and Co.).

Generation of Concanavalin A (Con A) Blast Cells. Spleen cells (2 × 10^7) were cultured with Con A at a concentration of 5 μg/ml for 3 d.

In Vitro Sensitization of Spleen Cells. Mixed lymphocyte tumor cell culture (MLTC) was performed by incubating responder spleen cells (5 × 10^7) with 5 × 10^6 mitomycin C (MMC)-treated BALBRL♂ 1 cells. MMC treatment was done by incubating cells with MMC at a concentration of 100 μg/ml for 30 min at 37°C. The culture medium was RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μM 2-ME. MLTC cells were harvested after 5 or 6 d culture and used as effector cells in assays of cell-mediated cytotoxicity.

Pretreatment of Effector Cells with MAb and Complement (C). Aliquots of MLTC cells (5 × 10^6) or cloned CTL (2 × 10^6) were incubated with mAb (diluted 1:20 with MEM) in a volume of 0.2 ml for 30 min at 4°C. The cells were then washed once and resuspended in 0.75 ml of preselected rabbit serum (diluted 1:10 with MEM) as a source of C. After incubation for 30 min at 37°C, the cells were washed three times before use.

Cell-mediated Cytotoxicity Assay. BALBRL♂ 1 or Con A blasts were labeled with ^{51}Cr by incubating 5 × 10^6 cells with 2 MBq of Na_2CrO_4 (New England Nuclear, Boston, MA) in 0.3 ml of medium for 45 min at 37°C in an atmosphere of 5% CO_2 in air. Then the cells were washed twice with culture medium and used as target cells. In direct assays, 2 × 10^4 labeled target cells (100 μl) were incubated with the effector cell suspension (100 μl). In antibody blocking assays, serially diluted mAb or antiserum (50 μl) was added to the culture of effector (50 μl) and labeled RL♂ 1 target cells (2 × 10^4) (50 μl) during assay. After incubation for 3 h at 37°C, the supernatants (100 μl) were removed and their radioactivity was measured in an Aloka ARC 300 γ counter. The percentage of specific lysis was calculated by the following equation: (a-b/c-b) × 100, where a is the radioactivity in the supernatant of target cells mixed with effector cells, b is that in the supernatant of target cells incubated alone, and c is that in the supernatant after lysis of target cells with NP-40.

Establishment and Maintenance of Cytotoxic T Lymphocyte Clones. Limited numbers of MLTC cells were cultured with 5 × 10^5 irradiated (2,000 rad) syngeneic spleen cells and 5 × 10^3 irradiated (10,000 rad) tumor cells in a volume of 0.2 ml in 96-well flat-bottomed microplates (Corning 25860; Corning Glass Works, Corning, NY). After culture for 14 d, cell growth was assessed microscopically and cytotoxicity was measured using half the culture (0.1 ml). Cells in wells that showed cytotoxicity were transferred to 24-well culture plates (Corning 25820; Corning Glass Works) and maintained by culture with 5 × 10^6 irradiated (2,000 rad) syngeneic spleen cells and 5 × 10^4 irradiated (10,000 rad) tumor cells in medium containing human recombinant IL-2 (Takeda

[1] *Abbreviations used in this paper:* B6, C57BL/6; bm1, B6.C-H-2^{bm1}; bm12, B6.C-H-2^{bm12}; MGS, mean graft survival; MLTC, mixed lymphocyte tumor cell culture; MMC, mitomycin C; TL, thymus leukemia.

Pharmaceutical, Osaka, Japan) at a concentration of 0.1 U/ml. After initial expansion, CTL were recloned and passaged every 5 d by transferring 10^5 cells to new wells of multiwell plates.

Tumor Assay. BALBRLO' 1 cells were harvested in MEM medium and washed twice. The hair of the back of mice was shaved with animal clippers and the tumor cells (2×10^6) were injected i.d. through a 27-gauge needle. Tumor diameters at right angles were measured with vernier calipers to calculate the mean diameter.

Antibody Administration. Mice were anesthetized with ether and 200 μl of antibodies (ascites diluted 1:8 with MEM) was injected through the retrobulbar venous plexus.

We found previously by antibody-mediated complement-dependent cytotoxicity assay that injections of anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb resulted in the elimination from the peripheral lymphoid tissues of $CD4^+$ cells and $CD8^+$ cells, respectively (34). Blocking of the functions of $CD4^+$ cells and $CD8^+$ cells in vivo by mAb treatment was monitored by skin graft rejection in combinations of H-2^b mutant strains and B6. A single injection of anti-L3T4 (CD4) mAb prolonged the mean graft survival (MGS) in B6 mice of B6.C-H-2^{bm12} (bm12) skin (MGS, 32.8 ± 12.1 d:MGS of MEM-treated mice, 12.8 ± 1.2 d [$p < 0.05$]), but not of B6.C-H-2^{bm1} (bm1) skin (MGS, 12.0 ± 1.0 d: MGS of MEM-treated mice, 15.0 ± 0.9 d). On the other hand, anti-Lyt-2.2 (CD8) mAb prolonged the survival in B6 mice of bm1 skin (28.4 ± 9.9 d [$p < 0.05$]), but not of bm12 skin (12.2 ± 0.8 d). In this study, to eliminate the $CD4^+$ or $CD8^+$ cells completely during the experiments, mAb's (25 μl of ascites) were administered every 4 d until tumors regressed.

Adoptive Transfer. Either CD4, or CD4 and CD8-depleted MLTC cells (6×10^6) were adoptively transferred to B6 *nu/nu* mice 5 d before challenge of the mice with BALBRLO' 1 tumor cells. To ensure depletion of $CD4^+$ cells, and of $CD4^+$ and $CD8^+$ cells, the mice were treated with anti-L3T4 (CD4) mAb, and both anti-L3T4 (CD4) and anti-Lyt-2.2 (CD8) mAb, respectively, during tumor assay.

Northern Blot Analysis. Total RNA's from MLTC cells, cell lines, and tumor cells were isolated by the guanidine thiocyanate/CsCl procedure (35). Fractionated RNA was blotted onto nitrocellulose filters (Schleicher & Schuell, Keene, NH) and hybridized with [^{32}P]dCTP-labeled probes in the presence of 50% formamide for 18 h at 45°C as described (36). Filters were washed and exposed to X-ray film (XAR-5; Eastman Kodak, Rochester, NY). The following probes were used: pSP64L3T4 for L3T4 (CD4) (37) (provided by Dr. D. R. Littman, University of California, San Francisco, CA), pLY2C-1 for Lyt-2 (38) (provided by Dr. H. Nakauchi, The Institute of Physical and Chemical Research, Tsukuba, Japan), pclca for TCR α (39) (provided by Dr. T. W. Mak, The Ontario Cancer Institute, Toronto, Canada), PL5 for TCR β (provided by Dr. D. Y. Loh, Washington University School of Medicine, St. Louis, MO), p8/10-2γ 1.1 for TCR γ (40) (provided by Dr. T. W. Mak), and RAD11C for TCR δ (41) (provided by Dr. Y. Yoshikai, Nagoya University School of Medicine, Nagoya, Japan).

Results

Effects of In Vivo Administration of Anti-L3T4 (CD4) MAb and, or Anti-Lyt-2.2 (CD8) MAb on Rejection of BALBRLO' 1 by B6 Mice. Inocula of 2×10^6 BALBRLO' 1 cells in the back of C57BL/6 (B6) mice grew to tumors with a maximum diameter of 7–11 mm on day 6–7, which then regressed and disappeared by day 10–12. The effects of administration of anti-L3T4 (CD4) mAb and, or anti-Lyt-2.2 (CD8) mAb to recipient mice on growth of tumors were investigated. As shown in Fig. 1, administration of either MEM (control), anti-L3T4 (CD4) mAb or anti-Lyt-2.2 (CD8) mAb resulted in rejection, although significant delay was observed in mice treated with anti-Lyt-2.2 (CD8) mAb. Administration of both

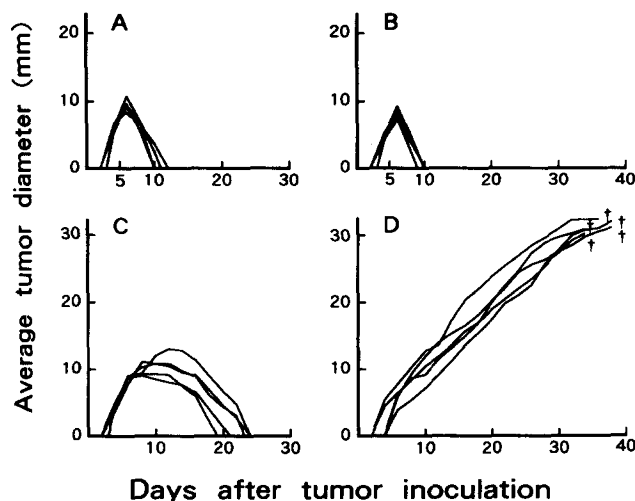

Figure 1. Effects of in vivo administration of MEM (control) (*A*), anti-L3T4 (CD4) mAb (*B*), anti-Lyt-2.2 (CD8) mAb (*C*) and a combination of both (*D*) on rejection of BALBRLO' 1 by B6 mice. Inocula of 2×10^6 BALBRLO' 1 cells were injected i.d., into the back of recipients. Volumes of 0.2 ml of mAb (ascites) (diluted 1:8 with MEM) were injected i.v. every 4 d.

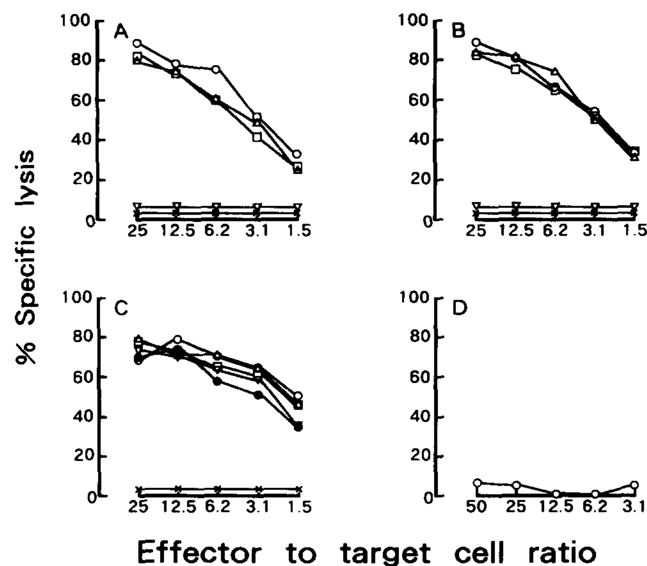

Figure 2. Generation and phenotypic analysis of CTL. Spleen cells (5×10^7) from B6 mice treated with MEM (control) (*A*), anti-L3T4 (CD4) mAb (*B*), anti-Lyt-2.2 (CD8) mAb (*C*) or a combination of both (*D*) obtained 7 d after rejection (*A–C*) or 12 d after inoculation (*D*) of BALBRLO' 1 tumor were cultured with 5×10^6 MMC-treated BALBRLO' 1 in a volume of 20 ml for 6 d. BALBRLO' 1 cytotoxicity on MLTC cells was tested by a 3-h chromium assay. The CTL phenotype was determined by elimination of cytotoxicity by pretreatment with mAb and C before assay. (×) anti-Thy-1.2 mAb; (□) anti-L3T4 (CD4) mAb; (▽) anti-Lyt-2.2 (CD8) mAb; (●) anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb; (△) C alone; (○) untreated.

anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb blocked rejection.

In Vitro Cytotoxic T Lymphocyte Responses of Spleen Cells from MEM (Control), Anti-L3T4 (CD4) MAb, or Anti-Lyt-2.2 (CD8) MAb-Treated B6 Mice that Rejected BALBRL♂ 1. CTL were generated by in vitro stimulation with mitomycin C (MMC)-treated BALBRL♂ 1 cells of spleen cells from B6 mice treated with MEM (control), anti-L3T4 (CD4) mAb or anti-Lyt-2.2 (CD8) mAb that had rejected the BALBRL♂ 1 tumor, but not on similar treatment of spleen cells from B6 mice treated with both mAbs and bearing BALBRL♂ 1 tumor (9–13 mm diameter) obtained 12 d after tumor inoculation (Fig. 2). The cytotoxicity generated in MLTC spleen cells from B6 mice treated with MEM (control) or anti-L3T4 (CD4) mAb was abolished by treatment with anti-Thy-1.2 mAb or anti-Lyt-2.2 (CD8) mAb, but not with anti-L3T4 (CD4) mAb and complement (C). On the other hand, the cytotoxicity generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice was abolished by treatment with anti-Thy-1.2 mAb, but not with anti-L3T4 (CD4) mAb or anti-Lyt-2.2 (CD8) mAb and C.

Specificity Analysis and Antibody Blocking of CTL. The $CD4^-CD8^+$ CTL generated in MLTC spleen cells from MEM (control) or anti-L3T4 (CD4) mAb-treated B6 mice that had rejected BALBRL♂ 1 and the $CD4^-CD8^-$ CTL generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRL♂ 1 showed similar specificities: these CTL lysed target cells that expressed either H-$2K^d$ or H-$2D^d$ (L^d) antigen (Fig. 3). Next we examined the blocking of CTL with antibody. $CD4^-CD8^+$ CTL were blocked by anti-CD3 mAb, anti-TCR β mAb, anti-Lyt-2.2 (CD8) mAb or anti-H-2^d serum, but not by anti-L3T4 (CD4) mAb, as shown by additions of these antibodies to cultures during assays (Fig. 4 *A*). On the other hand, $CD4^-CD8^-$ CTL were blocked by anti-CD3 mAb, anti-TCR β mAb or anti-H-2^d serum, but not by anti-L3T4 (CD4) mAb or anti-Lyt-2.2 (CD8) mAb (Fig. 4 *B*). Anti-H-2^d serum used in these experiments include B6 anti-BALBRL♂ 1, B6 anti-BALBMeth A and B6 anti-BALB/c spleen cells and the effect was essentially similar. No blocking of either type of CTL was observed with the anti-TL mAb, anti-Qa-2 mAb, or anti-Qa-3 mAb.

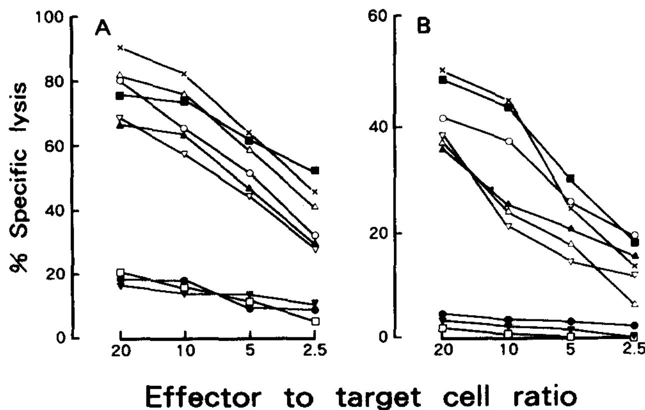

Figure 3. Comparison of specificities of $CD4^-CD8^+$ CTL generated in MLTC spleen cells of B6 mice treated with anti-L3T4 (CD4) mAb that had rejected BALBRL♂ 1 (*A*) and $CD4^-CD8^-$ CTL generated in MLTC spleen cells of B6 mice treated with anti-Lyt-2.2 (CD8) mAb that had rejected BALBRL♂ 1 (*B*). Con A stimulated spleen cells were used as target cells. (▼) B6 (K^bD^b); (■) BALB/c ($K^dD^dL^d$); (□) BALB.B (K^bD^b); (●) C3H/He (K^kD^k); (△) C3H.OH (K^dD^k); (▲) A/J ($K^kD^dL^d$); (×) B10.D2 ($K^dD^dL^d$); (▽) B10.HTG (K^dD^b); (○) B10.A ($K^kD^dL^d$).

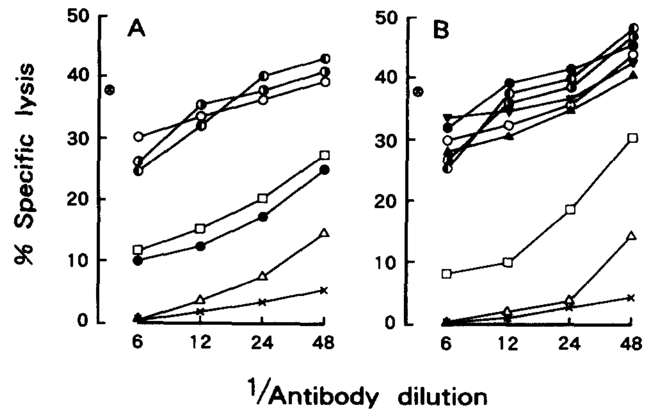

Figure 4. Antibody blocking of $CD4^-CD8^+$ CTL generated in MLTC spleen cells from anti-L3T4 (CD4) mAb-treated B6 mice that had rejected BALBRL♂ 1 (*A*) and $CD4^-CD8^-$ CTL generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRL♂ 1 (*B*). (○) anti-L3T4 (CD4) mAb; (●) anti-Lyt-2.2 (CD8) mAb; (□) anti-TCR β mAb (H57-597); (◑) anti-TL mAb (168); (◐) anti-TL mAb (177); (▲) anti-Qa-2 mAb; (▼) anti-Qa-3 mAb; (×) anti-CD3 mAb (145-2C11); (△) conventional anti-H-2^d serum; (⊗) none. The effector to target cell ratio was 2.5:1 in *A* and 7.5:1 in *B*.

Cytofluorometric Analysis. Cytofluorometric analysis showed that in vivo administration of anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb resulted in depletion of $CD4^+$ and $CD8^+$ cells, respectively, in peripheral lymphoid tissues. Depletion of $CD4^+$ cells in spleen and lymph node cells was shown by a lack of cells that stained with PE anti-L3T4 (CD4) mAb, and by a lack of Thy-1^+ cells that would double label with either FITC anti-rat Ig or anti-L3T4 (CD4) mAb plus FITC anti-rat Ig. Similarly, depletion of $CD8^+$ cells was evident by a lack of cells that stained with FITC rat anti-Lyt-2 (CD8) mAb, and by a lack of Thy-1^+ cells that would double label with either FITC anti-mouse Ig or anti-Lyt-2.2 (CD8) mAb plus FITC anti-mouse Ig.

The populations of $CD4^+$ and $CD8^+$ cells in 6-d cultures of MLTC spleen cells from MEM (control)-treated B6 mice that had rejected BALBRL♂ 1 were approximately 20 and 70%, respectively, of the total cells (Fig. 5 *A*). Scarcely any $CD4^+$ or $CD8^+$ cells were detected in cultures of MLTC spleen cells from B6 mice treated with anti-L3T4 (CD4) mAb or anti-Lyt-2.2 (CD8) mAb that had rejected BALBRL♂ 1 (Fig. 5, *B* and *C*). Since the cytotoxicity generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRL♂ 1 resided mainly in cells with the unusual Thy-1^+ $CD4^-CD8^-$ phenotype, as described above, TCR expression in this cell population was examined. MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice were depleted of both $CD4^+$ and $CD8^+$ cells by mAb

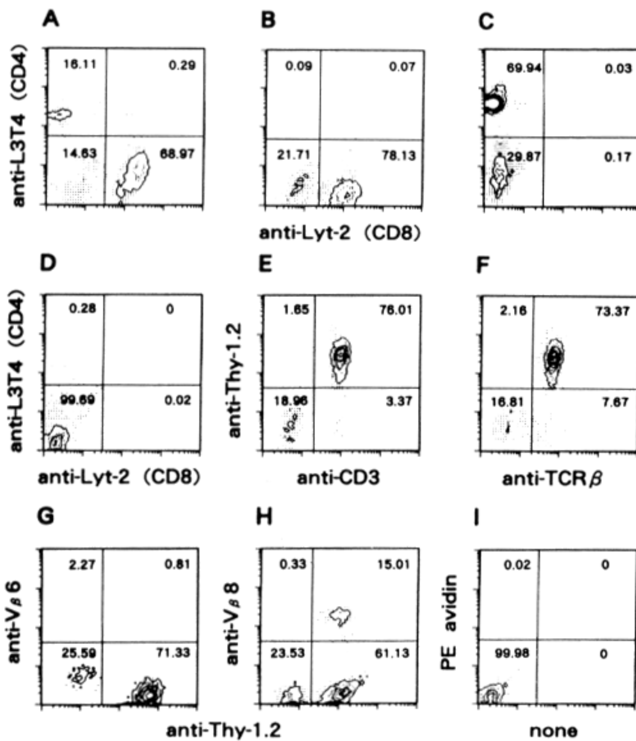

Figure 5. Cytofluorometric analysis. Spleen cells (5×10^7) from MEM (control) (*A*), anti-L3T4 (CD4) mAb (*B*) or anti-Lyt-2.2 (CD8) mAb (*C*)-treated B6 mice that had rejected BALBRL♂ 1 were cultured with 5×10^6 cells of MMC-treated BALBRL♂ 1 in a volume of 20 ml for 6 d. MLTC cells were stained with PE anti-L3T4 (CD4) mAb and FITC anti-Lyt-2 (CD8) mAb. MLTC cells in (*C*) were treated with anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb and C, and stained with PE anti-L3T4 (CD4) mAb and FITC anti-Lyt-2 (CD8) mAb (*D*), biotinylated anti-Thy-1.2 mAb with PE-avidin and FITC anti-CD3 mAb (145-2C11) (*E*) or anti-TCR β mAb (H57-597) (*F*) with FITC anti-hamster IgG, biotinylated anti-Vβ6 mAb (G) or anti-Vβ8 mAb (*H*) with PE avidin and FITC anti-Thy-1.2 mAb. (*I*) PE avidin alone.

and C (Fig. 5 *D*). Thy-1$^+$ CD3$^+$ cells constituted about 70–80% of the total CD4$^-$CD8$^-$ cells in MLTC (Fig. 5 *E*), and most of these were TCR αβ-positive cells (Fig. 5 *F*). TCR Vβ6- and Vβ8-positive cells constituted ~0% and ~20% of the Thy-1 positive cells, respectively (Fig. 5, *G* and *H*).

Rejection of BALBRL♂ 1 Inoculated into B6 nu/nu Mice by Adoptive Transfer of CD4$^-$CD8$^-$ Cells Obtained from Anti-Lyt-2.2 (CD8) MAb-Treated B6 Mice that Had Rejected BALBRL♂ 1. MLTC spleen cells from anti-L3T4 (CD4) mAb-treated B6 mice that had rejected BALBRL♂ 1 were treated with anti-L3T4 (CD4) mAb and C, and 6×10^6 cells were adoptively transferred to B6 *nu/nu* mice 5 d before challenge of the mice with BALBRL♂ 1 tumor. In these mice, BALBRL♂ 1 grew to a tumor, and then regressed (Fig. 6 *A*). For examination of the in vivo effector activity of Thy-1$^+$ CD4$^-$CD8$^-$ CTL, MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRL♂ 1 were treated with both anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb and C, and the same number of cells were similarly transferred to B6 *nu/nu* mice. To re-

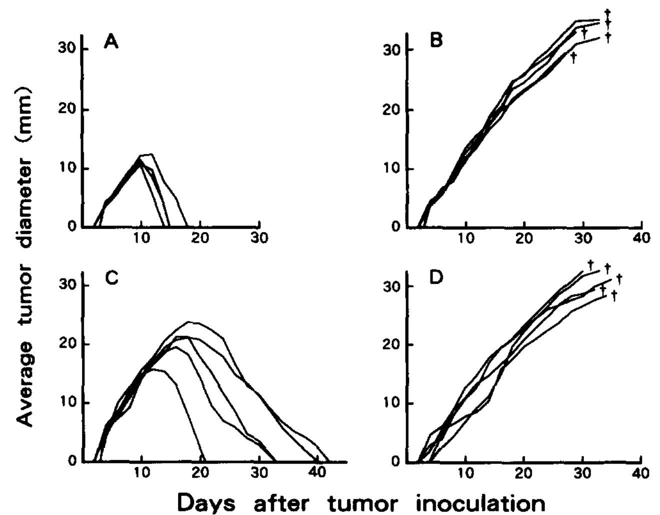

Figure 6. Adoptive transfer experiments. B6 *nu/nu* mice were injected i.p. with 6×10^6 MLTC spleen cells from anti-L3T4 (CD4) mAb-treated B6 mice that had rejected BALBRL♂ 1, which were depleted of L3T4 (CD4)$^+$ cells (*A* and *B*), and from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRL♂ 1, which were depleted of both L3T4 (CD4)$^+$ and Lyt-2.2 (CD8)$^+$ cells (*C* and *D*), 5 d before challenge with BALBRL♂ 1 tumor. Anti-L3T4 (CD4) mAb (*A* and *B*), and a combination of both anti-L3T4 (CD4) and anti-Lyt-2.2 (CD8) mAb (*C* and *D*) were administered to recipient B6 *nu/nu* mice to ensure depletion on days −5, 0, 10, 20, and 30. Anti-Lyt-2.2 (CD8) mAb (*B*) or anti-TCR β mAb (H57-597) (*D*) was administered on days 0, 10, 20, and 30.

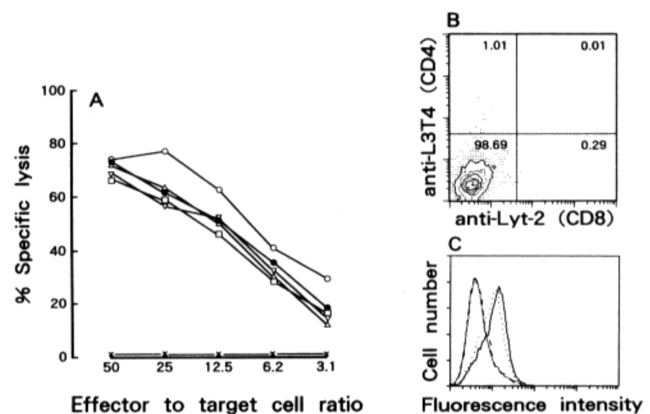

Figure 7. Spleen cells (5×10^7) from B6 *nu/nu* mice that had received adoptive transfer of CD4$^-$CD8$^-$ cells (shown in Fig. 6 *C*) were obtained 7 d after rejection of BALBRL♂ 1 and were cultured with 5×10^6 MMC-treated BALBRL♂ 1 in a volume of 20 ml for 6 d. BALBRL♂ 1 cytotoxicity was tested by a 3-h chromium assay. The CTL phenotype was determined by pretreatment with mAb and C before assay (*A*). (×) anti-Thy-1.2 mAb; (□) anti-L3T4 (CD4) mAb; (▽) anti-Lyt-2.2 (CD8) mAb; (●) anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb; (△) C alone; (○) no treatment. MLTC cells were depleted of L3T4 (CD4)$^+$ and Lyt-2.2 (CD8)$^+$ cells by treatment with mAb and C, and stained with PE anti-L3T4 (CD4) mAb and FITC anti-Lyt-2 (CD8) mAb (*B*) and anti-TCR β mAb (H57-597) (——), anti-TCR δ mAb (3A10) (······) or anti-CD3 mAb (145-2C11) (· · · ·) with FITC anti-hamster IgG (C). (– – –) FITC anti-hamster IgG alone.

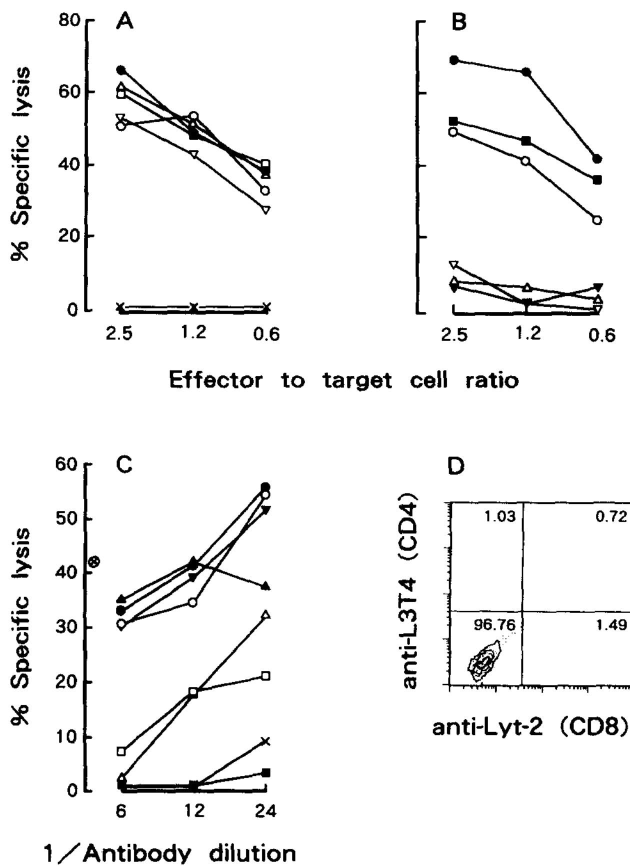

Figure 8. Elimination (*A*), direct cytotoxicity (*B*), antibody blocking (*C*), and cytofluorometric analysis (*D*) of CTL clone S 1. In *A*, S 1 cells were pretreated with mAb's and C before cytotoxicity assay. The target cells were BALBRLơ 1. (×) anti-Thy-1.2 mAb; (□) anti-L3T4 (CD4) mAb; (▽) anti-Lyt-2.2 (CD8) mAb; (●) anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb; (△) C alone; (O) untreated. In *B*, Con A stimulated spleen cells were used as target cells. (▼) B6 (K^bD^b); (■) BALB/c ($K^dD^dL^d$); (△) C3H.OH (K^dD^k); (▽) B10.HTG (K^dD^b); (O) B10.A (5R) ($K^bD^dL^d$); (●) B10.AQR ($K^qD^dL^d$). In *C*, the antibodies used for blocking were (O) anti-L3T4 (CD4) mAb; (●) anti-Lyt-2.2 (CD8) mAb; (□) anti-TCR β mAb (H57-597); (▲) anti-H-2K^d mAb (HB159); (▼) anti-H-2D^d mAb (HB102); (■) anti-H-2L^d mAb (30-5-7); (×) anti-CD3 mAb (145-2C11); and (△) conventional anti-H-2^d serum. (O) none. The effector to target cell ratio was 1:1. In *D*, S1 cells were stained with PE anti-L3T4 (CD4) mAb and FITC anti-Lyt-2 (CD8) mAb.

duce the possibility of involvement of CD4$^+$ and CD8$^+$ cells that escaped from depletion and proliferated, we added both anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb during tumor assay. Adoptive transfer of CD4$^-$CD8$^-$ cells resulted in rejection of BALBRLơ 1 tumors, although less efficiently than adoptive transfer of a CD4$^-$CD8$^+$ population (Fig. 6 *C*). Administration of anti-TCR β mAb (H57-597) abolished rejection by the CD4$^-$CD8$^-$ population, confirming the TCR $\alpha\beta$ phenotype of the effector cells (Fig. 6 *D*). Administration of anti-L3T4 (CD4) mAb and/or anti-Lyt-2.2 (CD8) mAb, or anti-TCR β mAb (H57-597) alone had no effect on BALBRLơ 1 tumor growth in B6 *nu/nu* mice. Thy-1$^+$ CD4$^-$CD8$^-$ CTL were recovered from MLTC spleen cells of B6 *nu/nu* mice that had rejected BALBRLơ 1 after adoptive transfer of a CD4$^-$CD8$^-$ population (Fig. 7).

Establishment of CD4$^-$CD8$^-$ TCR $\alpha\beta$ CTL Clones. MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRLơ 1 were treated with anti-L3T4 (CD4) mAb plus anti-Lyt-2.2 (CD8) mAb and C. The residual cells were plated at 1 cell/well in 96-well plates and cultured with irradiated BALBRLơ 1 and B6 spleen cells. In this way, CTL clones S 1 and 2 were obtained from individual mice. Both these clones were found to be Thy-1$^+$CD4$^-$CD8$^-$TCR$\alpha\beta^+$V$_\beta$6$^-$V$_\beta$8$^-$ by cytofluorometry and were specific for L^d (Fig. 8).

Northern Blot Analysis. The expression of L3T4 (CD4), Lyt-2 (CD8) and TCR genes in the MLTC spleen cells and the CTL clones S 1 and 2 were analyzed by Northern blotting. Transcripts of the L3T4 (CD4) and Lyt-2 (CD8) genes were detected in MLTC spleen cells from MEM-treated B6 mice that had rejected RLơ 1, but no transcripts of the Lyt-2 (CD8) gene were detected in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRLơ 1 (Fig. 9). Transcripts of the TCR α, β, and γ genes, but not of the TCR δ, L3T4 (CD4) or Lyt-2 (CD8) genes, were detected in both S1 and 2 clones.

Discussion

In the present study, we demonstrated that MHC class I specific CD4$^-$CD8$^-$ TCR $\alpha\beta$ CTL were generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice obtained after rejection of allogeneic BALBRLơ 1. CD4$^-$CD8$^-$ CTL were involved in mediating rejection of the tumor in these mice.

Inocula of BALBRLơ 1 were rejected in B6 mice treated with MEM (control), anti-L3T4 (CD4) mAb or anti-Lyt-2.2 (CD8) mAb and CTL against the tumor were generated in vitro. No rejection and no induction of CTL were observed in B6 mice treated with both anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) mAb. CTL with the classical Thy-1$^+$CD3$^+$CD4$^-$CD8$^+$ phenotype was generated in MLTC spleen cells from B6 mice treated with MEM (control) or anti-L3T4 (CD4) mAb, whereas CTL with an unusual Thy-1$^+$CD3$^+$CD4$^-$CD8$^-$ phenotype were generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice. The CD4$^-$CD8$^-$ phenotype of CTL was shown by elimination and blocking of cytotoxicity. Pretreatment of MLTC cells with anti-Thy-1 mAb, but not anti-L3T4 (CD4) mAb or anti-Lyt-2 (CD8) mAb and C abolished the cytotoxicity. Addition of anti-CD3 mAb, but not anti-L3T4 (CD4) mAb or anti-Lyt-2.2 (CD8) mAb blocked the cytotoxicity in the absence of added C. No difference was observed in the specificities of bulk CTL with the CD4$^-$CD8$^+$ and CD4$^-$CD8$^-$ phenotypes: both were reactive with either H-2K^d or D^d (L^d). These findings suggest that when CD4$^+$ cells were blocked by anti-L3T4 (CD4) mAb, CD8$^+$ CTL mediated rejection efficiently, and when CD8$^+$ cells were blocked by anti-Lyt-2.2 (CD8) mAb, CD4$^+$ cells were capable of mediating rejection, although less efficiently than CD8$^+$ cells, by inducing CD4$^-$CD8$^-$ CTL.

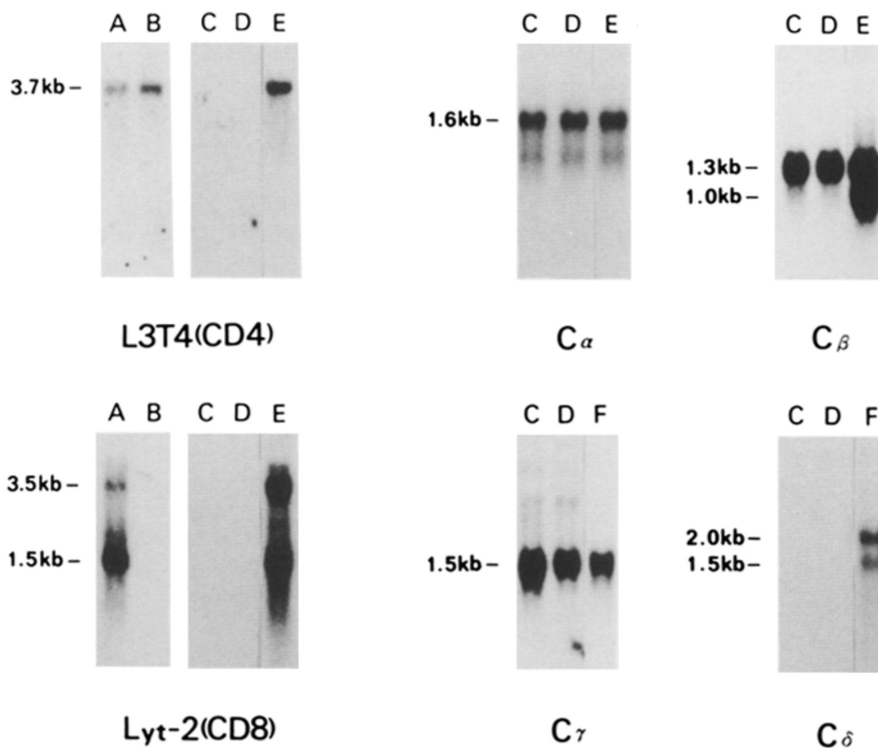

Figure 9. Northern blot analysis. mRNAs were obtained from MLTC spleen cells from MEM (control)-treated B6 mice that had rejected BALBRL♂ 1 (*A*), MLTC spleen cells from anti–Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRL♂ 1 (*B*), clone S 1 cells (*C*), clone S 2 cells (*D*), B6LK leukemia cells (TCR $\alpha\beta$ $CD4^+CD8^+$) (*E*) and K4D11 leukemia cells (TCR $\gamma\delta$ $CD4^-CD8^-$) (*F*). The DNA probes used were pSP64L3T4 for L3T4 (CD4), pLy2C-1 for Lyt-2 (CD8), pclca for TCR α, PL5 for TCR β, p8/10-2γ1.1 for TCR γ, and RAD11C for TCR δ.

The $CD4^-CD8^-$ CTL demonstrated in this study use TCR $\alpha\beta$ but not $\gamma\delta$. Anti-TCR β mAb (H57-597) blocked the in vitro cytotoxicity of $CD4^-CD8^-$ CTL and tumor rejection in anti–Lyt-2.2 (CD8) mAb-treated B6 mice (data not shown) or B6 *nu/nu* mice after adoptive transfer of $CD4^-CD8^-$ CTL. Cytofluorometric analysis revealed that Thy-1^+ cells from anti–Lyt-2.2 (CD8) mAb-treated B6 mice contained approximately 70–80% of CD4- and CD8-depleted MLTC cells and that most of the cells were TCR $\alpha\beta$-positive. Vβ6- and Vβ8-positive cells constituted ~0% and ~20% of the Thy-1^+ cells, their levels being similar to those in normal B6 thymocytes (42). The CTL clones S 1 and 2 with the Thy-$1^+CD3^+CD4^-CD8^-$ TCR $\alpha\beta$ phenotype were established from these MLTC cells.

The thymus was found to contain less than 1% of T cells with the $CD4^-CD8^-$ TCR $\alpha\beta$ phenotype (1). The finding that these cells respond to various stimuli and produce IL-2 (7) suggests that thymocytes with the $CD4^-CD8^-$TCR $\alpha\beta$ phenotype are mature. Furthermore, clonal deletion has been shown to occur in these cells (8, 18). Although thymocytes with this phenotype have been defined, no counterpart has been detected in the peripheral lymphoid tissues of normal mice. However, Guidos et al. (21) recently demonstrated that $V_\beta8^+$ cells are present in a $CD3^+CD4^-CD8^-$ population of lymph node cells. The present study demonstrated that $CD4^-CD8^-$ TCR $\alpha\beta$ T cells could be functionally involved in immune responses. The in vivo administration of anti-L3T4 (CD4) mAb with anti–Lyt-2.2 (CD8) mAb abolished both BALBRL♂ 1 rejection and the in vitro CTL response. These findings indicate that $CD4^+$ cells were required for generation of the $CD4^-CD8^-$ TCR $\alpha\beta$ T cell response, probably as helper cells. The effector cell mechanisms of allograft or tumor rejection mediated by $CD4^+$ cells are unknown (43, 44). One possible mechanism could be CD4-dependent induction of $CD4^-CD8^-$ TCR $\alpha\beta$ effector cells. In this regard, it is noteworthy that the accumulation of $CD4^-CD8^-$ $\alpha\beta$ cells observed in certain autoimmune mice has been shown to be dependent on $CD4^+$ cells. Treatment of MRL-*lpr/lpr* mice with anti-L3T4 (CD4) mAb prevented accumulation of $CD4^-CD8^-$ TCR $\alpha\beta$ T cells and the development of autoimmune disease (45). This finding suggests that $CD4^-CD8^-$ TCR $\alpha\beta$ cells that are normally present in these mice are somehow stimulated to proliferate by activation of $CD4^+$ cells. The fact that $CD4^-CD8^-$ cells in enlarged lymph nodes undergo clonal deletion suggests that these cells are derived from normal counterparts in the thymus (8, 18). Class I specific $CD4^-CD8^-$ $\alpha\beta$ CTL clones have been generated from lymph node cells from *gld* mice (46).

$CD4^-CD8^-$ effector cells were less efficient than $CD4^-CD8^+$ effector cells in mediating tumor rejection. Significant delay of BALBRL♂ 1 tumor rejection was observed in anti–Lyt-2.2 (CD8) mAb-treated B6 mice compared with that in MEM (control) or anti-L3T4 (CD4) mAb-treated B6 mice, and also in B6 *nu/nu* mice after adoptive transfer of CD4 and CD8-depleted MLTC spleen cells obtained from anti–Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALBRL♂ 1 compared with that in mice after adoptive transfer of CD4-depleted MLTC spleen cells obtained from anti-L3T4 (CD4) mAb-treated B6 mice. The exact reason for the difference between the efficiencies of $CD4^-CD8^-$ and $CD4^-CD8^+$ cells is unknown. $CD4^-CD8^-$ effector cells could be less efficient than $CD4^-CD8^+$ effector cells. Alternatively, the difference could be due to a lower number of $CD4^-CD8^-$ TCR $\alpha\beta$ CTL precursors.

$CD4^-CD8^+$ effector cells were stimulated predominantly and no significant involvement of $CD4^-CD8^-$ $\alpha\beta$ CTL was observed in MEM (control)-treated mice. Preferential stimulation of $CD4^-CD8^+$ effector cells could be explained by supposing that MHC class I antigen expressed on the BALBRLꝯ 1 tumor stimulated $CD4^-CD8^+$ $\alpha\beta$ CTL preferentially to $CD4^-CD8^-$ $\alpha\beta$ CTL, although the same antigen could be a ligand for both types of CTL. Preferential stimulation could simply be due to a much larger precursor pool of $CD4^-CD8^+$ CTL, as discussed above. Alternatively, $CD4^-CD8^+$ cells might suppress generation of $CD4^-CD8^-$ $\alpha\beta$ T cells. This problem requires further investigation.

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