CD4−CD8− T Cell Receptor αβ T Cells: Generation of an In Vitro Major Histocompatibility 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) αβ cytotoxic T lymphocytes (CTL) and their allogeneic tumor rejection were investigated. Inocula of BALB/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-1+CD3+CD4−CD8+ TCR αβ 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-1+CD3+CD4−CD8+ TCR αβ 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-2Kd and Dd (Ld) 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 αβ 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 BALB/1, resulted in rejection of BALB/1 inoculated into B6 nu/nu mice confirmed the above notion. CTL clones with the CD4−CD8− TCR αβ phenotype specific for Ld were established.

Thymocytes with the CD4−CD8− phenotype constitute about 5% of all cells in the thymus of adult mice (1). The TCR αβ-bearing subset constitutes 2–30% of the CD4−CD8− thymocytes in different mouse strains and displays a CD3+, HSA−, CD5high, IL-2R+, Pgp-1+, B220−, and Qa-2+ phenotype (2–6). Although these cells appear to have a functional TCR (7), the origin and developmental pathway of CD4−CD8− TCR αβ cells is currently unknown. Recently, however, it was shown that Vβ clonal deletion took place in CD4−CD8− TCR αβ thymocytes from mice with Msb+ and Msb− 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 αβ 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 αβ cells are found in the thymus, these cells are rarely detected in peripheral lymphoid tissues. However, CD4−CD8− TCR αβ 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 αβ cells in these mice also undergo Vβ clonal deletion (8, 18) and thymectomy prevents disease development (19, 20). These findings suggest that CD4−CD8− TCR αβ 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 αβ
CTL response specific for both H-2K<sup>d</sup> and D<sup>d</sup> (L<sup>d</sup>) class I antigen was generated in C57BL/6 (B6)<sup>1</sup> mice that had been treated with anti-Lyt-2.2 (CD8) mAb and had rejected the BALB/c<sup>1</sup> 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 (SR), 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/c mice were provided by Dr. H. Fujisawa (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. BALB/c<sup>1</sup> is a radiation-induced leukemia (22). B6Lk is a nitrosourea-induced thymic leukemia that expresses TCR αβ, CD4, and CD8 on the cell surface. K4D11 is a T cell lymphoma that developed in a C3H Tg Tla<sup>-3-1</sup> transgenic mouse. 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. Hamerling (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. Kudo (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. I.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 HDQα-2. Anti-Qa 3 mAb is a mouse IgG2a antibody produced by hybridoma Qa3-59. Anti-H-2K<sup>d</sup> mAb is a mouse IgG2a antibody produced by hybridoma HB159. Anti-H-2D<sup>d</sup> mAb is a mouse IgG2a antibody produced by a hybridoma HB102. Both these mAbs were obtained from the American Type Culture Collection (Rockville, MD). Anti-H-2L<sup>d</sup> mAb is a mouse IgG2a antibody produced by a hybridoma 30-5-7 (31). H-2<sup>d</sup> antibodies used are B6 anti-BALB/c<sup>1</sup> and B6 anti-BALB/c<sup>1</sup> spleen cells.

Cytotoxicity Assay. 1 (2<sup>1</sup>–10<sup>5</sup>) 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 3H-82) 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-Vα6 mAb (44-22-1) (32) and anti-V<sub>β</sub>8 mAb (F23.1.1) (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 IgG (G+M) F(ab<sup>2</sup>)<sub>2</sub> 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<sup>1</sup>–10<sup>5</sup>) 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<sup>1</sup>–10<sup>5</sup>) with 5<sup>10</sup> 10<sup>5</sup> mitomycin C (MMC)-treated BALB/c<sup>1</sup> 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 μM 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 MAbs and Complement (C). Aliquots of MLTC cells (5<sup>1</sup>–10<sup>5</sup>) or Con A blasts were labeled with 3<sup>1</sup>Cr by incubating 5<sup>1</sup>–10<sup>5</sup> cells with 2 MBq of Na<sub>2</sub>CrO<sub>4</sub> (New England Nuclear, Boston, MA) in 0.3 ml of medium for 45 min at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. Then the cells were washed twice with culture medium and used as target cells. In direct assays, 2<sup>1</sup>–10<sup>5</sup> 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<sup>1</sup> target cells (2<sup>1</sup>–10<sup>5</sup>) (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/(a-c-b)) x 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<sup>10</sup> 10<sup>5</sup> irradiated (2,000 rad) syngeneic spleen cells and 5<sup>10</sup> 10<sup>5</sup> 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<sup>10</sup> 10<sup>5</sup> irradiated (2,000 rad) syngeneic spleen cells and 5<sup>10</sup> 10<sup>5</sup> irradiated (10,000 rad) tumor cells in medium containing human recombinant IL-2 (Takeda
Pharmaceutical, Osaka, Japan) at a concentration of 0.1 U/ml. After initial expansion, CTL were recloned and passaged every 5 d by transferring 10^6 cells to new wells of multiwell plates.

**Tumor Assay.** BALB/c(Lo') 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^d^ 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^b^ (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^d^ (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 BALB/c(Lo') 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 [γ-^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), pI.Y2C-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), p8/10-2y1.1 for TCR y (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 BALB/c(Lo') 1 by B6 Mice.** Inocula of 2 × 10^6 BALB/c(Lo') 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...
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 BALB/c 1. CTL were generated by in vitro stimulation with mitomycin C (MMC)-treated BALB/c 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 BALB/c 1 tumor, but not on similar treatment of spleen cells from B6 mice treated with both mAbs and bearing BALB/c 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 BALB/c 1 and the CD4 -CD8 - CTL generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALB/c 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 α 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, 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 B). Anti-H-2 d serum used in these experiments included B6 anti-BALB/c, B6 anti-BALB/Meth 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.

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-Thy-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 BALB/c 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-Lyt-2.2 (CD8) mAb that had rejected BALB/c 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 BALB/c 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 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 included B6 anti-BALB/c 1, B6 anti-BALB/Meth 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.
Cytotoxicity analysis. Spleen cells (5 x 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 BALB/c (71 were cultured with 5 x 10^6 cells of MMC-treated BALB/c 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-TCRβ 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 BALB/c 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 BALB/c 1. MLTC spleen cells from anti-L3T4 (CD4) mAb-treated B6 mice that had rejected BALB/c 1 were treated with anti-L3T4 (CD4) mAb and C, and 6 x 10^6 cells were adoptively transferred to B6 nu/nu mice 5 d before challenge of the mice with BALB/c 1 tumor. In these mice, BALB/c 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 BALB/c 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 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 BALB.RL° 1. (△) anti-Thy-1.2 mAb; (□) anti-L3T4 (CD4) mAb; (○) anti-Lyt-2.2 (CD8) mAb and anti-Ly-2.2 (CD8) mAb; (Δ) C alone; (O) untreated. In B, Con A stimulated spleen cells were used as target cells. (V) B6 (Kbd); (■) BALB/c (KbdLd); (△) C3H.OH (Kbd); (○) B10.A (Kbd); (□) B10.AQR (Kbd). In C, the antibodies used for blocking were (O) anti-L3T4 (CD4) mAb; (△) anti-Lyt-2.2 (CD8) mAb; (□) anti-TCR a mAb (H57-597); (○) anti-H-2Kd mAb (HB159); (V) anti-H-2Ld mAb (HB102); (●) anti-H-2Ld nAb (30-5-7); (△) anti-CD3 mAb (145-2C11); and (Δ) conventional anti-H-2d 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.

Discussion

In the present study, we demonstrated that MHC class I specific CD4+CD8− TCR aβ CTL were generated in MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice obtained after rejection of allogeneic BALB.RL° 1. CD4+CD8− CTL were involved in mediating rejection of the tumor in these mice.

Inocula of BALB.RL° 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+CD4+CD8+ phenotype were 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-2Kd or Dd (L). 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.

BALB.RL° 1 after adoptive transfer of a CD4−CD8− population (Fig. 7).

Establishment of CD4−CD8− TCR aβ CTL Clones. MLTC spleen cells from anti-Lyt-2.2 (CD8) mAb-treated B6 mice that had rejected BALB.RL° 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 BALB.RL° 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 aβ+Vα6+Vβ8− by cytofluorometry and were specific for Ld (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 RLC° 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 BALB.RL° 1 (Fig. 9). Transcripts of the TCR a, b, and γ genes, but not of the TCR δ, L3T4 (CD4) or Lyt-2 (CD8) genes, were detected in both S1 and 2 clones.
The CD4-CD8- CTL demonstrated in this study use TCR αβ but not γδ. 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 αβ-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 S1 and 2 with the Thy-1+CD3+CD4-CD8+ TCR αβ phenotype were established from these MLTC cells.

The thymus was found to contain less than 1% of T cells with the CD4-CD8- TCR αβ phenotype (1). The finding that these cells respond to various stimuli and produce IL-2 (7) suggests that thymocytes with the CD4-CD8- TCR αβ 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 Vβ8+ cells are present in a CD3+CD4-CD8- population of lymph node cells. The present study demonstrated that CD4-CD8- TCR αβ 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 BALB.Ly1-1 rejection and the in vitro CTL response. These findings indicate that CD4+ cells were required for generation of the CD4-CD8- TCR αβ 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 αβ effector cells. In this regard, it is noteworthy that the accumulation of CD4-CD8- TCR αβ 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 αβ T cells and the development of autoimmune disease (45). This finding suggests that CD4-CD8- TCR αβ 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- αβ 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 BALB.Ly1-1 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 BALB.Ly1-1 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 αβ CTL precursors.
CD4−CD8+ effector cells were stimulated predominantly and no significant involvement of CD4−CD8+ αβ 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 BALB/c 1 tumor stimulated CD4−CD8+ αβ CTL preferentially to CD4−CD8− αβ 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− αβ T cells. This problem requires further investigation.

We thank Ms. M. Kobayashi and Ms. H. Hasegawa for expert technical assistance. We also thank members of the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine, for care of mice.

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Received for publication 3 October 1990 and in revised form 2 April 1991.

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