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Sequential T Cell Response Involved in Tumor Rejection of Sarcoma, Meth A, in Syngeneic Mice

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We investigated the type of T cell response involved in Meth A tumor rejection in primary immune and hyperimmune syngeneic mice. It was found that a CD4$^+$ T cell-mediated delayed-type hypersensitivity (DTH) response activating non-specific killer cells such as macrophages, NK and LAK cells, without a specific CD8$^+$ cytotoxic T lymphocyte (CTL) response, was the major immune response leading to Meth A tumor rejection in primary immune mice. In contrast, the specific CD8$^+$ CTL response was the major response leading to the tumor rejection, in addition to CD4$^+$ T cell-mediated DTH response, in hyperimmune mice. Analysis of CD4$^+$ T cell clones established from primary immune and hyperimmune spleen cells indicated that a CD4$^+$ T cell clone (C9) of primary immune mice (although only one clone was established) was of Th1 type, and induced cytotoxicity in accessory cells by classic DTH *in vitro*. Eight CD4$^+$ T cell clones were established from hyperimmune spleen cells. Six out of the eight clones were of the Th2 type and two were Th0-like. However, no Th1-type CD4$^+$ T cell clone was established from hyperimmune spleen cells. All of these CD4$^+$ T cell clones, even the Th2-type clones, were capable of inducing cytotoxicity *in vitro* in T cell-depleted accessory cells, as in an *in vitro* DTH response. We postulate on the basis of these results that the T cell response leading to Meth A tumor rejection *in vivo* sequentially changed from a CD4$^+$ T cell-mediated classic DTH response to a CD8$^+$ CTL response, in addition to a cellular response mediated probably by Th2-type cells, during the process of repeated immunization.

Key words: T cell response — Meth A tumor rejection — CD4$^+$ T cell clones

The chemically induced murine sarcoma Meth A raised in a BALB/c mouse[1] has been well studied by many investigators.[2–10] Meth A can be rejected by syngeneic immune mice which have been prepared by repeated immunization with attenuated Meth A tumor cells.[1, 3, 7, 11, 12] It is believed that such rejection of syngeneic tumors is mainly due to cell-mediated immunity, in particular, activation of CD8$^+$ cytotoxic T lymphocytes (CTL). Recently, owing to the development of molecular biology techniques, so-called tumor rejection antigens (TRA) capable of inducing anti-tumor resistance in mice[4, 5, 7, 13] have been purified, and furthermore tumor antigenic peptides recognized by CTL clones have been sequenced, even in Meth A sarcoma.[4, 5, 7] However, the type of T cell response which causes tumor rejection in immune syngeneic mice has not been analytically characterized in detail. We describe here the type of T cell responses to Meth A involved in tumor rejection in primary immune and hyperimmune syngeneic mice.

MATERIALS AND METHODS

Animals Six- to 8-week-old BALB/c female mice were used for these experiments. These mice were propagated in our animal breeding facility.

Tumors Chemically induced sarcoma, Meth A raised in a BALB/c mouse, was a gift of Dr. T. Toko at the Institute of Taiho Pharmaceutical Ltd. (Tokushima). Methylcholanthrene-induced sarcoma, MS61391c was induced in a BALB/c mouse in our lab. Other methylcholanthrene-induced sarcomas, MS922BR, MS1114D2 and MS829AY, were raised in B10.BR, B10.D2 and A.BY mice, respectively, in our lab. Those chemically induced murine sarcoma cells were adapted to grow *in vitro* in stationary culture with RPMI1640 medium (Life Technologies, Inc., Long Island, NY) supplemented with 5% fetal calf serum (FCS, Filtron, Brooklyn, NY) and 100 μg/ml of Kanamycin (Meiji Seika Co., Ltd., Tokyo); this is referred to as complete RPMI medium.

Antibodies Hamster anti-CD3 (145-2C11), rat anti-CD4 (L3T4a, GK1.5), anti-CD8 (Lyt-2.2, 3.155 and 2.43), anti-IL-2 (S4B6), and anti-IL-4 (11B11), and mouse anti-I-A^d (25-9-17), and anti-I-E^d (31-1-4) monoclonal antibodies (mAbs) were kind gifts from Dr. N. Shinohara (Cellular Immunology Division, Mitsubishi Life Science Institute, Kanagawa). Hamster anti-TCRβ (H57-597), rat anti-CD45RA (14.8), anti-CD45RB (MB23G2), anti-ICAM-1 (YN1/17.4), anti-VLA-4 (PS/2), anti-LFA-1 (FD441.8), anti-IFN-γ (R4-6A2), and anti-mouse NK cell subset (4D11) mAbs were purchased from American Type Cul-

ture Collection (ATCC, Rockville, MD). Mouse anti-Thy-1.2 (HO-13-4) mAb was a gift from Dr. H. Ishikawa (Department of Microbiology, Keio University, Tokyo). Rat anti-CD44 (KM114) mAb was kindly supplied by Dr. A. Tominaga (Department of Biology, Kochi Medical School, Kochi). Anti-IFN-γ (XMG1.2) mAb was purchased from Endogen Inc. (Woburn, MA). These antibodies were purified by Protein G column (Pharmacia, Uppsala, Sweden) chromatography, as described in our previous report.[14]

Preparation of animals immune to Meth A Primary immune mice were prepared by the surgical removal of growing Meth A tumors (diameter: about 1 cm) which had been inoculated into normal syngeneic mice 10 days before. About 2 weeks later, these mice were confirmed to be immune on the basis of their ability to reject a 1×10^6 cell rechallenge with Meth A. Hyperimmune mice were prepared by repeatedly rechallenging primary immune mice with 1×10^6 live Meth A tumor cells, 4 times at 10-day intervals.

Preparation of accessory cells Accessory cells were prepared as T cell-depleted splenocytes from either normal or tumor-bearing mice. The T cell-depleted splenocytes were prepared by the treatment of spleen cells with anti-Thy-1.2 mAb and rabbit complement. One hundred million spleen cells/ml were treated with 10 μg/ml of anti-Thy-1.2 mAb for 30 min at room temperature, washed with phosphate-buffered saline (PBS) once, and then incubated with 1/10 rabbit complement for 60 min at 37°C. The cells were washed with PBS, then resuspended at the desired concentration in complete RPMI medium, and exposed to 30 Gy of γ-irradiation. The T cell-depleted splenocytes from normal and tumor-bearing mice were used as normal accessory cells (N-AC) and tumor-bearing host accessory cells (TBH-AC) for *in vitro* experiments.

***In vivo* treatment of immune mice with monoclonal antibodies to T cell subsets** In order to deplete $CD4^+$ or/and $CD8^+$ cells from either primary immune or hyperimmune mice at Meth A tumor cell challenge, these mice were intraperitoneally given 0.5 mg per mouse of rat anti-CD4 (GK1.5) and/or of rat anti-$CD8^+$ (2.43) mAbs four times, on the day before a tumor cell challenge (day −1), at the time of the tumor challenge (day 0), and on the first day (day 1) and the third day (day 3) after the tumor challenge. Total depletion of the $CD4^+$ and/or $CD8^+$ cell population in spleen cells of treated immune mice selected from each group was confirmed by flow cytometric analyses. The Meth A tumor cell challenge was performed by subcutaneous inoculation of 1×10^6 cells into the center of the backs of these immune mice. Tumor size was measured with a vernier caliper in terms of two diameters at right angles. Tumor growth was expressed as tumor area (mm^2). Normal rat IgG was used as a control for these rat monoclonal antibodies.

Adoptive cell transfer To detect *in vivo* anti-tumor activity of immune spleen cells, immune spleen cells were prepared from either primary immune or hyperimmune mice 10 days after final immunization. Twenty million primary immune or hyperimmune mouse spleen cells were transferred i.v. into syngeneic mice a total of 3 times, once every 4 days after s.c. inoculation of 1×10^6 Meth A cells into the back.

Establishment of $CD4^+$ T cell clones To establish Meth A-specific $CD4^+$ T cell clones, $CD8^+$ T cell-depleted spleen cells obtained from either primary or hyperimmune mice were restimulated with mitomycin C (MMC) (Kyowa Hakko Co., Ltd., Tokyo)-treated Meth A cells for five days *in vitro* in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum (FCS), 100 μg/ml of Kanamycin, 5×10^{-5} *M* 2-mercaptoethanol (2-ME) and 20 m*M* HEPES, according to our original method described elsewhere.[14, 15] The activated spleen cells from immune mice were further cultured in RPMI 1640 supplemented with 5% FCS, 5×10^{-5} *M* 2-ME, 100 μg/ml of Kanamycin and concanavalin A (Pharmacia)-stimulated rat spleen cell culture supernatant as a T cell growth factor; this is referred to as maintenance medium. These cells were restimulated with TBH-AC cells at 1/25 of cultured spleen cells at 2–3 week intervals. After a 2-month culture, T cell clones were prepared by the limiting dilution technique, by plating at a cell concentration of 0.3–0.1 cells/well in 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark). This procedure was repeated at least twice to establish T cell clones.

Cell lines The C5.3 T cell clone specific for MS61391c was established from MS61391c-immune BALB/c mouse spleen cells in our lab and is an IL-2- and IL-4-sensitive $CD8^+$ CTL clone. The C5.3 T cell clone was used for bioassay to detect IL-2 and IL-4. L929 was purchased from the Japanese Cancer Research Resources Bank (JCRB, Tokyo) and used for measurement of TNF.

Cell culture supernatants Culture supernatants were harvested from cultures of established Meth A-specific $CD4^+$ T cell clones after stimulation with solid-phase anti-CD3 mAb. $CD4^+$ T cell clones were washed twice and resuspended in a total volume of 4 ml of the complete RPMI medium at a cell concentration of 2×10^6/ml and cultured in 6-well tissue culture plates (Nunc) for 48 h. After culture, the supernatants were harvested by centrifugation at 2000 rpm for 10 min then filtered through a 0.22 μm Millipore filter (Nihon Millipore, Yonezawa). The filtrate was kept at 4°C until use.

Cytotoxicity assay Cytotoxic activity was measured by means of a 16 h ^{51}Cr release assay as described previously.[14, 15] Briefly, 1×10^4 ^{51}Cr-labeled target cells and various numbers of effector cells at different effector/tumor (E/T) ratios in a total volume of 0.2 ml were dis-

tributed to each well of 96-well round-bottomed microplates in quadruplicate and incubated for 16 h at 37°C in 5% CO_2. After centrifugation at 900 rpm for 5 min, a 0.1 ml aliquot of the supernatant was taken from each well, and its radioactivity was counted in a Packard Auto-Gamma 5000 gamma scintillation counter (Meriden, CT). The maximum releasable counts amounted to 85–90% of the total radioactivity incorporated into target tumor cells, as determined by treatment with 1.25% Saponin (Nacalai Tesque, Inc., Kyoto). The spontaneous release from ^{51}Cr-labeled target tumor cells in the wells averaged less than 20% of the maximum release.

Cell proliferation assay Antigen-specific cell proliferation of established $CD4^+$ T cell clones was assayed as follows. $CD4^+$ T cell clones were harvested from culture dishes, washed with PBS three times and then resuspended in the complete RPMI medium. The cells were plated in a 96-well flat-bottomed microplate at a cell concentration of 5×10^4/0.2 ml in each well and 30-Gy-irradiated, T cell-depleted syngeneic Meth A tumor-bearing mouse spleen cells were added as an antigen-presenting cell source at a cell concentration of 5×10^4 cells/0.2 ml/well. As controls, 30-Gy-irradiated, T cell-depleted spleen cells of either normal syngeneic mice or irrelevant tumor-bearing mice were added instead. The cell mixtures were incubated for 36 h at 37°C in a 5% CO_2 atmosphere, and then the cells were pulsed with 0.5 μCi/well of [^{3}H]dThd for a further 12 h. After culture for a total of 48 h, the cells from each well were harvested and washed separately. Their radioactivity was counted on a liquid scintillation counter (LKB-Wallac Betaplate 1205-012, Wallac, Oy, Turku).

Cytokine assay Cytokine production of established $CD4^+$ T cell clones in their culture supernatant after solid-phase anti-CD3 mAb stimulation in culture was detected by the following methods. Interleukin (IL)-2, -4 and tumor necrosis factor (TNF) were detected by bioassay according to the methods described previously.[14] Briefly, the IL-2- and IL-4-dependent cell line C5.3, which was established as a $CD8^+$ CTL clone from BALB/c spleen cells in our lab, was used for detection of IL-2 and IL-4 in terms of inhibition of C5.3 cell proliferation with either anti-mouse IL-2 (S4B6) or anti-mouse IL-4 (11B11) mAbs. Bioassay detection of TNF was performed according to a reported method.[16] Interferon (IFN)-γ in culture supernatants of $CD4^+$ T cell clones was measured using a sandwich ELISA. Briefly, anti-mouse IFN-γ (XMG1.2) mAb was used as the coating mAb and alkaline phosphate-conjugated anti-mouse IFN-γ (R4-6A2) mAb as the detection reagent.

Flow cytometry Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cells were stained with appropriate antibodies. For direct staining, 5×10^5 cells were treated with 100 μl of properly diluted FITC- or biotin-conjugated antibody for 30 min on ice then washed with staining buffer (0.1 *M* PBS containing 2% bovine serum albumin, 0.1% sodium azide). In the case of biotin-conjugated antibody-treated cells, the cells were further treated with properly diluted FITC-conjugated avidin for another 30 min, then washed and resuspended in 500 μl of the staining buffer. For indirect staining, 5×10^5 cells were treated with 100 μl of appropriate primary antibody for 30 min on ice, then washed and stained with 100 μl of properly diluted FITC-conjugated second antibody for another 30 min on ice. Both directly and indirectly stained cells were washed and resuspended in 500 μl of staining buffer and subjected to FACScan analyses.

RESULTS

***In vivo* analysis of T cell subsets involved in tumor rejection in primary immune and hyperimmune mice** To analyze specific T cell subsets involved in Meth A tumor rejection in primary immune and hyperimmune syngeneic mice, growth or rejection of tumor cells inoculated into these immune mice either untreated or treated with monoclonal antibodies to T cell subsets was observed.

In primary immune mice, only tumor cells inoculated into $CD4^+$ cell-depleted immune mice prepared by the i.v. injection of anti-CD4 (GK1.5) mAb were not rejected; the tumor cells were rejected even in immune mice depleted of $CD8^+$ cells by treatment with anti-CD8 (2.43) mAb, as shown in Fig. 1A. These results indicate that the tumor rejection was mainly mediated by $CD4^+$ T cells, and $CD8^+$ T cells were not major effector cells in primary immune mice with the Meth A tumor system.

In hyperimmune mice, however, tumor cells grew in $CD4^+$- as well as $CD8^+$-depleted immune mice prepared by the i.v. injection of both anti-CD4 and anti-CD8 mAbs, whereas the tumor cells were rejected in either $CD4^+$ cell-depleted or $CD8^+$ cell-depleted immune mice, as in untreated immune mice (Fig. 1B). These results indicate that both $CD4^+$ and $CD8^+$ T cells are directly involved in the tumor rejection in hyperimmune mice.

***In vivo* effect of primary and hyperimmune T cell subsets passively transferred into TBH on tumor growth** Immune spleen cells from primary immune mice significantly inhibited the tumor growth when they were passively i.v. transferred into TBH 4 days after tumor inoculation. As shown in Fig. 2A, however, $CD4^+$ T cell-depleted immune cells *in vitro* did not efficiently inhibit the tumor growth, whereas $CD8^+$ T cell-depleted immune cells inhibited the tumor growth in the same manner as untreated immune spleen cells (ISC alone).

On the other hand, in the case of ISC from hyperimmune mice, only depletion of both $CD4^+$ and $CD8^+$ T

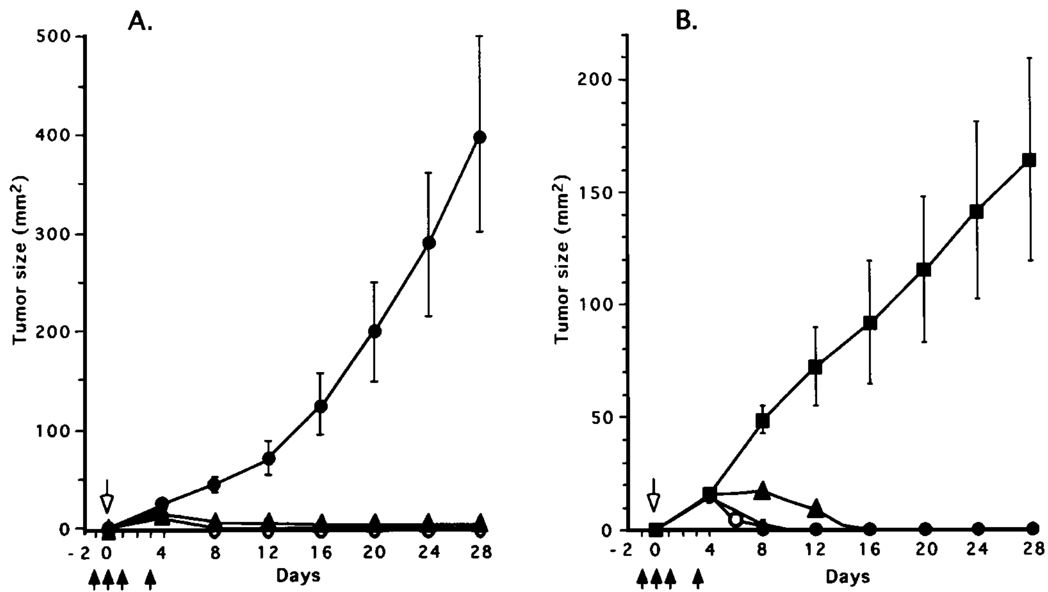

Fig. 1. A. Meth A tumor rejection or growth in primary immune mice treated by i.p. injection of 0.5 mg of normal rat IgG as a control (○) or anti-CD4 (GK1.5) mAb (●) or anti-CD8 (2.43) mAb (▲). Each group consisted of 5 mice and received i.p. injection 4 times as indicated by arrows (↑). The time of Meth A tumor cell inoculation is indicated by an arrow (↓). The tumor rejection was abrogated only in the group treated with anti-CD4 mAb. B. Meth A tumor rejection or growth in hyperimmune mice treated in the same way as primary immune mice with the addition of i.p. injection of both 0.5 mg of anti-CD4 and anti-CD8 mAbs (■). The tumor rejection was only abrogated in the group treated with both anti-CD4 and anti-CD8 mAbs.

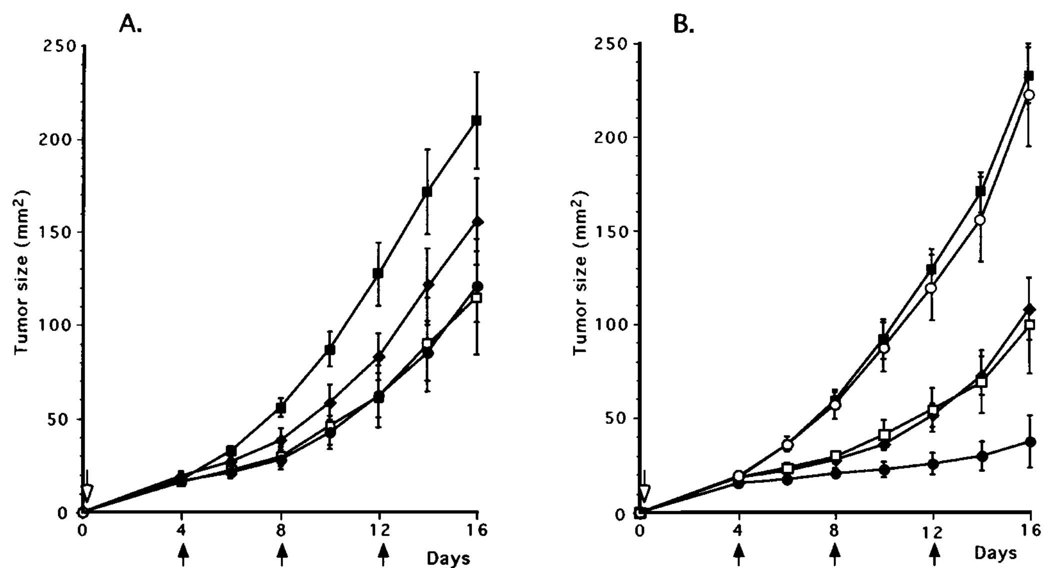

Fig. 2. Effect on tumor growth *in vivo* of adoptive transfer of immune spleen cells from either primary immune mice (A) or hyperimmune mice (B) into Meth A tumor-bearing mice. Tumor growth curves in mice which received i.v. inoculation of 2×10^7 normal spleen cells (■), untreated immune spleen cells (●), CD4 cell-depleted immune spleen cells treated with anti-CD4 (GK1.5) mAb and complement (◆), and CD8 cell-depleted immune spleen cells treated with anti-CD8 (3.155) mAb and complement (□). In the case of immune spleen cells from hyperimmune mice, an additional adoptive transfer of both CD4 and CD8 cell-depleted immune spleen cells treated with anti-CD4 and anti-CD8 mAbs and complement (○) was performed. Adoptive cell transfer was performed 3 times starting 4 days after tumor cell inoculation (↓), as indicated by arrows (↑).

cells abrogated the tumor growth-inhibitory activity, whereas depletion of either $CD4^+$ or $CD8^+$ T cells alone did not affect the inhibition of tumor growth, as shown in Fig. 2B. The results of these passive cell transfer experiments were consistent with the *in vivo* tumor rejection experiments in immune mice, as shown in Fig. 1.

***In vitro* analysis of effector cells induced in spleen cells of either primary immune or hyperimmune mice** To characterize effector cells *in vitro*-induced in ISC from primary immune and hyperimmune mice, these ISC were restimulated *in vitro* with MMC-treated Meth A cells for 5 days to induce cytotoxic effector cells directed to Meth A cells.

As shown in Fig. 3A, cytotoxic activity induced in primary ISC was mediated by both T cells and accessory cells since treatment with either anti-Thy-1.2 mAb and complement or with a nylon wool column significantly reduced the cytotoxicity. Furthermore, anti-CD3 as well as anti-CD4 mAb relatively blocked their cytotoxic killing, whereas anti-CD8 mAb did not block it. However, the killing activity was not specific for Meth A (Fig. 3A).

In contrast, cytotoxic activity induced in the hyperimmune spleen cells was mediated by $CD8^+$ T cells specific for Meth A, as shown in Fig. 3B since their cytotoxic activity was removed by treatment with anti-Thy-1.2, as well as anti-CD8 mAb and C. Moreover, either anti-CD3 or anti-CD8 mAb blocked the cytotoxicity.

All these results indicate that the cytotoxic effector cells induced *in vitro* in primary immune spleen cells were different from those in hyperimmune spleen cells.

Characteristics of $CD4^+$ T cell clones specific for Meth A established from either primary or hyperimmune spleen cells To elucidate the precise mechanisms of Meth A tumor rejection in primary immune and hyperimmune mice, specific $CD4^+$ T cell clones for Meth A were established from both primary and hyperimmune spleen cells. In the case of primary ISC, only one $CD4^+$ T cell clone, C9, was established in spite of considerable efforts. C9 showed a specific proliferative response to Meth A using T cell-depleted and irradiated Meth A-TBH-AC as antigen stimulation. This proliferative response was not induced by N-AC or MS61391c (an irrelevant tumor)-TBH-AC. Moreover, this response was blocked by either anti-CD4 mAb or anti-I-A^d mAb, as shown in Fig. 4A. The cell surface phenotype was $CD3^+$, $TCR\beta^+$, $CD4^+$, $CD8^-$, $CD45RA^+$, $CD45RB^-$, $LFA1^+$, $ICAM1^+$, $CD44^+$, $VLA4^+$

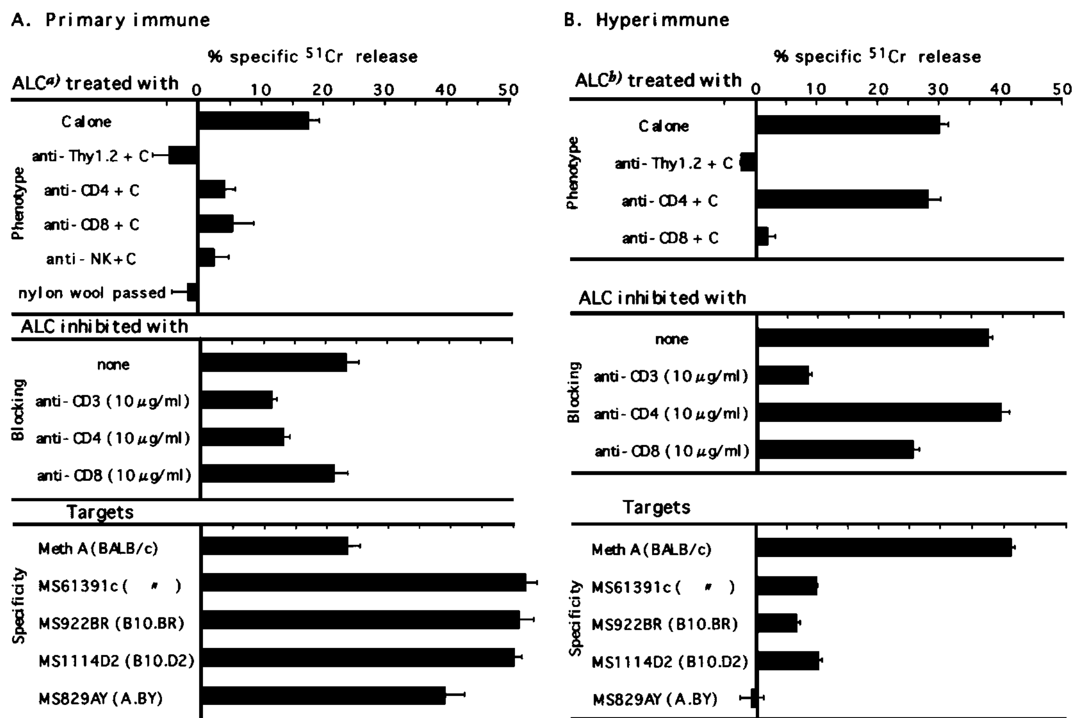

Fig. 3. Phenotype of *in vitro*-activated lymphoid cells (ALCs) having cytotoxic activity from either primary immune (A) or hyperimmune mice (B). ALCs obtained from either primary immune (*a*) or hyperimmune (*b*) mice were treated with anti-Thy-1.2 (HO-13-4), anti-CD4 (GK1.5), anti-CD8 (3.155) or anti-NK cell subset (4D11) mAbs and rabbit complement. In the case of ALCs from primary immune mice, ALCs[a)] were passed through a nylon wool column. Blocking of cytotoxic activities of ALCs by anti-CD3 (145-2C11), anti-CD4 (GK1.5) or anti-CD8 (2.43) mAb was examined.

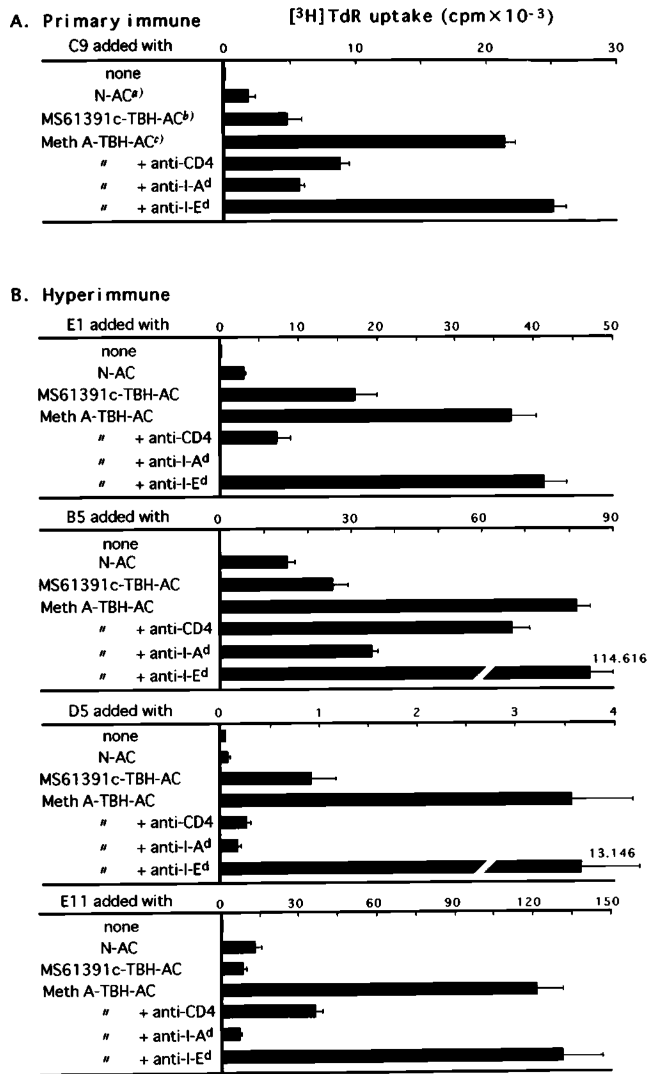

Fig. 4. Antigen-specific proliferation of CD4+ T cell clone (C9) established from primary immune spleen cells and 4 representative CD4+ T cell clones (E1, B5, D5 and E11) out of 8 clones established from hyperimmune spleen cells. Fifty thousand cells of the clones were incubated with the same number of spleen accessory cells from *a*) T cell-depleted normal host (N-AC) or *b*) irrelevant tumor MS61391c tumor-bearing host (MS61391c-TBH-AC) as controls, or *c*) T cell-depleted Meth A tumor-bearing host (Meth A-TBH-AC) as antigen stimulator cells. Specific proliferative response was blocked with 10 μg/ml of either anti-CD4 (GK1.5), anti-I-A^d (25-9-17), or anti-I-E^d (31-1-4) mAbs. N-AC, MS61391c-TBH-AC, and Meth A-TBH-Ac were prepared by treatment with anti-Thy-1.2 mAb and complement, followed by 30 Gy of irradiation.

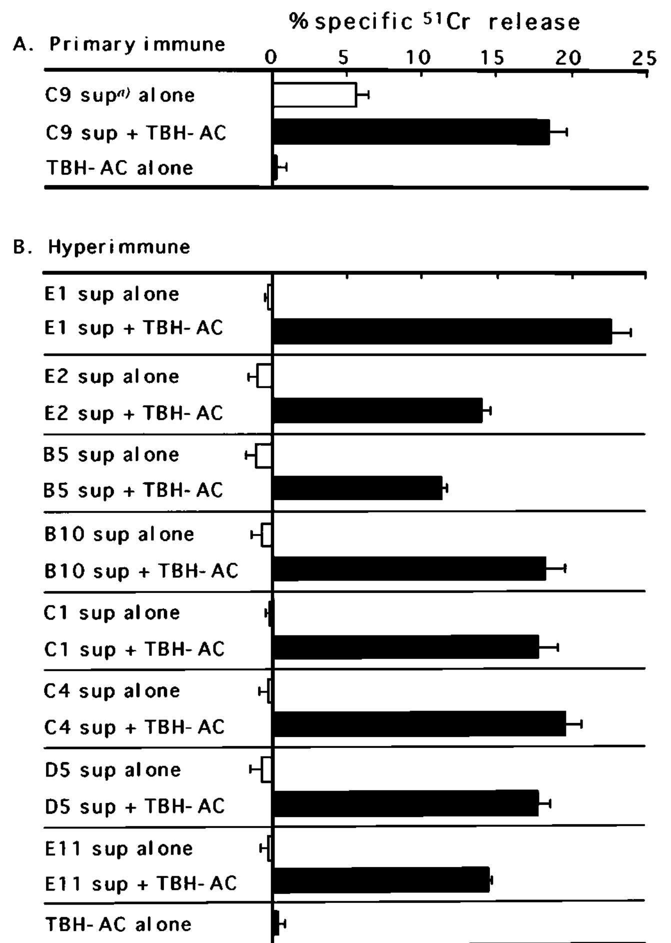

Fig. 5. Induction of cytotoxicity in spleen accessory cells (TBH-AC) of T cell-depleted Meth A tumor-bearing host with culture supernatants of CD4+ T cell clones at the effector phase. Culture supernatant of each CD4+ T cell clone was harvested separately after antigen stimulation. Each culture supernatant was added to a well at one-fourth of the total volume. Two hundred thousand TBH-AC were mixed with 1×10^4 ^{51}Cr-labeled Meth A cells in a total volume of 200 μl in each well of 96-well microplates in quadruplicate and their cytotoxicity was measured by 16 h ^{51}Cr release assay. Effector/target ratio=20. *a*) Culture supernatant harvested from 2×10^6 cells/ml of each established CD4+ T cell clone 2 days after stimulation with solid-phase anti-CD3 (145-2C11) mAb.

by FACScan flow cytometry, as shown in Table I. As regards the cytokine profile of C9, IFN-γ, TNF and IL-2 were detected in its culture supernatant (Table II). Furthermore, the supernatant induced cytotoxic activity in Meth A-TBH-AC although the supernatant itself showed almost no cytotoxic activity towards Meth A cells, just like the classical delayed-type hypersensitivity (DTH) response *in vitro* (Fig. 5A).

In contrast, a total of eight CD4+ T cell clones were established from hyperimmune spleen cells. All established clones were shown to proliferate specifically in

Table I. Cell Surface Phenotype [a] of Established $CD4^+$ T Cell Clones from Either Primary Immune or Hyperimmune Spleen Cells Analyzed by FACScan Flow Cytometry

	Clone	TCRβ	CD3	CD4	CD8	CD45RA	CD45RB	ICAM-1	LFA-1	CD44	VLA-4
Primary	C9	+	+	+	−	+	−	+	+	+	+
Hyper-immune	E1	+	+	+	−	+	−	+	+	+	−
	E2	+	+	+	−	+	−	+	+	+	+
	B5	+	+	+	−	+	−	+	+	+	−
	B10	+	+	+	−	+	+	+	+	+	+
	C1	+	+	+	−	+	+	+	+	+	+
	C4	+	+	+	−	+	+	+	+	+	−
	D5	+	+	+	−	+	+	+	+	+	−
	E11	+	+	+	−	+	+	+	+	+	−

a) Phenotypic analysis was performed by FACScan analysis as described in "Materials and Methods."

Table II. Cytokine Profiles [a] of Established $CD4^+$ T Cell Clones from Primary Immune or Hyperimmune Spleen Cells

	Clone		IL-2	IL-4	IFN-γ (Unit/ml)	TNF (Unit/ml)
Primary	C9	(Th1)	+	−	23	64
Hyper-immune	E1	(Th0)	−	+	196	ND
	E2	〃	−	+	80	256
	B5	(Th2)	−	+	0	ND
	B10	〃	−	+	0	512
	C1	〃	−	+	0	ND
	C4	〃	−	+	0	ND
	D5	〃	−	+	0	1024
	E11	〃	−	+	0	ND

a) IL-2, IL-4 and TNF were detected by bioassay, and IFN-γ was measured by ELISA as described in "Materials and Methods."

response to Meth A-TBH-AC as an antigen stimulation. Specific proliferative responses of four representative clones, B5, D5, E1 and E11, are shown in Fig. 4B. These proliferative responses were inhibited by anti-CD4 or anti-I-A^d mAb. The cell surface phenotypes of all eight clones as found by flow cytometric analyses are shown in Table I and the cytokine profiles are shown in Table II. We also examined the cytokine profiles of established $CD4^+$ T cell clones from hyperimmune spleen cells. As shown in Table II, six $CD4^+$ T cell clones, B5, B10, D5, E11, C1 and C4, out of eight clones secreted IL-4 but not IL-2 or IFN-γ, and seemed to be of Th2 type. The other two clones, E1 and E2, secreted both IL-4 and IFN-γ and seemed to be Th0-like.

Furthermore, as shown in Fig. 5B, the culture supernatants of all eight CD4 T cell clones induced cytotoxic activity in Meth A-TBH-AC, like C9.

DISCUSSION

In this study, we investigated the type of T cell response involved in Meth A tumor rejection in immune syngeneic mice during the process of repeated immunization. In primary immune mice, $CD4^+$ T cells but not $CD8^+$ T cells were involved in rejection of Meth A. In *in vitro* experiments with cells from primary immune mice, nonspecific cytotoxic cells such as macrophages and NK cells, as well as LAK cells, were induced by the activation of specific $CD4^+$ T cells without activation of specific $CD8^+$ CTL. These results are supported by several reports showing that inhibition of Meth A growth is mediated by mainly $CD4^+$ T cells but not by $CD8^+$ cytotoxic T lymphocyte (CTL).[10–12] Furthermore, although only one specific $CD4^+$ T cell clone (C9) was established from the primary ISC, it was confirmed that C9 secreted IL-2, IFN-γ, and TNF, and its culture supernatant induced cytotoxic activity to Meth A in Meth A-TBH-AC, as occurs in the classic DTH response *in vitro*. In contrast, both $CD4^+$ and $CD8^+$ T cells were essentially involved in Meth A tumor rejection in hyperimmune mice prepared by repeated immunization. Specific $CD8^+$ CTL to Meth A were induced in the hyperimmune spleen cells *in vitro*. These results suggest that the immune response ranges from specific $CD4^+$ T cell-mediated DTH response to specific $CD8^+$ CTL response, in addition to $CD4^+$ T cell-mediated DTH response, in mice subjected to repeated immunization. Previously, it was thought that tumor rejection was mediated by specific $CD4^+$ T cells and there was no involvement of specific $CD8^+$ CTL in the tumor rejection in the case of the Meth A system.[11, 12] However, recently TRA of Meth A was identified[5–7,17–20] and $CD8^+$ CTL clones for Meth A were established[6, 19, 20] by repeated immunization with TRA. Furthermore, several Meth A tumor antigen peptide sequences recognized by CTL were also identified.[19, 20] Our study seems to resolve the dis-

crepancy between the earlier and the recent results in the Meth A system. However, it is still unclear what kinds of factors are directly involved in changing the type of T cell response to Meth A during repeated immunization. To characterize the type of specific CD4+ T cells induced in hyperimmune spleen cells, eight specific CD4+ T cell clones were established. Six of the eight clones secreted IL-4 but not IFN-γ, so these clones seem to be of Th2 type. As the other two clones secreted both IL-4 and IFN-γ, but not IL-2, these two clones are considered to be Th0-like. However, no Th1-type cell was found in established CD4+ T cell clones. It might be considered from these results that T cell response to Meth A changes sequentially from Th1-type-mediated DTH in the primary immune state to Th2- and Th0-mediated T cell response and specific CD8+ CTL response as major effector responses in the hyperimmune state. No direct evidence that Th2 is capable of inducing specific CD8+ CTL for Meth A was obtained in this study. However, since specific CD8+ CTL were so strongly activated that CD8+ CTL were induced even in CD4+ T cell-depleted hyperimmune spleen cells *in vitro* by MMC-treated Meth A stimulation, the predominance of specific CD8+ CTL response to Meth A tumor rejection in hyperimmune mice may be due to IL-4-secreting CD4+ T cells (Th2 and Th0 type), because IL-4 selectively activates primed specific CD8+ CTL rather than non-specific NK or LAK cells,[21, 22] especially in the presence of primed CD8+ CTL.

It has long been postulated that Th2-type CD4+ T cells mainly mediate humoral immune response and do not mediate DTH response, whereas Th1-type cells mediate cellular immune response.[23, 24] However, we reported recently that a Th2-type CD4+ T cell clone was directly involved in syngeneic tumor rejection via activation of primed CD8+ CTL directed to the syngeneic sarcoma[14] and we have also found that culture supernatant as well as IL-4 could replace the Th2 clone's activity for inducing CD8+ CTL in CD4-depleted spleen cells from A/J mice immune to S1509a sarcoma (unpublished data). Furthermore, in this study, the culture supernatant of any Th2-type clone or Th0-type clone after stimulation could induce cytotoxic activity in the T cell-depleted tumor-bearing host's spleen accessory cells, like the Th1-type clone (C9). These results indicate that not only the Th1 type, but also some of the Th2 and Th0 subsets are able to induce *in vitro* a DTH-like response in the absence of a T cell population. It still remains to be elucidated why a specific CD8+ CTL response can not be induced in primary immune spleen cells even in the presence of specific Th1-type CD4+ T cells in the case of Meth A. One possibility is that the immunogenicity of Meth A might be too weak for induction of enough specific CD8+ CTL in the primary immunization, or there may be some regulatory mechanisms present between DTH response and CTL response.

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