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Cyclophosphamide Given After Active Specific Immunization Augments Antitumor Immunity by Modulation of Th1 Commitment of CD4+ T Cells

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Background and Objectives: In order to evaluate the regulatory effect of cyclophosphamide (CPA) on active specific immunization (ASI)-induced antitumor immunity, we examined the timing of CPA (100 mg/kg) with ASI, and focused on whether CPA given after ASI augments antitumor immunity by modulation of Th1 commitment of $CD4^+$ T cells.

Methods: We examined the effect of CPA combined with ASI using sonicated tumor supernatant (SS) and recombinant interleukin-1 β (rIL-1 β).

Results: Survival of i.p. tumor inoculated mice after ASI (days −12, −9, and −6) followed by 100 mg/kg CPA (day −3) (ASI-CPA) was significantly prolonged compared with that of mice treated with ASI alone, whereas CPA (day −15) treatment before ASI (CPA-ASI) completely abrogated the survival prolongation by ASI alone. In early stage (day 0) after ASI-CPA treatment, the $CD4^+$ T cells were determined to play an important role in the protective immunity for the following reasons: 1) the $CD4^+/CD8^+$ ratio of spleen cells from immunized mice was higher than that of the control or CPA alone treated group; and 2) the tumor neutralizing activity of fresh spleen cells was abrogated by $CD4^+$ T-cell depletion in vitro. $CD4^+$ T cells of mice treated with ASI-CPA produced more interferon (IFN)-γ and IL-2 and less IL-4 than those of the ASI alone group.

Conclusions: These results suggest that the protective immunity induced by ASI was augmented through the modification of the Th1 and Th2 balance by CPA injection after ASI.

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KEY WORDS: cyclophosphamide; active specific immunization; Th1 type $CD4^+$ T cells; Th2 type $CD4^+$ T cells

INTRODUCTION

For augmenting specific antitumor immunity, antigens and some adjuvants have been used in active specific immunization (ASI) [1,2]. Our previous studies demonstrated that sonicated tumor supernatant (SS) as an antigen source could be used to induce autologous tumor-specific cytotoxic T cells (CTL) in in vitro cocultured systems with lymphocytes from patients with malignant disease [3]. In murine tumor models, when combined with interleukin-1 (IL-1) as an adjuvant [4], administra-

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tion of SS could include tumor-specific protective immunity and $CD4^+$ T cells have been revealed to be the main effectors [5].

On the other hand, ASI might not only induce an antitumor effect but also augment tumor growth by activation of certain suppressor cells during the immunizing process [1]. The anticancer drug cyclophosphamide (CPA) has been shown to be able to augment cell-mediated immunity through a selective reduction of host suppressor T cells or their precursors [6–8]. Suppression or augmentation of host immunity by CPA is determined by dose and timing of the administration [9,10]. Our previous study demonstrated that low dose CPA augmented the antitumor effects which were induced by IL-1 in tumor bearing mice [11]. In order to evaluate the regulatory effect of CPA on ASI-induced antitumor immunity, we examined the timing of CPA (100 mg/kg) with ASI, and focused on whether CPA given after ASI augments antitumor immunity by modulation of Th1 commitment of $CD4^+$ T cells.

MATERIALS AND METHODS

Animal and Tumor Cells

Inbred male BALB/c mice weighing 20–25 g were purchased from Japan SLC Co. Ltd. (Shizuoka, Japan) and used at 8–12 weeks of age. The tumor cell line MOPC-104E, which is a syngeneic plasmacytoma to BALB/c mice, was maintained serially in vivo by intraperitoneal (i.p.) passage. Tumor cells were used for experiments 6–8 days after inoculation.

Reagents

Recombinant human interleukin-1 β (rIL-1 β) 71 ser mutant was supplied by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). The lymphocyte activating activity of rIL-1 β is 2×10^7 U/mg protein. CPA was obtained from Shionogi Pharmaceutical Co. Ltd. (Osaka, Japan) and dissolved with sterile saline immediately before i.p. injection.

For in vitro negative selection of $CD4^+$ and $CD8^+$ T-cell subpopulations, the anti-Lyt2.2 (CD8) and anti-L3T4 (CD4) monoclonal antibody (mAb) were purchased from Cedarlane Laboratories Ltd. (Ontario, Canada).

Preparation of SS

Preparation of SS was described previously [12]. Briefly, tumor cells were separated from ascitic fluid followed by red blood cell lysis with Tris-NH_4Cl, and resuspended in RPMI-1640 at a concentration of 2×10^7 cells/ml. After sonication for 90 sec (20 kHz, 105 W) with an ultrasonic disrupter (Tomy Seiko, Tokyo, Japan), they were centrifuged for 90 min at 15,000*g*. The supernatants were passed through a 0.22 μm filter and stored at −80°C until use.

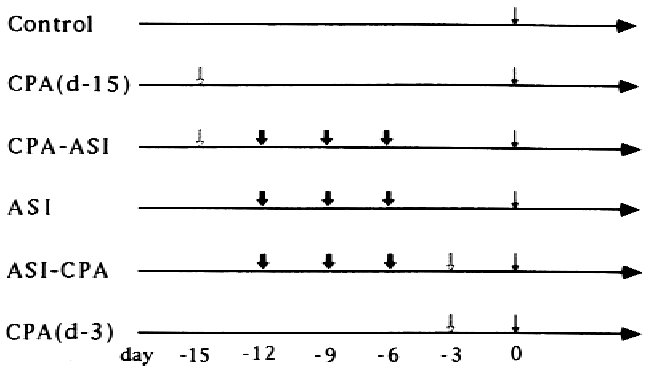

Fig. 1. Scheme of protocols for in vivo treatments (↓) 1×10^5 MOPC-104E tumor cells per mouse i.p.; (⇩) CPA (100 mg/kg) i.p.; (⬇) rIL-1 β (1.0 μg) and SS (1.0 ml) i.p.; sterile saline was used as a control.

In Vivo Experimental Protocols

The six protocols of immunization in vivo with rIL-1 β plus SS and/or CPA are shown in Figure 1. The control group was treated by sterile saline 1.0 ml/mouse at the time point; CPA (day −15) means a single CPA (100 mg/kg) i.p. 15 days before tumor inoculation; CPA-ASI is CPA (day −15) treatment plus ASI immunization with rIL-1 β (1.0 μg/mouse) and SS (1.0 ml) i.p. on days 12, 9, and 6 prior to tumor inoculation (i.e., days −12, −9, and −6); ASI means rIL-1 β and SS treatment alone; CPA (day −3) is a single CPA i.p. 3 days before tumor inoculation; and ASI-CPA means ASI plus CPA (day −3) treatment. After these treatments, mice received i.p. inoculation on day 0 with 1×10^5 MOPC-104E tumor cells, and their survival was observed daily until day 100.

Preparation of the Spleen Cells

Spleens from treated mice were aseptically removed on day 0, minced, and passed through a No. 100 stainless steel mesh. After erythrocytes were lysed with 0.83% Tris-NH_4Cl, the spleen cells were washed three times with Hank's balanced salt solution (HBSS) and suspended in HBSS.

Tumor Neutralizing Assay and Cell Depletion

The antitumor effect of splenocytes was investigated by tumor neutralizing assay. The fresh splenocytes from treated mice on day 0 were mixed with 5×10^5 MOPC-104E tumor cells at a ratio of 30:1 and inoculated subcutaneously (s.c.) in a volume of 0.2 ml into the dorsum of recipient normal mice. Tumor diameter was measured twice per week for 21 days. For in vitro cell depleted treatment, fresh splenocytes (1×10^7/ml) were coincubated with anti-CD4 or anti-CD8 mAbs at 4°C for 60 min and centrifuged. The pellet was treated with Low-Tox-M rabbit complement at ×10 dilution (Cedarlane Laboratories Ltd.) at 37°C for 60 min. After three washings, these cells were used for tumor neutralizing assay in vivo.

Flow Cytometric Analysis

The following mAbs were purchased from Pharmingen (San Diego, CA): fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 (CD3), anti-L3T4 (CD4), and anti-Ly2 (CD8). Fresh splenocytes (1×10^6) were stained for 30 min at 4°C with corresponding mAbs. The stained cells were washed three times with phosphate-buffered saline (PBS) and examined for fluorescence using a FACScan instrument (Becton Dickinson, Mountain View, CA). Analysis of data was determined by counting 1×10^4 viable cells.

Isolation of $CD4^+$ T-Cell Subset From Fresh Spleen Cells

Fresh splenocytes were depleted of B cells and other adherent cells by anti-IgM panning. The non-adherent cells were collected and resuspended at 1×10^7 cells/ml. They were treated with anti-CD8 mAb for 60 min at 4°C before they were incubated with rabbit complement for 60 min at 37°C. The final cell suspensions were washed three times with RPMI-1640 and used for experiments as $CD4^+$ T cells. The phenotypes of enriched $CD4^+$ T-cell suspensions were 96% of $CD3^+$ and 89% of $CD4^+$.

$CD4^+$ T-Cells Proliferation Assay and Cytokine Assay

To assess the proliferation of $CD4^+$ T cells, enriched $CD4^+$ T cells (2×10^5/well) from in vivo treated mice were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; BioWHITTAKER, Verviers, Belgium), 100 μg gentamicin, and 0.2 μg/ml fungizone [complete medium (CM)] at a final volume of 0.2 ml in a 96-well flat-bottomed microtest plate (Nunc, Roskilde, Denmark). Three days later, ^{3}H-thymidine (1.0 μCi/well) was added to each well for the last 6 hr and cells were harvested onto a 96-well UniFilter plate GFC96 by a Filtermate cell harvester (Packard, Meriden, CT). Their radioactivity was measured by a liquid scintillation counter (Packard) and mean count per minute (cpm) was calculated from triplicate wells.

For cytokine assay, the enriched $CD4^+$ T cells (2×10^6/ml) were incubated in CM in a 12-well culture plate (Nunc) and 5 days later their supernatants were collected and stored at −80°C until test. The interferon (IFN)-γ and IL-4 concentrations of these cultured supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) (Mouse Titer Screen III EIA, PerSeptive Diagnostics, Inc., Cambridge, MA). Minimum detection levels of IFN-γ and IL-4 were 11.7 and 1.0 pg/ml, respectively. The IL-2 production was analyzed by a bioassay using an IL-2 sensitive cells line, CTLL2 (American Type Culture Collection, Rockville, MD).

Statistical Analysis

The generalized Wilcoxon test and Cox-Mantel test were used to compare survival data. Comparison of tumor diameters was performed using the Student's *t*-test. $P < 0.05$ was defined as significant.

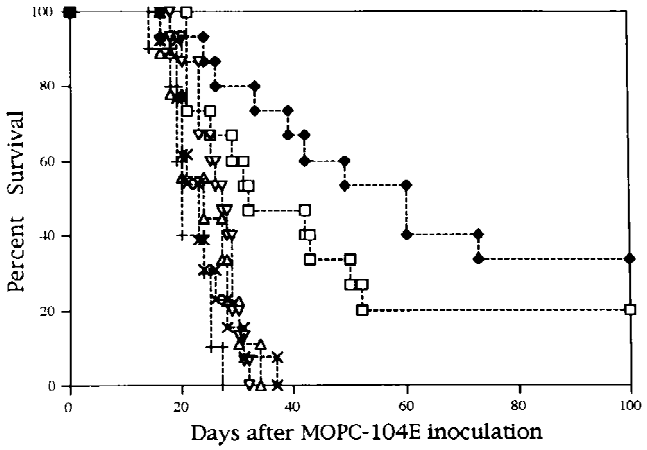

Fig. 2. Survival of the mice inoculated with MOPC-104E tumor cells (1×10^5) i.p. on day 0 after pretreatment with (▽) control [median survival time (MST) = 23 days, n = 13]; (+) CPA (day −15) (MST = 20 days, n = 9); (×) CPA-ASI (MST = 26 days, n = 13); (□) ASI alone (MST = 32 days, n = 15); (△) CPA (day −3) (MST = 24 days, n = 9); (♦) ASI-CPA (MST = 59 days, n = 15). Differences of survival were calculated by the generalized Wilcoxon test: $P < 0.01$: control vs. ASI and control vs. ASI-CPA; $P < 0.05$: ASI vs. ASI-CPA.

RESULTS

CPA After, But Not Before, ASI Augments the Protective Immunity Against the Following Tumor Challenge

In order to study antitumor immunity induced by ASI and/or CPA, BALB/c mice received six protocols of treatments as shown in Figure 1. After each treatment, they were i.p. inoculated with 1×10^5 MOPC-104E cells on day 0, and their survival was monitored for 100 days. As shown in Figure 2, the immunized mice with ASI experienced longer survival times than control mice ($P < 0.01$). Moreover, a single i.p. administration of CPA after the ASI (ASI-CPA) significantly prolonged their survival time when compared with that of ASI alone ($P < 0.05$). In contrast, CPA before ASI (CPA-ASI) abolished the effect of ASI. Survival of those mice with CPA alone on day −15 or on day −3 was comparable to that of control mice. These results indicated that ASI could induce antitumor immunity in vivo, and CPA after, but not before, ASI augmented this protective immunity, though CPA alone did not have any effect.

Tumor Neutralizing Activity of Fresh Spleen Cells From Immunized Mice

To examine antitumor activity of immunized spleen cells, we performed the tumor neutralizing assay. Spleen cells from each group on day 0 were mixed with tumor cells and inoculated in the dorsum of normal mice. Subsequent tumor diameters were measured and each tumor neutralizing activity was estimated as a mean value. As

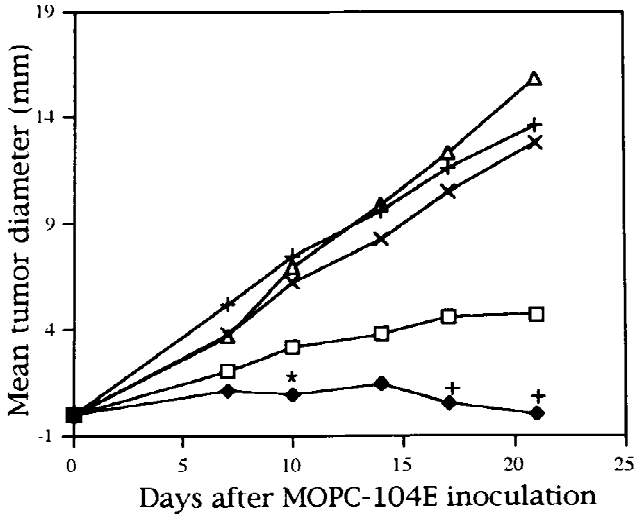

Fig. 3. Tumor neutralizing activity of fresh spleen cells. MOPC-104E tumor cells (5×10^5) were inoculated s.c. mixed with none (△, n = 10), or 1.5×10^7 spleen cells (effector to target ratio = 30) from normal mice (+, n = 10), CPA-ASI (×, n = 9), ASI alone (□, n = 10), and ASI-CPA (◆, n = 10) treated mice, respectively. Statistical values were compared by Student's *t*-test: $*P < 0.05$: ASI vs. ASI-CPA; $+P < 0.01$: ASI-CPA vs. other groups and ASI vs. other groups.

shown in Figure 3, spleen cells from the mice treated with ASI alone exhibited higher tumor neutralizing activity than those of control group. ASI-CPA significantly enhanced the effect of ASI, while CPA-ASI eliminated the antitumor activity induced by ASI. These results were exactly compatible with survival data in Figure 2, which confirmed that ASI could induce the strong antitumor immunity in vivo and CPA after ASI augmented this systemic host defense mechanism. Next, we examined cytotoxicity against tumor cells in vitro, but neither fresh nor rIL-2 cultured spleen cells showed any direct lytic activities (data not shown). These results suggest that ASI may not exert biological activity on effectors but may affect the induction phase of antitumor immunity.

Phenotypic Analysis of Fresh Spleen Cells From Immunized Mice and Subpopulation Analysis

To determine the relationship between antitumor activity and change of T-cell subsets in spleen cells of immunized mice, we analyzed fresh spleen cells from each group on day 0. After counting total cell number, spleen cells from each group were single color stained with anti-CD3, anti-CD4, and anti-CD8 mAbs before flow cytometric analysis. Data in Table I are shown as mean ± SD for three individual mice. Total cell number and $CD4^+/CD8^+$ ratio of spleen from the mice immunized with ASI were significantly higher than those of control mice ($P < 0.01$). The mice which received ASI-CPA treatment showed a comparable $CD4^+/CD8^+$ ratio to the ASI alone group, although their total spleen cells decreased in number. These results suggest that ASI treatment efficiently increased $CD4^+$ T cells and that CPA did not exert any effect on the $CD4^+/CD8^+$ ratio although it reduced the total cell number. To gain insight into functions of $CD4^+$ T cells, spleen cells from the mice immunized with ASI-CPA were depleted of $CD4^+$ or $CD8^+$ T cells in vitro before tumor neutralizing assay. Tumor neutralizing activity induced by ASI-CPA was abolished by depletion of $CD4^+$ T cells, but not $CD8^+$ T cells (Fig. 4). These results strongly suggest that the $CD4^+$ T cells might play an important role in this CPA-combined vaccination model.

CPA Augments the Proliferation of $CD4^+$ T Cells and Preferentially Increases Th1 Type Cytokines of $CD4^+$ T Cells

ASI-CPA significantly augments antitumor immunity compared to ASI alone, but the $CD4^+/CD8^+$ T-cell ratio did not change. To address the effect of CPA on $CD4^+$ T cells, we first examined the proliferation of $CD4^+$ T cells. Fresh spleen cells from each treatment group were depleted of B cells and $CD8^+$ T cells as described in Materials and Methods, which consisted of 89% $CD4^+$ T cells. These CD4-enriched spleen cells were cultured in CM alone for 3 days followed by pulsation with ^{3}H-thymidine. As shown in Figure 5, $CD4^+$ T cells from immunized mice with ASI-CPA exhibited apparent proliferative potential when compared to those from the control or ASI groups.

Finally, we examined the effect of cytokine profile of $CD4^+$ T cells. $CD4^+$ T-enriched spleen cells were cultured for 5 days in CM alone and the culture supernatants were assayed for IL-4 or IFN-γ production by ELISA and for IL-2 by CTLL2 bioassay. ASI strongly induced IL-4 production of $CD4^+$ T cells, while CPA shifted the cytokine profile of these $CD4^+$ T cells from IL-4 to IFN-γ and IL-2, i.e., from Th2 to Th1 type (Fig. 6).

DISCUSSION

In our IL-1 plus MOPC-SS (ASI) model, preimmunized mice became resistant to tumorigenicity of MOPC and developed long-time survival. Moreover, this protective immunity was significantly enhanced by CPA after, but not before, ASI. Subpopulation analysis showed that $CD4^+$ T cells in the immunized mice were involved in tumor neutralizing activity. These results indicated that $CD4^+$ T cells seem to play a major role in this protective immunity induced by ASI alone [5] or ASI combined with CPA, which might modulate the function of $CD4^+$ T cells in an unknown manner. Therefore, this study has focused on the analysis of $CD4^+$ T-cell populations in fresh spleen cells from immunized mice and examined the effect of CPA on $CD4^+$ T cells.

IL-1 was first defined as a mediator of host inflammatory response and secreted mainly from mononuclear phagocytes. It is well known that IL-1 consists of two

TABLE I. Phenotypic Analysis of Fresh Spleen Cells From Immunized Mice by ASI and/or CPA†

Treatment groups	Total no.	Nos. of staining lymphocytes per spleen ($\times 10^{-5}$)			CD5/CD8 ratio
		Thy1.2 (CD3)	L3T4 (CD4)	Ly2 (CD8)	
Control	663.3 ± 169.2	212.7 ± 24.9 (32.1%)[a]	165.6 ± 27.3 (25.0%)	49.3 ± 7.0 (7.4%)	3.4 ± 0.3
CPA (day −3)	158.0 ± 26.3	103.5 ± 19.2 (65.5%)	77.8 ± 15.2 (49.2%)	24.5 ± 5.6 (15.5%)	3.2 ± 0.2
ASI	1,780.0 ± 113.7	518.4 ± 80.9 (29.1%)	516.4 ± 63.9 (29.0%)	89.3 ± 13.5 (5.0%)	5.9 ± 0.6*
ASI-CPA	452.7 ± 78.0	166.4 ± 25.6 (36.8%)	158.6 ± 27.5 (35.0%)	32.1 ± 6.2 (7.1%)	5.0 ± 0.4*

†In each treatment group, fresh spleen cells from three mice were analyzed by flow cytometry on day 0. Results are represented as mean ± SD.
[a]Percentage to total number.
*$P < 0.01$: ASI or ASI-CPA vs. control or CPA (day −3) on CD4/CD8.

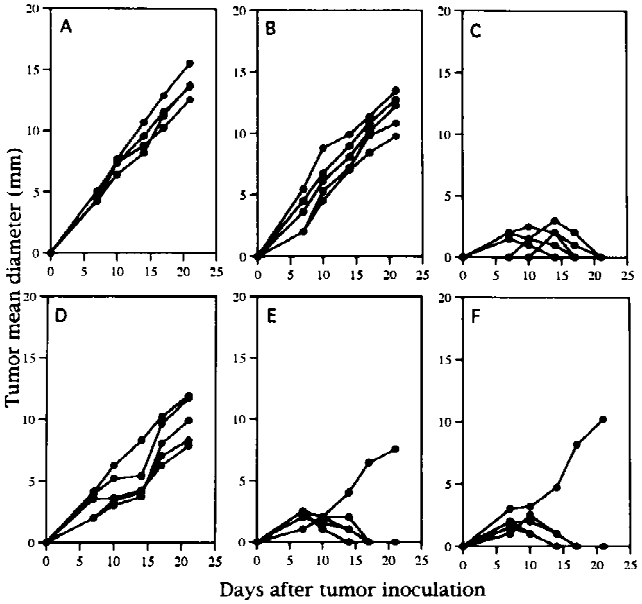

Fig. 4. Tumor neutralizing activity of in vitro mAb treated fresh spleen cells from ASI-CPA treated mice. Mice were inoculated s.c. with **(A)** 5×10^5 MOPC-104E tumor cells alone, admixed with 1.5×10^7 spleen cells from **(B)** normal mice, **(C)** ASI-CPA treated mice, or those cells with **(D)** CD4 depletion, **(E)** CD8 depletion, and **(F)** complement alone.

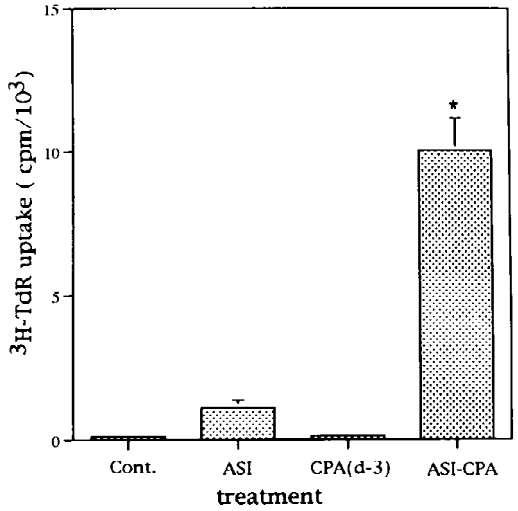

Fig. 5. Proliferation of $CD4^+$ cells from spleens of treated mice. Enriched $CD4^+$ cells (1×10^5/0.2 ml/well) were cultured in CM for 3 days. After 6 hr ^{3}H-thymidine uptake, mean cpm was calculated from triplicate wells. *$P < 0.01$: others vs. ASI-CPA.

isoforms, i.e., IL-1 α and IL-1 β, and the latter exerts its main biological activity in the circulation. A report indicated that IL-1 has not only direct cytotoxicity but also an adjuvant effect with tumor vaccine [4]. The precise mechanism of this antitumor immunity remains obscure, but our previous study revealed that IL-1 modulates some $CD4^+$ T-cell populations because antitumor activity induced by IL-1 and SS was abrogated by $CD4^+$ T-cell depletion both in vivo and in vitro [5]. Although IL-1 receptors are present on almost all cells, $CD4^+$ T cells share their receptors selectively on Th2, but not on Th1 [13,14]. In this study, $CD4^+$ T cells from mice immunized with IL-1 (ASI and ASI-CPA) produced significantly more IL-4 than those from control mice, which is compatible with the distribution of IL-1 receptors.

IL-4 is originally identified as a growth and differentiation factor for B cells which are responsible for humoral immunity and may antagonize the cell-mediated immune response, which is mainly involved in tumor rejection. However, among the experiments in which various cytokine genes were transduced into the tumor cells, it was reported that an IL-4 producing tumor cell line could be effectively rejected by a syngeneic host and showed therapeutic effect against established tumor [15,16]. Histologic examination suggested that this rejection process was associated with the infiltration of macrophages rather than lymphoid cells [15]. Moreover, it was also reported that macrophages expressed IL-4 receptors and IL-4 could activate macrophages for increased tumoricidal activity [17]. In this study, ASI increased IL-4 production of $CD4^+$ T cells which might cause the immunized mice to develop antitumor immunity. Although there is no way to explain the exact function of macrophages, it is quite reasonable that macro-

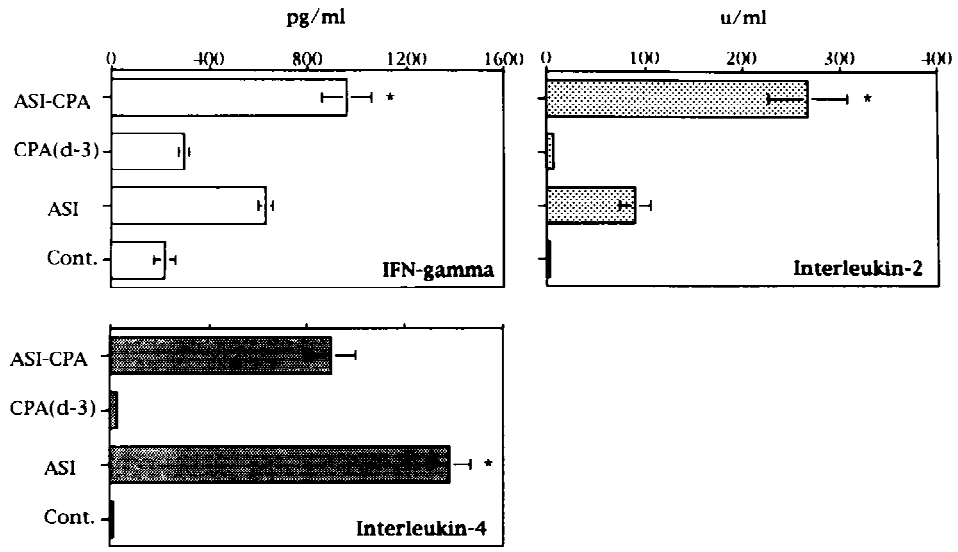

Fig. 6. Lymphokine production of $CD4^+$ T cells of the mice pretreated with ASI and/or CPA. The 2×10^6 $CD4^+$-enriched spleen cells were cultured in CM for 5 days and the supernatant was assayed for IFN-γ, IL-4, and IL-2 production by ELISA and CTLL2 bioassay, respectively. *$P < 0.01$: vs. other groups.

phages activated by IL-4 efficiently process tumor antigen in association with major histocompatibility complex class II since MOPC cells do not express class II antigen [18] which is necessary for $CD4^+$ T cells to recognize extrinsic antigen.

Next, we addressed the effect of CPA on the host defense mechanism. CPA has an immunomodulating effect as well as direct cytotoxicity. In murine systems, many studies have reported that CPA can activate the host immune response by selective elimination of suppressor T cells or their precursors [6,7,10,19]. We also previously reported that a combination of CPA and IL-1 exerted a curative effect in tumor bearing mice [11]. Activation of $CD8^+$ T or natural killer (NK) cells might be responsible for this effect, but the real mechanism has not been elucidated. In our ASI model, $CD4^+$ T cells played a major role and the $CD4^+/CD8^+$ ratio of spleen cells from immunized mice with ASI was higher than that of control mice. However, mice which received ASI plus CPA showed the same $CD4^+/CD8^+$ ratio as the ASI alone group, although their total splenocytes apparently decreased in number. These results, however, were observed only when CPA was injected after ASI, while CPA before ASI did not contribute to survival prolongation or tumor neutralizing activity. We speculate from these results that CPA before ASI is toxic to the naive immune system, but CPA after ASI modulates the immune cells which had been activated previously by ASI. Moreover, the proliferation assay disclosed that $CD4^+$ T cells were remarkably stimulated by CPA administration after ASI. How did CPA regulate the function of $CD4^+$ T cells? As mentioned above, ASI enhanced IL-4 production of $CD4^+$ T cells, but the cytokine profile of $CD4^+$ T cells when combined with CPA preferentially shifted to IFN-γ and IL-2. This means that CPA up-regulates expansion of IFN-γ and IL-2 producing Th1 cells. It is well known that one helper T-cell subset cross-regulates the development of the other subset [8,20]. Since ASI selectively expanded IL-4 producing Th2 cells rather than Th1 cells, abundant IL-4 might suppress development of Th1 cells. However, when combined with CPA, this suppression is eliminated, and improved IFN-γ and IL-2 producing Th1 cells may provide rather strong antitumor immunity by recruitment of $CD8^+$ or NK cells [21]. This regulation of Th1/Th2 balance by CPA was also reported in a non-obese diabetic mice model. In these autoimmune mice, inducible nitric oxidase synthetase, which is associated with the Th1 subset, was elevated after CPA administration although Th2 is dominant in themselves [22]. Collectively, these results indicate that CPA may modulate Th1 commitment of $CD4^+$ T cells to enhance the host defense against tumor cells.

CONCLUSIONS

The protective immunity can be driven by preimmunization with rIL-1 β plus SS, and the anti-tumor activity was augmented by injection of CPA (100 mg/kg) after, but not before, the immunization. $CD4^+$ T cells played a major role not only in ASI alone, but also in the ASI-CPA treated group. The effective $CD4^+$ T cells from the ASI-CPA treated group can proliferate greatly by autocrine secreting factors. The compositions of cultured supernatants from the ASI-CPA group contained more IFN-γ and IL-2 of Th1 cytokines and less IL-4 of Th2 cytokines compared with those of the ASI alone group. The protective effects of ASI-CPA might be mediated

through modification of the cytokine balance between the Th1 and Th2 subset of $CD4^+$ T cells.

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