Journal of Surgical Oncology 62:78–85 (1996)

A New Model of Active Specific Immunotherapy Using Interleukin-1 and Sonicated Tumor Supernatant in Murine Tumor System

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The possibility of active specific immunotherapy using interleukin-1 (IL-1) plus sonicated tumor supernatant (SS) was examined in a murine tumor model. The growth of intraperitoneally or subcutaneously inoculated plasmacytoma MOPC104E, which is syngeneic to BALB/c mice, was significantly suppressed by intraperitoneal pretreatment with IL-1 and SS from MOPC104E cells (MOPC-SS), on days 10, 7, and 4 before tumor inoculation. Pretreatment with IL-1 plus MOPC-SS or MethA-SS (SS from MethA cells) suppressed the growth of subcutaneous tumor of only the corresponding tumor cells, indicating the development of tumor-specific immunity in vivo. The splenic cells of immunized mice with IL-1 and MOPC-SS showed tumor neutralizing activity. However, their tumor neutralizing activity was abrogated when they were treated in vitro with anti-Thy1.2 or anti-L3T4 plus complement. Moreover, when combined with indomethacin per oral, IL-1 plus MOPC-SS significantly suppressed the growth of established subcutaneous tumor and prolonged survival of postoperative mice. These results suggest that this new type of active specific immunotherapy could be a useful method for cancer immunotherapy, especially when combined with oral indomethacin.

KEY WORDS: **active specific immunotherapy, interleukin-1, sonicated tumor supernatant, vaccine, murine plasmacytoma**

INTRODUCTION

In recent years, clinical trials of active specific immunotherapy using various types of cancer vaccines have been reported in the treatment of cancer, including colon cancer [1], renal cell carcinoma [2], and malignant melanoma [3,4] with encouraging results. Most of the vaccines consist of a source of tumor antigen and an adjuvant. As a source of tumor antigen, many types of vaccine, such as irradiated tumor cells [1,2], vaccinia oncolysate of tumor cell line [5], mechanical lysates of tumor cell lines [4], and carcinoembryonic antigen (CEA) in vaccinia virus [6], have been investigated. We have been employing sonicated tumor supernatant (SS) as a source of tumor antigen for in vitro sensitization of cultured lymphocytes to be transferred for the treatment of cancer patients [7]. It is easy to prepare SS and keep its activity. The antigenicity of SS was elucidated in detail in our previous studies [8,9].

In addition, various types of adjuvants were investigated to augment the immunogenicity of the relevant

Abbreviations: SS, sonicated tumor supernatant; IL-1, interleukin-1; IND, indomethacin; PG, prostaglandin; MLTR, mixed lymphocyte tumor reaction; APC, antigen presenting cell.

Accepted for publication January 23, 1996.

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antigen in cancer vaccine, such as Bacillus Calmette-Guérin (BCG) [1], Detox (myobacterial cell wall skeleton and monophosphoryl lipid A) [10], or *Corynebacterium parvum (Propionibacterium acnes)* [2]. McCune and Marquis [11] demonstrated that interleukin-1 (IL-1) was highly effective as an adjuvant with irradiated tumor cells in a murine model of active specific immunotherapy. It has become clear that several adjuvants, such as muramyl dipeptide [12], lipopolysaccharide [13], and bacterial peptideglycan [14] are potent inducers of IL-1, which is involved in optimal antigen-driven proliferation of T cells.

In addition to adjuvant activity, it has been reported that IL-1 induces the production of prostaglandin E_2 (PGE_2) in macrophage. This product suppresses further production of IL-1 [15] and inhibits T-cell proliferation as well as macrophage Ia expression [16]. Many biological activities of IL-1, such as fever and inflammation, are linked to stimulation of PGE_2 release [17]. Moreover, it was reported that the production of prostaglandins, especially PGE_2, from host macrophages or certain tumors may have pansuppressor effects against effector cells in the tumor-bearing host [18]. Indomethacin (IND), an inhibitor of prostaglandin synthesis, could affect the toxicity and the therapeutic efficacy of IL-1 in advanced tumor burden [19].

In the present study, we examined the effectiveness of IL-1 plus SS as a cancer vaccine and whether or not IND augments the effect of IL-1 plus SS in a murine model of active specific immunotherapy.

MATERIALS AND METHODS

Animals and Tumor Cells

Inbred male BALB/c mice weighing 20–25 g were obtained from Japan SLC Co. Ltd. (Shizuoka, Japan) and used at 8–12 weeks of age. The plasmacytoma cell line MOPC104E and fibrosarcoma cell line MethA, which are syngeneic to BALB/c mice, were used and maintained serially in vivo by intraperitoneal (i.p.) passage. Tumor cells were collected 3 or 4 days after inoculation and used for experiments.

Interleukins and SS

Recombinant human interleukin-1β (IL-1β) 71 Ser mutant was supplied by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan) (lymphocyte activating activity: 2×10^7 U/mg protein). Recombinant human interleukin-2 (IL-2) [20] was supplied by Takeda Chemical Industries Ltd. (Osaka, Japan). The specific activity of IL-2 was 1.4×10^7 JRU/mg protein. For the preparation of SS, tumor cells were separated from ascitic fluid of mice inoculated with MOPC104E or MethA cells by treatment with Tris–NH_4Cl and resuspended in RPMI1640 at a concentration of 2×10^7 cells/ml. After sonication for 90 s (20 kHz, 105 W) with a ultrasonic disruptor (Tomy Seiko, Japan), cells were centrifuged for 90 min at 15,000*g*, and the supernatant was passed through a 0.22-μm filter and stored at −80°C until use. After its protein concentration was measured, SS was used for active specific immunotherapy and assay.

In Vivo Immunization Before or After Tumor Inoculation

For preimmunization, 1 μg/head of IL-1 and/or 1 ml of MOPC-SS or MethA-SS were injected intraperitoneally into mice on days 10, 7, and 4 prior to tumor inoculation. After preimmunization, mice received subcutaneous (s.c.) inoculation with 5×10^5 MOPC104E cells or 1×10^6 MethA cells into the dorsum. The tumor diameters were measured twice a week for 21 days and expressed as the mean ± SE. In another experiment, preimmunized mice received intraperitoneal inoculation with 1×10^5 MOPC104E cells, and their survival was checked daily until death. In some experiments, the concentration of IL-1 and volume of MOPC-SS were reduced to examine their dose response for protective immunity.

For in vivo immunization after tumor inoculation, mice given subcutaneous transplantation with MOPC104E cells on day 0 were injected intraperitoneally on days 5, 8 and 11 with 1 μg/head of IL-1 and/or 1 ml of MOPC-SS with or without per oral indomethacin (IND) (Research Biochemicals Inc., Natick, MA) from day 5 to day 33 and the solid tumor diameters were measured up to 39 days. In another experiment, mice inoculated into right foot pad with 1×10^6 MOPC104E cells on day −21, had the right foot amputated on day 0. They were treated with IL-1 and MOPC-SS on days 4, 7, and 11 with or without per oral IND from day 4 until death, and their survival was checked until death. IND was initially dissolved in absolute ethanol (10 mg/ml) and finally diluted 250 times in water, to provide a concentration of 40 μg/ml of drinking water. Control mice received an identical concentration (0.4%) of ethanol only in the drinking water [21].

Preparation of the Splenocytes

Spleens from mice preimmunized on days −10, −7, and −4 were aseptically removed on day 0, minced and passed through a #100 stainless steel mesh. After erythrocytes were lysed with 0.83% Tris–NH_4Cl, splenocytes were washed three times with Hank's balanced salt solution (HBSS) and suspended in an appropriate medium.

Tumor Neutralizing Assay and In Vitro and In Vivo Antibody Treatment of Splenocytes

The antitumor effect of splenocytes was investigated by tumor neutralizing assay [22]. The splenocytes were mixed with 5×10^5 MOPC104E cells at a ratio of 30:1 and inoculated subcutaneously in a volume of 0.2 ml into the dorsum of recipient normal mice. Tumor diameter

was measured twice a week for 21 days and expressed as the mean ± SE.

For in vitro antibody treatment, anti-Thy1.2 (CD3), anti-Lyt2.2 (CD8), and anti-L3T4 (CD4) were used at dilutions of ×200, ×200, and ×20 (Cedarlane Laboratories Ltd. Hornby, Ontario, Canada), respectively. Anti-asialo GM1 (Wako Pure Chemicals, Osaka, Japan) was used at ×40 dilution. Fresh splenocytes (1×10^7 cells/ml) were treated with antibodies at 4°C for 60 min and centrifuged. The pellet was treated with complement at ×10 dilution (Low-Tox-M rabbit complement, Cedarlane, Canada) at 37°C for 60 min. After three washings, viable cells were recounted with trypan-blue dye exclusion test and used for tumor neutralizing assay.

For in vivo treatment of antibody, mice were injected intravenously with anti-asialo GM1 (20μl/mouse, days −10, −3, and 4), anti-CD8 (anti-Lyt2.2, 100 μl/mouse, day −10), or anti-CD4 (GK1.5, 50 μl/mouse, days −10, −6, and −3) in addition to the treatment with IL-1 plus MOPC-SS (days −10, −7, and −4) [23]. MOPC104E cells (5×10^5/mouse) were injected subcutaneously on day 0.

IL-2-Enhanced Mixed Lymphocyte Tumor Reaction (IL-2 Enhanced MLTR) (9)

Splenocytes (5×10^5/well) were cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS), 100 μg/ml gentamicin, 0.2 μg/ml Fungizone (Gibco, Grand Island, NY), 45 JRU/ml IL-2 with a graded concentration of MOPC-SS (0–11.1%), at a final volume of 0.225 ml in a 96-well flat-bottomed microtest plate for 4 days at 37°C in a humidified 5% CO_2 atm. Cultures were performed in triplicate and [^{3}H]-thymidine (0.5 μCi) was added to each well for the last 18 hr of incubation. The cells were harvested onto a glass-fiber filter (Labo Science Co., Tokyo, Japan) by a semiautomatic cell harvester (Labo Mash, Labo Science Co.), and their radioactivity (cpm) was counted by a liquid scintillation counter. The proliferative response to MOPC-SS was expressed as a stimulation index. The stimulation index was calculated as follows: stimulation index = ([^{3}H]-thymidine uptake in IL-2 plus MOPC-SS)/([^{3}H]-thymidine uptake in IL-2 alone).

Statistics

The generalized Wilcoxon test and Cox–Mantel test were used in comparing survival data. Comparison of tumor diameters was performed using Student's *t*-test and Wilcoxon test. A *P*-value of <0.05 was defined as significant.

RESULTS

Rejection of Tumor Challenge in Pretreated Mice With IL-1 and SS

For preimmunization, four groups of BALB/c mice that received IL-1 plus MOPC-SS, IL-1 alone, MOPC-SS alone, or 0.9% NaCl solution on days −10, −7, and −4, were injected intraperitoneally with MOPC cells on day 0. When preimmunized with IL-1 plus MOPC-SS, mean survival time was significantly longer than those of three other groups (IL-1 plus MOPC-SS, 46.7 days; IL-1, 20.3 days; MOPC-SS, 20.8 days; 0.9% NaCl solution, 20.9 days) (Fig. 1A). Similarly, the mice preimmunized with IL-1 plus MOPC-SS showed protective immunity against the subcutaneous challenge of MOPC cells because tumor growth on day 21 was completely suppressed (Fig. 1B).

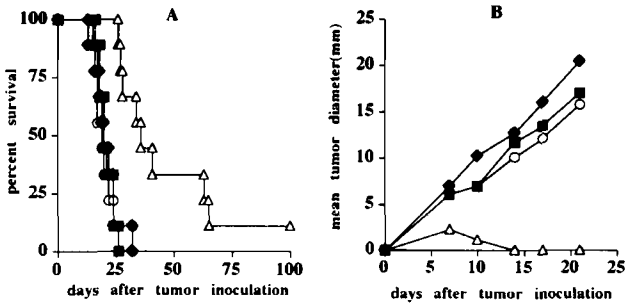

Fig. 1. Protective immunity with IL-1 plus MOPC-SS. For preimmunization, 1 ml of 0.9% NaCl solution (♦); 1 μg of IL-1 alone (○); 1 ml of MOPC-SS alone (■); or IL-1 plus MOPC-SS (△) were injected i.p. into mice (n = 5) on days 10, 7, and 4 before tumor inoculation. After preimmunization, mice received i.p. (1×10^5 cells, **(A)**) or s.c. (5×10^5 cells, **(B)**) inoculation with MOPC104E cells. Survival time (A) and mean tumor diameters (B) were represented. A: (△) versus (♦), $P = 0.00008$ by the Cox-Mantel test, B: (△) versus (♦), $P = 0.005$ by the Wilcoxon test.

TABLE I. Specific Antitumor Immunity of Preimmunized Mice

Tumor inoculated[a]	Preimmunization		
	IL-1 + MOPC-SS	IL-1 + MethA-SS	Control[b]
MOPC104E	0 ± 0[c,d]	15.6 ± 0.9[c,e]	13.2 ± 2.0[c]
MethA	11.1 ± 2.1[c,e]	0 ± 0[c,d]	10.2 ± 4.2[c]

[a]MOPC104E cells (5×10^5) or MethA cells (1×10^6) were inoculated subcutaneously on day 0 into preimmunized mice (n = 5) with IL-1 and MOPC-SS or MethA-SS on days −10, −7, −4 in the same manner as described in Fig. 1B.
[b]Mice received IL-1 alone.
[c]Mean tumor diameter (mm) ±SE on day 21.
[d]Significantly different from control using Wilcoxon test ($P = 0.005$).
[e]Value is not significant compared with control.

To investigate the specificity of antitumor activity induced by IL-1 and SS, we tested the effect of MOPC-SS or MethA-SS on subcutaneous challenge of MOPC cells or MethA cells. Immunization with IL-1 and MOPC-SS inhibited the growth of MOPC cells completely, while no inhibition on MethA growth was observed. Immunization with IL-1 and MethA-SS also inhibited the growth of corresponding MethA cells alone, showing that immunization with SS plus IL-1 is able to induce tumor specific immunity in vivo (Table I).

We next determined the minimum effective dose of

TABLE II. Dose-Response Study With Interleukin-1 and MOPC-SS on Induction of Protective Immunity

MOPC-SS (ml)	IL-1 (μg)	Mean tumor diameter ±SE (mm) on day 21	
Exp. 1			
1	1	0 ± 0[a]	(n = 10)
1×10^{-1}	1	4.4 ± 2.3[b]	(n = 10)
1×10^{-2}	1	3.6 ± 2.0[b]	(n = 9)
1×10^{-3}	1	12.1 ± 3.0[c]	(n = 5)
1×10^{-4}	1	12.2 ± 1.6[c]	(n = 5)
1×10^{-5}	1	14.9 ± 1.3[c]	(n = 5)
0	1	13.9 ± 0.7	(n = 10)
Exp. 2			
1×10^{-2}	0	12.0 ± 1.3	(n = 5)
1×10^{-2}	1×10^{-1}	0 ± 0[d]	(n = 5)
1×10^{-2}	1×10^{-2}	0 ± 0[d]	(n = 5)
1×10^{-2}	1×10^{-3}	0 ± 0[d]	(n = 5)
1×10^{-2}	1×10^{-4}	5.0 ± 3.3[e]	(n = 5)
1×10^{-2}	1×10^{-6}	6.0 ± 3.6[e]	(n = 5)
1×10^{-2}	1×10^{-8}	6.1 ± 3.1[e]	(n = 5)

[a]Significantly different from control with IL-1 alone using Student's *t*-test ($P < 0.001$).
[b]Significantly different from control with IL-1 alone using Student's *t*-test ($P < 0.01$).
[c]Not significant compared with control with IL-1 alone by Student's *t*-test.
[d]Significantly different from control with MOPC-SS alone using Wilcoxon test ($P = 0.005$).
[e]Not significant compared with control with MOPC-SS alone by Student's *t*-test.

TABLE III. Tumor Neutralizing Activity and In Vitro Monoclonal Antibody Treatment of Splenocytes of Preimmunized Mice

Preimmunization	In vitro treatment of splenocytes	Mean tumor diameter ±SE on day 21 (mm)	
Exp. 1[a]			
IL-1 + SS	—	3.4 ± 2.1[c]	(n = 5)
SS	—	14.6 ± 1.4[d]	(n = 5)
IL-1	—	16.3 ± 0.5[d]	(n = 5)
RPMI	—	11.1 ± 0.5	(n = 5)
Exp. 2[b]			
RPMI	—	12.2 ± 1.1	(n = 5)
IL-1 + SS	anti-Thy1.2 + C′[e]	10.4 ± 3.0[f]	(n = 5)
IL-1 + SS	anti-L3T4 + C′	8.6 ± 1.7[g]	(n = 7)
IL-1 + SS	anti-Lyt2.2 + C′	0 ± 0[h]	(n = 5)
IL-1 + SS	anti-AsialoGM1 + C′	1.1 ± 1.1[h]	(n = 5)
IL-1 + SS	C′	1.8 ± 1.7	(n = 8)
IL-1 + SS	—	0 ± 0	(n = 4)

[a]Splenocytes from preimmunized mice were mixed with MOPC104E cells at the E/T ratio of 30:1 and inoculated into normal mice (Exp. 1). E/T ratio = Effector-to-target ratio.
[b]Splenocytes from preimmunized mice were treated in vitro with monoclonal antibodies and mixed with MOPC104E cells at the E/T ratio of 40:1 and inoculated into normal mice (Exp. 2).
[c]Significantly different from RPMI alone using Student's *t*-test ($P < 0.02$).
[d]Not significant compared with RPMI alone using Student's *t*-test.
[e]Complement.
[f]Significantly different from complement alone using Student's *t*-test ($P < 0.02$).
[g]Significantly different from complement alone using Student's *t*-test ($P < 0.05$).
[h]Not significant compared with complement alone using Student's *t*-test.

IL-1 and MOPC-SS for induction of protective immunity. First, when immunized with 1 μg of IL-1, 0.01 ml of MOPC-SS was sufficient for induction of protective immunity (Table II, Exp. 1). Second, when immunized with minimum effective dose (0.01 ml) of MOPC-SS, 1×10^{-3} μg of IL-1 was sufficient for induction of protective immunity (Table II, Exp. 2).

Tumor Neutralizing Activity of Immunized Splenocytes and Subpopulation Analysis

The antitumor effect of immunized splenocytes was tested by tumor neutralizing assay. Splenocytes were obtained from four groups of mice which had received IL-1 plus MOPC-SS, IL-1, MOPC-SS, or RPMI alone, respectively. Only when preimmunized with IL-1 plus MOPC-SS, splenocytes showed tumor neutralizing activity (Table III, Exp. 1). Neither immunization with IL-1 alone nor MOPC-SS alone induced the activity.

We then determined the subpopulation of splenocytes responsible for tumor neutralizing activity. Splenocytes obtained from mice immunized with IL-1 plus MOPC-SS were treated with antibodies and complement and their tumor neutralizing activity was evaluated. Tumor neutralizing activity was abrogated when splenocytes were treated with anti-Thy1.2 or anti-L3T4 plus complement. On the contrary, treatment with anti-Lyt2.2 or anti-asialo GM1 did not interfere with the activity (Table III, Exp. 2). Thus, L3T4 (CD4) positive cells were responsible for this tumor neutralizing activity.

Moreover, we determined the subpopulation responsible for induction of the protective immunity with in vivo treatment using monoclonal antibodies. Only when treated with anti-CD4 monoclonal antibody, protective immunity did not develop, while treatment with anti-CD8 or anti-asialo GM1 did not inhibit its induction (Table IV). This result shows that the CD4-positive population has an important role in induction of protective immunity.

IL-2-Enhanced MLTR

We examined whether SS-dependent proliferative response of splenocytes could be induced by immunization with IL-1 plus MOPC-SS. When immunized with IL-1 alone, [^{3}H]-thymidine uptake higher than other groups regardless of addition of IL-2 or MOPC-SS. When immunized with IL-1 plus MOPC-SS, [^{3}H]-thymidine uptake was significantly increased by addition of MOPC-SS at a concentration from 0.4% to 3.7% (3.7% and 1.2%, $P < 0.001$; 0.4%, $P < 0.02$; Table V). Moreover, the stimulation index of this group was higher than three other groups. But, no enhancement of [^{3}H]-thymidine

TABLE IV. Simultaneous In Vivo Monoclonal Antibody Treatment With Preimmunization*

Preimmunization	In vivo treatment with antibody	Mean tumor diameter ±SE on day 21 (mm)	
IL-1 + SS	Antimouse IgG[a]	0 ± 0	(n = 5)
IL-1 + SS	Anti-CD4(GK1.5)[b]	9.2 ± 2.5[e]	(n = 5)
IL-1 + SS	Anti-CD8(Lyt2.2)[c]	2.5 ± 2.4[f]	(n = 4)
IL-1 + SS	Anti-AsialoGM1[d]	0 ± 0[f]	(n = 4)
RPMI	RPMI	10.6 ± 1.9	(n = 5)

*Mice were injected intraperitoneally with IL-1 and SS on days −10, −7, and −4 and inoculated s.c. with MOPC cells (5×10^5) on day 0.
[a]Mice were injected intravenously with rat antimouse IgG (50 μl/mouse, day −10).
[b]Mice were injected intravenously with GK1.5 (50 μl/mouse, days 10, −6, and −3).
[c]Mice were injected intravenously with Lyt2.2 (100 μl/mouse, day −10).
[d]Mice were injected intravenously with anti-asialo GM1 (20 μl/mouse, day −10, −3, and 4).
[e]Significantly different from IgG alone using Wilcoxon test ($P = 0.005$).
[f]Not significant compared with IgG alone.

uptake by MOPC-SS was observed if no IL-2 added. Thus, these results showed that immunization with IL-1 alone could activate lymphocyte proliferation nonspecifically, but immunization with IL-1 plus MOPC-SS could activate SS dependent lymphocyte proliferation in the presence of IL-2.

Therapeutic Activity of IL-1 Plus SS Against Established Tumor in the Presence of IND

We determined whether IL-1 plus MOPC-SS could inhibit the growth of established subcutaneous tumor. Immunization on days 5, 8, and 11 with IL-1 plus MOPC-SS could suppress established subcutaneous tumor inoculated with 2.5×10^5 MOPC cells on day 0. The mean tumor diameter on day 39 of mice treated with IL-1 plus MOPC-SS was significantly smaller than three other groups, but its suppressive effect was limited, and only two of 9 mice were tumor free (Fig. 2A).

Moreover, we examined whether IND could augment the antitumor activity induced by IL-1 plus MOPC-SS. Mice inoculated subcutaneously with MOPC cells of 3×10^5 were treated with IL-1 plus MOPC-SS with or without per oral IND. The mean tumor diameter on day 33 of mice treated with IL-1 plus MOPC-SS in combination with IND was significantly smaller than that of the group treated with IL-1 plus MOPC-SS alone (IL-1 plus MOPC-SS with IND, 7.8 ± 2.6 mm; IL-1 plus SS alone, 16.4 ± 2.8 mm; Fig. 2B). Five of 10 mice treated with IL-1 plus MOPC-SS in combination with IND were tumor free.

In another experiment, mice had their tumor-bearing feet amputated and postoperative treatment was performed. When treated with IL-1 plus MOPC-SS in combination with per oral IND, the mean survival time was significantly longer than those of groups treated with 0.4% ethanol or IND alone (IL-1 plus MOPC-SS with IND, 58.8 days; IL-1 plus MOPC-SS alone, 45 days; IND alone, 39.6 days; 0.4% ethanol alone, 38.9 days; Fig. 3). Moreover, there was a tendency for a life-prolonging effect of IL-1 plus SS in combination with IND compared with IL-1 plus SS alone (Fig. 3). These data indicate that IND augments antitumor activity of IL-1 plus SS.

DISCUSSION

In the present study, we demonstrated that IL-1 plus SS could induce specific antitumor immunity in a preimmunization model and in therapy model especially when combined with IND.

Several reports indicated that IL-1 has cytotoxic effects against tumor cell both in vivo and in vitro [24–27]. IL-1 was also reported to be a potent activator of T and B lymphocytes and to be critical in amplifying the response to specific antigen and mitogens [15,16]. Since the activity of IL-1 as an immunologic adjuvant was demonstrated first by Staruch and Wood in 1983 [28], many researchers have investigated adjuvant activity of IL-1 in conjugation with infectious or tumor antigens [11,29,30]. McCune and Marquis [11] employed IL-1 or its peptide as an adjuvant with antitumor vaccine consisting of irradiated tumor cells. These investigators showed that mice treated with vaccine plus IL-1 became 70–100% tumor free, whereas mice treated with vaccine alone were only 0–20% tumor free.

Our experiment differs from theirs on two points. First, we did not employ tumor cells but sonicated tumor supernatant as the source of tumor antigen [31]. The comparison between irradiated tumor cells (2×10^7) and SS prepared from 2×10^7 cells showed no significant difference in the induction of protective immunity (data not shown). In addition, SS prepared from 2×10^5 was sufficient for its induction (Table II, Exp. 1). The application of SS, which is easy to prepare and contains autologous tumor antigen, enables us to overcome the ethical or technical problems accompanied by clinical use of tumor cells. Furthermore, it became feasible to immunize tumor bearers repeatedly and whenever needed in the same quality. The antigenic activity of crude sonicated lysates after centrifugation existed in supernatant, but not in pellet. Moreover, only fraction of SS over 30,000 of M_r could induce protective immunity (data not shown). Second, minimal effective dose of IL-1 (1 ng × 3/mouse) in our model necessary for protective immunity was much lower than their effective dose (120 ng × 8/mouse) [11]. So our therapy model is safer because IL-1 at higher dose may have severe toxicities [15]

The potent effector mechanism of protective immunity induced by preimmunization with IL-1 plus SS was evalu-

TABLE V. ^{3}H-Thymidine Uptake in IL-2 Enhanced Mixed Lymphocyte Tumor Reaction*

Pretreatment	Concentration of SS (IL-2 45 JRU/ml) 0%	0.4%	1.2%	3.7%	11%	Con A alone (2 μg/ml)
IL-1 + SS	4.8 ± 0.3[a]	10.1 ± 1.6[c] (2.1 ± 0.3)[b,e]	8.8 ± 0.2[d] (1.8 ± 0.1)	9.9 ± 0.2[d] (2.0 ± 0.1)[f]	0.2 ± 0.02 (0.04 ± 0.01)	22.3 ± 0.5
IL-1	9.7 ± 1.8	11.2 ± 1.1 (1.2 ± 0.1)	12.9 ± 2.1 (1.3 ± 0.2)	13.9 ± 0.8 (1.4 ± 0.1)	1.8 ± 1.2 (0.2 ± 0.1)	20.4 ± 1.4
SS	2.8 ± 0.2	4.8 ± 0.6[c] (1.7 ± 0.2)[e]	3.1 ± 0.8 (1.1 ± 0.3)	2.2 ± 0.1 (0.8 ± 0.04)	0.2 ± 0.06 (0.07 ± 0.02)	24.8 ± 2.0
RPMI	0.9 ± 0.1	1.0 ± 0.2 (1.0 ± 0.2)	1.4 ± 0.3 (1.5 ± 0.3)	0.7 ± 0.04 (0.8 ± 0.04)	0.2 ± 0.008 (0.2 ± 0.01)	20.2 ± 1.4

*Spleens from preimmunized mice with IL-1 plus SS, IL-1 alone, SS alone or RPMI days 10, 7, and 4 prior to their harvest were removed. Splenocytes (5×10^5/well) were cultured in complete medium with 45 JRU/ml IL-2 with a graded concentration of MOPC-SS in a 96-well flat bottomed microtest plate for 4 days, and [^{3}H]-thymidine was added to each well for the last 18 h of incubation.

[a] ^{3}H-thymidine uptake (cpm ±SE/10^3).

[b] Stimulation index (±SE).

[c] Significantly different compared with control group without SS ($P < 0.02$).

[d] Significantly different compared with control group without SS ($P < 0.001$).

[e] Significantly different compared with control group with RPMI alone ($P < 0.02$).

[f] Significantly different compared with control group with RPMI alone ($P < 0.001$).

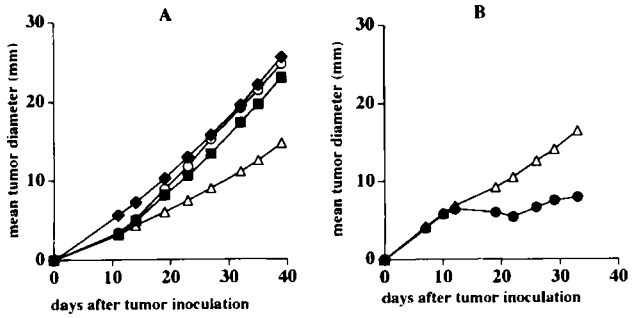

Fig. 2. Therapeutic effect of IL-1 plus SS and augmentation by IND on s.c. tumor. **A:** Mice inoculated s.c. with 2.5×10^5 MOPC104E cells on day 0 were injected i.p. with 1 ml of RPMI (n = 8, ♦); IL-1 alone (n = 5, ○); SS alone (n = 5, ■); IL-1 plus SS (n = 9, △) on days 5, 8, and 11. Established tumor diameters were measured until day 39. (♦) versus (△), $P < 0.02$ by Student's t-test. **B:** Mice inoculated s.c. with 3×10^5 MOPC104E cells on day 0 were injected i.p. with IL-1 plus SS on days 5, 8, and 11 with (n = 10, •) or without p.o. IND (n = 10, △). Established tumor diameters were measured until day 33. IND was added in drinking water at a concentration of 40 μg/ml. (•) versus (△), $P < 0.05$ by Student's t-test. Tumor diameter in IL-1 plus SS without IND was not significantly different from those in RPMI with or without IND (data not shown).

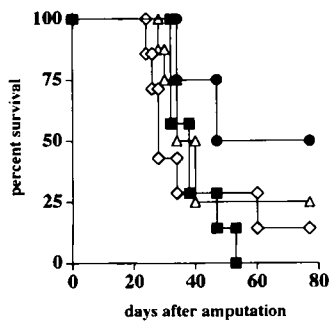

Fig. 3. Therapeutic effect of active specific immunotherapy and IND in postoperative therapy model. Mice were inoculated s.c. with 1×10^6 MOPC cells on day −21; their tumor-bearing feet were amputated on day 0. They were treated as follows; 1 ml of RPMI alone (days 4, 7, 10, i.p.), ■; 1 ml of RPMI + IND, ◇; IL-1 (1μg) plus SS (1ml) (days 4, 7, 10, i.p.), △; IL-1 (1 μg) plus SS (1 ml) + IND, •. IND was added in drinking water at the concentration of 20 μg/ml. (•) versus (◇), $P = 0.022$; (•) versus (■), $P = 0.022$; (•) versus (△), $P = 0.064$ by generalized Wilcoxon test.

ated by both in vitro and in vivo depletion models. When splenocytes of immunized mice with IL-1 plus SS were treated with anti-Thy1.2 or anti-L3T4, their tumor neutralizing activity was abrogated (Table III, Exp. 2). Moreover, in vivo depletion of the CD4-positive subpopulation inhibited the induction of protective immunity (Table IV). It has been suggested that the expression of IL-1 receptor is restricted to the L3T4-positive subset of mature T lymphocytes [32]. These results indicated that the main population operating at induction phase or transplantability of protective immunity in this therapy was not Lyt2.2-positive (CD8) cells but L3T4-positive (CD4) cells. However, in vivo antibody treatment of preimmunized mice after tumor inoculation suggested that a CD8-positive subpopulation was involved in tumor eradication (data not shown).

We speculate the mechanism as follows: first, helper T cells (CD4+) are activated by SS and antigen presenting cells (APCs), where exogenous IL-1 plus SS enhances SS-specific T-cell activation and proliferation in collaboration with endogenous IL-1, and second, helper T cells activate other populations that act as the final tumoricidal effector, including Lyt2.2+ cells. It was reported that IL-1 activates T-cell proliferation but does not affect recognition of T cell [33]. But the presence of IL-1 receptors on dendritic cells has been postulated because IL-1 appeared to amplify the function of dendritic

cells in mice [34] and human [35]. Further studies on the direct effect of IL-1 on APCs are needed.

In the therapeutic model, IL-1 plus SS had only marginal effect on subcutaneous inoculation of no more than 2.5×10^5 MOPC cells (Fig. 2A). However, IL-1 plus SS combined with IND was found to display apparent antitumor activity on both subcutaneous tumor and the postoperative therapeutic model (Figs. 2B and 3). Two possible mechanisms are suggested: (1) SS might be presented preferably by APCs as compared with viable tumor in tumor-bearing mice, which are supposed to be immunosuppressive; or (2) PGE_2 production, which might be induced in the tumor-bearing host, is inhibited by IND. Thus the effective antitumor immunity could be induced and augmented with IND in the tumor-bearing host.

Thus, it is suggested that there are some differences between immunization and the therapeutic model, including toxicity of IL-1 and SS. For clinical application, further studies on combined therapy with other agents, including chemotherapeutic agents, cytokines, or biological response modifiers, in addition to IND, will be needed to augment the efficacy of active specific immunotherapy.

CONCLUSION

The immunization using IL-1 plus sonicated tumor supernatant was a highly effective method of active specific immunotherapy, especially in combination with IND. In the near future, immunization using IL-1 plus SS will become a valuable basis for a new active specific immunotherapy against human cancer.

ACKNOWLEDGMENTS

We thank the staff at the Animal Experiment Center, Kyoto University and Radioisotope Research Center, Kyoto University, where part of this study was performed. We also thank Ms. Aiko Tanaka for her assistance.

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