Augmentation of Anti-tumor Immunity in Low-responder Mice by Various Biological Response Modifiers: Analysis of Effector Mechanism

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In order to elucidate the role of biological response modifiers (BRMs) in anti-tumor immunotherapy, we examined their effect on the induction of anti-tumor immunity in low-responder mice which hardly exhibit anti-tumor resistance against syngeneic Rous sarcoma virus (RSV)-induced tumors, such as B10 or B10.BR mice. The anti-tumor immunity induction in the low-responder mice was 0% on immunization with mitomycin C-treated syngeneic tumor cells alone. However, if BRMs were used as an adjuvant, BCG cell wall skeleton, OK-432 or lentinan augmented the induction of anti-tumor immunity to 50%, 33% and 33%, respectively. In the low-responder mice treated with BRMs, the anti-tumor immune cells had antigen-specificity at the induction phase of *in vitro* restimulation but not at the effector phase of target cell lysis by the stimulated cells. When T cells were depleted from immune spleen cells just before *in vitro* stimulation, cytotoxicity was not induced. Furthermore, cytotoxicity was not induced if accessory cells were removed from immune spleen cells at the induction phase. However, cytotoxicity at the effector phase was not mediated by T-lymphocytes, but by non-T cells. These results suggested that the induced cytotoxicity in low-responder mice was associated with the delayed-type hypersensitivity-like effector mechanism.

Key words: Anti-tumor immunity — BRM — Tumor immunotherapy

Using Rous sarcoma virus (RSV)-induced tumor raised in B10 congenic mice, we have previously reported that the immune response against virus-induced tumor was controlled by a gene(s) mapped in the major histocompatibility complex (MHC). B10 H-2 congenic and recombinant mice can be divided into two types as judged from their ability to induce anti-tumor immunity and cytotoxic T lymphocyte (CTL) response against RSV-induced syngeneic tumor. One is designated as high-responder mice, capable of inducing anti-tumor resistance *in vivo* as well as CTL response *in vitro* on immunization with attenuated syngeneic RSV-induced tumor, and the other as low-responder mice which can hardly induce anti-tumor immunity either *in vivo* or *in vitro*. The high-responder strain had H-2^d haplotype in the H-2D region and the low-responder strain had H-2 haplotype other than H-2^d in the H-2D region. However, anti-tumor immunity was hardly induced in the low-responder mice even after repeated immunization with attenuated syngeneic RSV-induced tumor cells. Furthermore, the cytotoxic activity of effector cells in low-responder mice did not show any target cell specificity.[1] It was reported that anti-tumor immunity was mediated not only by cytotoxic T cells but also by non-cytotoxic T cells in some experimental systems. Recent studies have revealed that anti-tumor immunity *in vivo* can be transferred by CD4-positive T cells which have been considered to be a T cell subset with helper/delayed-type hypersensitivity (DTH) function but not with killer/suppressor function.[2-5] Lymphokines derived from CD4-positive T cells can activate macrophages resulting in cytocidal cells against tumors.[6, 7] Various biological response modifiers (BRMs), especially immunomodulators such as streptococcal preparation (OK-432), Bacillus Calmette-Gúerin cell wall skeleton (BCG-CWS) and polysaccharide extracted from the edible mushroom *Lentinus edodes* (lentinan), have been widely used as anti-tumor agents both experimentally and clinically.[8-12] Although the mechanism(s) of BRMs action is not precisely clear, it is generally believed that the anti-tumor activity of BRM is mediated through its immunologically augmentative properties, such as stimulation of lymphocytes or monocytes to produce lymphokines or monokines, resulting in the induction of activated macrophages, natural killer (NK) cells, CTLs, etc.[13-16] It is known that not only immunological responses to various antigens but also host responses to BRMs are genetically regulated.[17, 18] Therefore, we have used congenic strains of mice and syngeneic tumor systems to evaluate the mode of BRM action on the anti-tumor immunity. In the present study, we examined the effect of BRMs on the induction of anti-tumor immunity against RSV-induced tumors, and analyzed the effector mechanism(s) of anti-tumor immunity in low-responder mice.

MATERIALS AND METHODS

Mice and tumors Male B10 congenic mice were purchased from Shizuoka Agriculture Cooperative Association for Laboratory Animals (Hamamatsu). Ten- to fourteen-week-old mice were used for experiments. The RSV-induced tumors, S1018B10(H-2^b), S623BR(H-2^k) and S908D2(H-2^d) were originally raised in syngeneic newborn mice by injection of the Schmidt-Ruppin strain of RSV-infected chicken tumor cells. EL-4(H-2^b) leukemia induced by dimethylbenzanthracene in C57BL/6, YAC-1(H-2^a) induced by mouse leukemia virus in A/Sn and P815(H-2^d) mastocytoma in DBA/2 were maintained by stationary culture in RPMI-1640 medium (Flow Laboratories, Inc., McLean, VA) supplemented with 4% fetal bovine serum (FBS; Bocknek Laboratories Inc., Toronto), 50 μg/ml of kanamycin sulfate (Banyu Pharm. Co., Ltd., Tokyo).

MMC treatment Tumor cells were washed once with Eagle's minimum essential medium (MEM; Flow Laboratories, Inc.), suspended in MEM and then incubated with 100 μg/ml of MMC (Kyowa Hakko Kogyo Co., Ltd., Tokyo) at 37°C for 45 min at a cell concentration of 1×10^7/ml. After incubation, tumor cells were washed three times with MEM, and used for *in vivo* immunization and *in vitro* stimulation.

Immunization procedure Mice were divided into 13 groups, each of which contained at least 6 mice (see Table I). Mice subcutaneously received 1×10^6 MMC-treated syngeneic tumor cells as the immunization antigen with or without various BRMs four times at two-week intervals. Two to four weeks after the last immunization, mice were challenged subcutaneously with 5×10^5 viable syngeneic tumor cells.

BRM treatment BCG-CWS was kindly supplied by Dr. I. Azuma (Institute of Immunological Science, Hokkaido University). Oil-attached BCG-CWS was prepared according to the method of Azuma et al.[19] Briefly, 6 mg of BCG-CWS were ground with 3 drops of oil (Drakeol 6VR) in a hand homogenizer and suspended in 3 ml of sterile saline which contained 0.2% Tween 80 (Tween-saline) to make an oil-in-water emulsion. Then 0.05 ml of this emulsion was mixed with 0.05 ml of MMC-treated tumor cells (2×10^7/ml) and injected into mice.

Dried streptococci (OK-432, Chugai Pharm. Co., Ltd., Tokyo), 0.1 mg, equivalent to 1.0 KE (Klinische Einheit), were suspended in 0.05 ml of Tween-saline, mixed with 0.05 ml of MMC-treated tumor cells (2×10^7/ml) and injected into mice.

Lentinan, 1 mg (Yamanouchi Pharm. Co., Ltd., Tokyo), was dissolved in 10 ml of sterile distilled water. Following each immunization, 10 ml/kg body weight/day of lentinan solution was given intraperitoneally on five successive days.

In vitro restimulation of immunized spleen cells Single cell suspension of spleens from the immunized mice was prepared, washed with MEM and resuspended in Dulbecco's modified MEM (DMEM; Flow Laboratories Inc.) supplemented with 5% FBS, 20 mM HEPES, 5 × 10^{-5} M 2-mercaptoethanol and 100 μg/ml of kanamycin. The immune spleen cells were cultured with MMC-treated tumor cells at a ratio of spleen to tumor cells of 300 to 1 at 37°C in a humidified CO$_2$ incubator for 5 days.

Cytotoxicity assay The *in vitro* restimulated spleen cells were harvested and tested for their cytotoxic activity by ^{51}Cr release assay. Target cells were labeled with Na$_2$^{51}CrO$_4$ (New England Nuclear, Boston, MA) for 45 min at 37°C in a humidified CO$_2$ incubator. The labeled tumor cells were washed three times with MEM and resuspended in the supplemented DMEM. ^{51}Cr-labeled target cells (1.5×10^4) and serial dilutions of effector cells in a total volume of 200 μl were plated in 96-well round-bottomed micro titer plates (Linbro, Flow Laboratories, Inc.) and incubated for 12 h at 37°C in a humidified CO$_2$ incubator. After the incubation, the supernatants were taken from the reaction mixture by centrifugation at 500g for 5 min and released radioactivity was measured in a well-type gamma counter (Aloka Co. Ltd., Model ARC-301, Tokyo). The cytotoxic activity was expressed in terms of percentage of specific release and calculated by use of the following formula;

$$\% \text{ specific release} = \frac{\text{cpm(stimulated cells)} - \text{cpm(normal cells)}}{\text{cpm(maximal release)} - \text{cpm(normal cells)}} \times 100.$$

The maximum count was determined by disrupting the target cells in 1.25% saponin solution, and amounted to more than 90% of the total radioactivity incorporated into the target cells.

Antibody treatment GK1.5 monoclonal antibody-producing hybridoma line,[20] specific for L3T4a determinant of the L3T4 molecule, was a gift from Dr. F. Fitch, the University of Chicago, IL, through Dr. D. H. Sachs, National Institutes of Health, Bethesda, MD, and Dr. K. Saito, Keio University School of Medicine, Tokyo. Ascitic fluids were obtained from BALB/c *nu/nu* mice which were pristane-primed and inoculated intraperitoneally with GK1.5 hybridoma cells. Gamma globulin fraction was prepared by precipitation at 40% saturation with ammonium sulfate and used as monoclonal antibody fraction. The monoclonal anti-Thy-1.2 antibody (Clone HO13.4.9)[21] was the gift of Dr. H. Ishikawa, Keio University School of Medicine, Tokyo. Spleen cells were treated with monoclonal anti-Thy-1.2 or anti-L3T4 antibody at a dilution of 1:100 or 1:50, respectively. Cells were then washed and incubated for 45 min at 37°C with a 1:15 dilution of rabbit complement (C).

Treatment of spleen cells with nylon wool or Sephadex G-10 column Spleen cells (1×10^8 cells/ml) were passed through either a nylon wool or a Sephadex G-10 column according to Julius et al.[22] or Ly and Mishell,[23] respectively.

Preparation of splenic macrophages Splenic macrophages were enriched on a bovine serum albumin (BSA) gradient according to the method described by Glimcher et al.[24] Briefly, the gradient was prepared by layering 2.5 ml of 11% BSA containing 2×10^8 spleen cells over 2.5 ml of 35% BSA and centrifuging in an RPS50 rotor in a Hitachi 65P centrifuge (Hitachi, Tokyo) at 4°C for 30 min. Cells banding between 35% and 11% were collected. They were comprised of about 25% macrophages as measured by latex bead phagocytosis.

RESULTS

Augmentation of anti-tumor immunity by the use of BRMs We examined whether or not BRMs were able to augment the anti-tumor immunity in such low-responder mice. As shown in Table I, when the low-responder mice were immunized with MMC-treated syngeneic RSV-induced tumor cells alone, anti-tumor immunity was not induced. However, if BRMs were co-administered to the low-responder mice with MMC-treated tumor cells,

BRMs were able to generate anti-tumor immunity. The numbers of B10 and B10.BR mice which acquired immunity on day 21 were 4/7 and 3/6 of BCG-CWS-treated mice, 3/6 and 2/6 of OK-432-treated mice, 2/6 and 2/6 of lentinan-treated mice, respectively, whereas the mice treated with BRM alone did not show anti-tumor immunity. The augmenting effect of BRMs on inducibility of anti-tumor immunity was clearly found in low-responder mice, and it seemed to be dose-dependent. On the other hand, BRMs were not so effective in the augmentation of anti-tumor immunity in high-responder mice, such as B10.D2.

Specificity of effector cells induced in low-responder mice treated with BRMs In order to characterize the effector cells which were induced in low-responder mice by immunization with syngeneic RSV-induced tumor cells together with various BRMs, immune spleen cells from mice which had become resistant to challenge with syngeneic RSV-induced tumor were tested for their specific cytotoxic activity against the target tumor cells. As shown in Table II, the spleen cells from immune B10 or B10.BR mice which induced anti-tumor immunity showed cytotoxic activity against not only syngeneic RSV-induced tumor, such as S1018B10 or S623BR, but also unrelated tumors, such as EL-4, YAC-1 and P815. There was neither MHC-restricted nor antigen-specific

Table I. Effect of BRMs on the Induction of Anti-tumor Immunity against RSV-induced Tumor in B10 Congeneic Mice

Treatment[b]	Anti-tumor immunity[a]		
	S1018B10	S623BR	S908D2
None	0/12	0/12	0/11
Vehicle	0/11	0/11	0/11
MMC-tumor alone	0/12	0/12	7/11
BCG-CWS 100 μg alone	0/6	0/6	0/6
BCG-CWS 100 μg + MMC-tumor[c]	4/7[e]	3/6[e]	4/6
OK-432 1.0 KE alone	0/6	0/6	0/6
OK-432 1.0 KE + MMC-tumor	3/6[e]	2/6[f]	5/6
OK-432 0.25 KE + MMC-tumor	2/6[f]	2/6[f]	2/6
OK-432 0.1 KE + MMC-tumor	2/6[f]	0/6	3/6
LNT 1.0 mg/kg/day alone	0/6	0/6	0/6
LNT 1.0 mg/kg/day + MMC-tumor[d]	2/6[f]	2/6[f]	2/6
LNT 0.25 mg/kg/day + MMC-tumor	0/6	1/6	4/6
LNT 1.0 mg/kg/day + MMC-tumor	2/6[f]	0/6	4/6

a) Number of tumor-free mice/number of mice inoculated.

b) B10, B10.BR or B10.D2 mice were immunized with MMC-treated tumor cells (1×10^6) with or without BRM four times at two-week intervals and two weeks after the last immunization, mice were challenged with syngeneic tumor cells (5×10^5).

c) A mixture of each BRM and MMC-tumor cells was sc injected.

d) Lentinan (LNT) was administered ip for 5 successive days after each immunization period.

e, f) Significantly different from the group immunized with MMC-treated tumor alone ($P < 0.01$, $P < 0.05$ by χ^2-test respectively).

Table II. Specificity of Cytotoxic Effector Cells Induced in B10 Congeneic Mice

Effector[a]		% specific ^{51}Cr release from target[b]					
		RSV-induced tumor			P815	YAC-1	EL-4
Mice	Treatment	S1018B10	S623BR	S908D2			
B10	BCG-CWS	11.2	5.8	55.6	20.6	35.1	20.8
	OK-432	13.8	8.6	17.1	17.8	26.4	20.4
	LNT	25.1	25.4	22.7	13.6	19.8	13.1
B10BR	BCG-CWS	12.3	12.9	35.6	25.0	63.3	14.5
B10D2	BCG-CWS	−2.8	−0.4	17.2	2.0	2.4	3.0
	OK-432	−7.0	−3.3	15.6	−3.6	2.8	2.6
	LNT	−4.6	−3.3	13.5	−3.7	−0.1	1.7

a) The splenocytes obtained from immunized mice were restimulated *in vitro* with their syngeneic RSV-induced tumor for five days.
b) The effector:target ratio was 20:1.

Table III. Effect of Treatment with Anti-Thy-1.2 Monoclonal Antibody or G-10 Column on the Cytotoxic Activity Induced in B10 Congeneic Mice

	Effector	Target	Treated with	% specific ^{51}Cr release[a]
Exp. 1	B10	EL-4	None	29.4±0.4
			C[b]	30.3±0.9
			a-Thy1.2 +C[c]	28.5±1.1
	B10BR	EL-4	None	30.1±0.9
			C	28.8±1.1
			a-Thy1.2 +C	31.7±0.7
	B10D2	S908D2	None	19.0±1.8
			C	17.6±1.8
			a-Thy1.2 +C	−1.7±0.6
Exp. 2	B10	EL-4	None	8.4±0.2
			G-10 passed[d]	−0.5±0.5

a) The effector:target ratio was 20:1.
b) C means rabbit complement.
c) Restimulated splenocytes were treated with anti-Thy1.2 plus rabbit complement and washed with medium.
d) Restimulated splenocytes were passed through a Sephadex G-10 column and washed with medium.

cytotoxic activity toward target cells in low-responder strains. On the other hand, effector cells, which were induced in B10.D2 high-responder mice by the same immunization procedure as that in low-responder mice, showed specific cytotoxic activity to syngeneic RSV-induced tumor (S908D2).

Characteristics of effector cells induced by BRMs in low- or high-responder mice Because the cytotoxic effector cells induced in low-responder mice showed H-2 non-restricted and antigen non-specific killing, the characteristics of the effector cells were examined. To test whether or not the effector cells are T cells, the induced effector cells were treated with anti-Thy-1.2 antibody and complement. As shown in Exp. 1 of Table III, their cytotoxic

activity was not removed in low-responder strains. However, in the case of cytotoxic effector cells in high-responder mice, their cytotoxic activity was completely removed by the treatment. These results indicated that the effector cells induced in low-responder mice by BRMs were a non-T cell population, but effector cells induced in high-responder mice by BRMs were T cells.

Specificity of primed spleen cells of low-responder mice at the induction phase As the effector cells induced in low-responder mice by BRMs showed non-specific cytotoxic activity and non-T cell characteristics, it was assumed that the cytotoxicity was associated with a population of either macrophages or NK cells. Although it is known that BRMs augment NK cell activity as well as
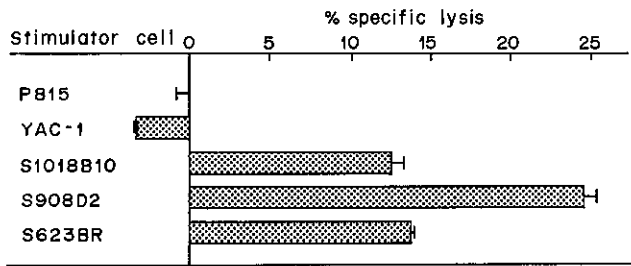
Fig. 1. Specific activation of killer cells in low-responder mice. Immune spleen cells, obtained from immunized B10 mice simultaneously treated with BCG-CWS, were cultured for 5 days with each indicated tumor cells line at a responder:stimulator ratio of 300:1. The cytotoxic activity was measured by 16 h ^{51}Cr release assay at an effector: target ratio of 20:1.
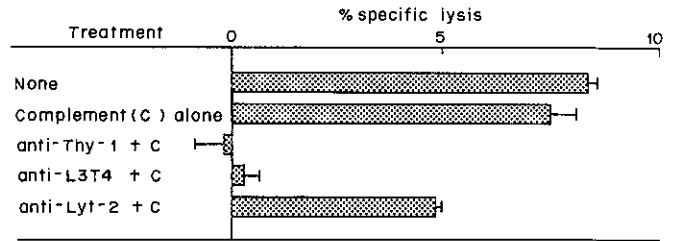

Fig. 2. Effect of antibodies on the induction of cytotoxic activities. Immune spleen cells, obtained from immunized B10 mice simultaneously treated with BCG-CWS, were treated with monoclonal antibodies and rabbit complement. The final dilution of antibody was 100:1 for anti-Thy-1.2 or 50:1 for anti-L3T4. The treated spleen cells were stimulated with MMC-treated S1018B10 for 5 days. The cytotoxic activity was measured by 16 h ^{51}Cr release assay at an effector:target ratio of 20:1.

macrophge activity,[13, 25-27] our previous report suggested that the effector cells induced in low-responder mice were not NK cells but macrophages,[1] because the cytotoxic activity of the effector cells induced in low-responder mice was not removed by treatment with anti-brain associated T cell antigen (anti-BAT) which can remove NK cells in the presence of rabbit complement. If so, some lymphokines released from antigen-primed T cells by antigenic stimulation might be required for activation of macrophages. Thus, the requirement of specific antigen stimulation for the primed spleen cells was examined at the induction phase. As shown in Fig. 1, only RSV-induced tumor cells were able to induce cytotoxic effector cell activity, whereas all other tumors were unable to do so. As the tumor-associated antigen is common among the different tumor cells induced by the same virus,[28, 29] the primed spleen cells might receive the common antigen-specific stimulation of RSV-induced tumor cells. Moreover, as shown in Exp. 2 of Table III, although the total killing activity was low, the induced cytotoxic activity was completely removed by passage through a G-10 column. Therefore, we concluded that the non-specific cytotoxic activity induced by BRMs might be due to macrophages which were activated by the antigen-specific immune cells.

Phenotype of primed spleen cells which can induce the non-specific effector cells in low-responder mice Since the specific antigen stimuration of primed spleen cells with RSV-induced tumor cells was required for the activation of non-specific cytotoxic effector cells, it was considered that specific immune T cells might be involved in this activation process. In order to examine this possibility, the primed spleen cells were treated with monoclonal antibodies directed to various T cell markers and complement just before *in vitro* stimulation. As shown in Fig. 2, the cytotoxic activity was not induced in the
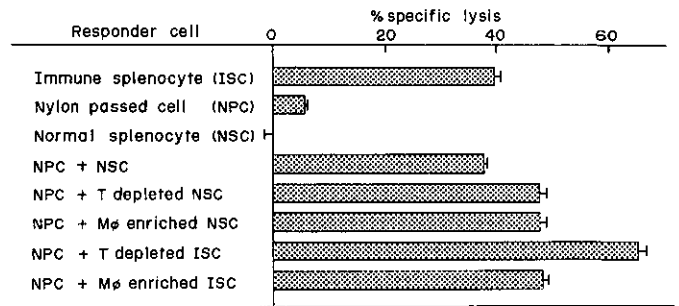

Fig. 3. Requirement of splenic adherent cells to achieve the induction of cytotoxic activity. Immune spleen cells obtained from B10 mice (see the legend to Fig. 1) were passed through a nylon wool column (NPC). Splenic adherent cells, which were added back to the NPC at a ratio of add-back-cell:NPC= 25:1, were collected from the immune splenocytes by the use of density gradient centrifugation or a nylon wool column. The treated spleen cells were stimulated with MMC-treated S1018B10 for 5 days. The cytotoxic activity was measured by 16 h ^{51}Cr release assay at an effector:target ratio of 20:1.

immune spleen cells treated with either anti-Thy-1.2 or anti-L3T4 antibody and complement, but not with anti-Lyt-2.2 antibody. These results indicate that at least specific T cells having L3T4-positive and Lyt-2-negative phenotype are involved in the activation mechanism of the non-specific cytotoxic effector cells in low-responder mice.

Requirement of both nylon wool column-passed immune T cells and macrophages for the induction of killer cells In order to confirm the necessity of both immune T cells and macrophages to generate the non-specific cytotoxic effector cells in low-responder mice, nylon-passed

immune T cells with or without either T cell-depleted spleen cells or enriched splenic adherent cells were stimulated with MMC-treated RSV-induced tumor cells at the induction phase and cultured for 5 days. As shown in Fig. 3, T lymphocytes enriched by passage through a nylon wool column did not show any significant cytotoxic activities. With the addition of either T cell-depleted normal or immune spleen cells or enriched normal or immune splenic adherent cells to the T cell enriched fraction, induction of cytotoxic activity was restored. Therefore, both antigen-primed T cells and macrophages were essential for the induction of non-specific cytotoxic effector cells in the low-responder strains.

DISCUSSION

As we previously reported, it was hard to induce both CTL activity *in vitro* and anti-tumor immunity *in vivo* against RSV-induced tumor in low-responder mice such as B10 or B10.BR.[1] However, when we used some BRMs as adjuvants, we could augment the anti-tumor immunity induction (Table I). On the other hand, anti-tumor immunity was easy to induce in the high-responder mice such as B10.D2 without using BRMs either *in vivo* or *in vitro*. Thus, it was considered that the anti-tumor resistance was associated with the CTL induction capability in the high-responder mice. However, we were not successful in detecting antigen-specific cytotoxicity in the low-responder mice which had become resistant to syngeneic tumor by BRMs treatment. Therefore, we considered that the anti-tumor resistance in low-responder mice was due to the induction of non-specific effector cells other than the induction of CTL. The activation of non-specific cytotoxic cells required antigen-specific stimulation of RSV-induced tumor cells at the induction phase, and the cytotoxic activity was not directly associated with T cells at the effector phase. From the above results, it was concluded that the final effector cells belonged to neither the NK cell nor lymphokine-activated killer cell population. Moreover, the cytotoxic activity was removed by passage through a G-10 column, thus suggesting that the final effector cells were macrophages.

It was reported that the non-cytolytic T lymphocytes which had CD4-positive phenotype could eradicate tumor cells *in vivo*[30, 31] and also that CD4-positive lymphocytes could release the macrophage activating factor.[6, 32] We also found that antigen-specific CD4-positive T cells were an essential requirement for the induction of cytotoxic activity in low-responder mice.

Based on these results, we hypothesized that the anti-tumor immunity induced by BRMs in low-responder mice consisted of the following three steps; first, CD4$^+$8$^-$ T cells (probably DTH-T cell) recognize the tumor-associated antigen, such as RSV-related determinants which are expressed on the antigen-presenting cells; second, such DTH-T cells release some lymphokine(s) resembling macrophage activating factor, such as interferon γ, etc.[30-32] for the activation of macrophages; third, the activated macrophages as final effector cells kill the tumor cells. As BRMs are able to stimulate both T cells and macrophages,[13-16] BRMs may play an important role in either the stimulation stage of DTH-T cells to produce the lymphokines or the activation stage of macrophages to kill the tumor in the low-responder group.

According to the results shown in Table I as well as in our previous report, we assumed that the anti-tumor immunity which was caused by such DTH-type T cells and macrophage system might have little potency to eradicate the tumor.[1] Therefore, in order to raise the anti-tumor activity in the genetically low-responder mice, it should be necessary to use some kind of BRM as an adjuvant.

It has been generally recognized that tumor-associated antigens induced by an oncogenic virus, such as RSV used here, are expressed on the tumor cells.[28, 29] In spite of the presence of such an antigenic determinant(s) on the RSV-induced tumor cells, some types of tumors were able to escape from the host defense mechanism due to the genetically low responsiveness of the host to such antigens. It is possible to assume that the same situation might be present in humans. Even patients having highly antigenic tumors might not be able to respond to their own tumors due to their genetic background. In this paper, we have suggested that BRMs are able to augment anti-tumor immunity depending on the activation of either DTH-type T cells or macrophages in low-responder mice.

Although the BRMs have not been so effective as expected in clinical trials in cancer patients,[33] we have to wait to evaluate their efficacy until the precise mechanisms of BRM action are established. As we have described in this report, both anti-tumor CTL induction and host responsiveness to BRM are highly dependent on genetically determined background. If we can detect whether or not the cancer patient is a genetically low responder to his own tumor and is sensitive to some BRMs, the patient can be selected as a candidate for BRM treatment. If this can be done, we predict from our present study that the clinical efficacy of BRMs will be remarkably improved.

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