TUMOR IMMUNITY INDUCED BY BCG-TUMOR CELL MIXTURES IN SYNGENEIC MICE

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(Received: May 22, 1973)

SUMMARY: Effects of mixing living BCG with living tumor cells on the growth of the tumor cells in the intradermally inoculated mice and induction of the tumor immunity were investigated with various syngeneic and allogeneic mouse tumor systems. Tumor immunity was assessed from transplant resistance against challenge tumor grafts, macrophage migration inhibition and peritoneal macrophage disappearance. The effects of BCG-tumor cell mixtures varied depending upon the tumor lines and also upon the experimental conditions employed. From the modes of the effect, the tumors used were classified into the following three types: (1) rejecting the primary tumor grafts and inducing the tumor-specific, cell-mediated immunity; (2) rejecting the primary tumor grafts but inducing no detectable tumor immunity; and (3) not rejecting the primary tumor grafts even in BCG-sensitized mice. These types seemed to correlate with the levels of antigenicity and the growth rates of the tumor cells. Number of the tumor cells in the inoculum, in addition to the tumor lines, largely influenced the effects. For the optimal suppression of the growth of tumor cells in the primary inocula, generally required were the following factors: (1) prior sensitization of mice to BCG, (2) direct contact between BCG and tumor cells, and (3) intradermal route of injection.

INTRODUCTION

Zbar et al. (1970 a, 1971 b) reported that the intradermal growth of syngeneic hepatocarcinoma (line-10) in guinea pigs (strain 2) was suppressed when inoculated with living BCG. The BCG-tumor cell mixture also induced the tumor-specific immunity, characterized by delayed cutaneous hypersensitivity and rejection of challenged tumor cells of the same line. Tanaka and Tokunaga (1971) stated in a preliminary note that these might generally be true in syngeneic murine tumors. Bartlett, Zbar and Rapp (1972 a), however, showed markedly contrasted effects of a BCG-tumor cell mixture on the murine and the guinea pig systems: namely injection with a mixture of BCG and murine tumor cells (line-1038) did not induce any higher tumor immunity than with the tumor cells alone.

In this investigation, various syngeneic and allogeneic murine tumors have been

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examined to find whether or not the intradermal injection with BCG-tumor cell mixture could always suppress the growth of the inoculated tumor cells and induce systemic and tumor-specific immunity.

MATERIALS AND METHODS

Animals: Male Swiss mice (SWM/Ms) were obtained from an inbred subline maintained at the National Institute of Health of Japan (Tokyo, Japan) or from a breeding colony at the Ohmura Experimental Animals (Zama, Kanagawa, Japan). These substrains were separated respectively in 1971 and 1966 from a colony maintained at the National Institute of Genetics (Mishima, Shizuoka, Japan). Female C3H/He mice were obtained from a breeding colony at the Funabashi Farm Co. Ltd. (Funabashi, Chiba, Japan). Female ddY mice were obtained from a closed colony at the Shizuoka Experimental Animal Union (Hamamatsu, Shizuoka, Japan). CF#1 mice were supplied from a closed colony at the National Institute of Health. F1 hybrids of DBA and Cd mice were provided by the Department of Antibiotics, National Institute of Health.

Mice at 5 to 8 weeks of age were used for the experiments. Groups of 10 or less animals were housed in metal cages and fed on CMF pellet purchased from the Oriental Yeast Co. Ltd. Bedding was changed twice a week.

Tumors: Designations and origins of the tumors employed are listed in Table I. Tumors, K1, K2, K3, K5, MCA-1 and MCA-2, were induced in male SWM/Ms or female C3H/He mice by a single subcutaneous injection with 0.5 mg of 20-methylcholanthrene (MCA) suspended in olive oil. Tumor 4NQO-1 was induced by subcutaneous injections with 4-nitroquinoline 1-oxide dissolved in propylene-glycol once a week for 7 weeks. Tumor M3 was transferred from mammary tumor spontan-

TABLE I

Tumors employed

Tumor	Mouse	Origin
K1	C3H/He	induced with MCA at NIH* in 1971
K2	C3H/He	induced with MCA at NIH in 1971
K3	SWM/Ms	induced with MCA at NIH in 1971
K5	SWM/Ms	induced with MCA at NIH in 1971
MCA-1	SWM/Ms	induced with MCA at NCCRI** in 1970
MCA-2	SWM/Ms	induced with MCA at NCCRI in 1970
4NQO-1	SWM/Ms	induced with 4NQO at NCCRI in 1968
M3	SWM/Ms	obtained from spontaneous mammary tumor at NIH in 1971
L1210	(DBA×Cd)F ₁	
S180	ddY	
Ehrlich	CF#1 or ddY	

* NIH: Department of Tuberculosis, National Institute of Health, Tokyo, Japan.

** NCCRI: National Cancer Center Research Institute, Tokyo, Japan.

eously developed in an SWM/Ms mouse. These tumors were maintained by passages in the syngeneic mouse systems. For the passage, a solid tumor was removed from a donor mouse and minced finely with a pair of scissors without enzymic treatment or any other mechanical homogenization. The pieces were inoculated subcutaneously with a syringe. L1210 leukaemia and S180 ascites tumor were supplied by Department of Antibiotics, National Institute of Health, and Ehrlich ascites tumor was given by National Cancer Center Research Institute. These allogeneic tumors were maintained as ascites types.

Preparation of tumor cell suspension: The method used by Rapp et al. (1968) in the guinea pig system was employed. Solid tumor was removed from a donor mouse and trimmed off from the connective tissue and any grossly visible necrotic or hemorrhagic areas. The viable portions were then minced finely and suspended in Hanks' balanced salt solution (HBSS). Minced tumor was digested by pronase (Reagent E; Kakenkagaku Co. Ltd., Tokyo, Japan) and DNase (Worthington Biochem. Corp., U.S.A.) at final concentrations of 0.15 % and 0.6 $\mu\text{g/ml}$, respectively. The material was mixed at room temperature with a magnetic stirrer for several minutes. The tumor clumps were allowed to settle and the supernatant fluid decanted. The residual tumor was resuspended in the enzyme solution and digestion of the tumor repeated until sufficient cells were obtained. The tumor cells were collected by centrifugation, washed twice in HBSS and resuspended in medium 199 (Chiba Serum Institute, Ichikawa, Japan).

Counts for viable tumor cells: An aliquot of a tumor cell suspension was diluted 1:50 with saline containing trypan blue in 0.12 %. Unstained cells were counted in a hemacytometer.

BCG: Lyophilized BCG manufactured by the Japan BCG Laboratory (Tokyo, Japan) was employed. Viable cell counts showed that an 80-mg portion of the suspension contained 1 to 2×10^9 colony-forming units. Prior to use, a cell suspension of a given concentration was prepared by appropriately diluting the original suspension with physiological saline or Hanks' solution.

Prior sensitization to BCG: Mice were injected subcutaneously with about 1×10^7 viable cells of BCG. In about 3 weeks, these were considered to be BCG-sensitized animals.

Evaluation of tumor growth: After injected with tumor cells, mice were examined twice a week, and lesions at the injection sites measured with a calliper. Results were expressed in average tumor diameter squared in mm^2 ($\pm$ S.D.). Tumor incidence was expressed as the ratio of number of mice bearing a lesion larger than 5 mm in diameter at the challenge site to that of mice injected, or its percentage.

Preparation of BCG-tumor cell mixtures for injection: Equal volumes of a suspension of tumor cells at a specified cell concentration and of BCG at a concentration, in most cases, of 4×10^8 viable units/ml were mixed at room temperature and the mixture was injected as quickly as possible. Routinely, each mouse received intradermally with 0.05 ml of the mixture.

Preparation of antigenic suspension of tumors: Tumor cells suspended in HBSS at a concentration of 2×10^7 viable cells/ml were disintegrated by repeated freeze-thawings and employed as the antigenic suspension of the tumor to be used in the macrophage migration inhibition test and the peritoneal macrophage disappearance test.

Macrophage migration inhibition: Peritoneal exudate cells were obtained by an intradermal injection with 1 ml of Drakeol (Pennsylvania Refining Co., Butler, Penn., U.S.A.) four days before harvesting the cells. Five ml of chilled HBSS containing 15 U heparin/ml was injected intraperitoneally and the fluid containing peritoneal cells was harvested. This procedure was repeated again. The harvested cells were pooled and washed twice in HBSS containing no heparin. Washed cells were finally suspended in Eagle's Minimum Essential Medium (MEM; Nissui Seiyaku Co. Ltd., Tokyo, Japan) containing 20 % fetal calf serum (GIBCO, Berkeley, Calif., U.S.A.). Peritoneal cells, alone or mixed with an equal volume of an antigenic suspension of tumor, were packed into glass capillary tubes by centrifugation; the capillary tubes were cut at the cell-fluid interface. The packed cell portion was fixed on the surface of glass culture dishes. The dishes were filled with Eagle's MEM with or without addition of an antigenic suspension in 20 % and incubated at 37 C in a CO₂ incubator. Migration was expressed by the distance (mm) of "outgrowth" from the edge of capillary tube measured in 24 hr of incubation. The mean distance $\pm$ standard deviation was calculated from the values of six capillary tubes.

Peritoneal macrophage disappearance test: The method of Nelson and Boyden (1962) was employed with minor modifications. One milliliter of 12.5 % casein sodium (Nutrose; Wako Pure Chemical Ind., Ltd., Osaka, Japan) suspended in physiological saline was injected into the peritoneal cavity. In 4 days, 1 ml of an antigenic solution (10 μ g/ml of PPD or an antigenic suspension of K3 or K5) was injected into the peritoneal cavity. In 4 hr, animals were sacrificed by exsanguination under chloroform narcosis and 5 ml of HBSS was introduced into the peritoneal cavity. After gentle agitation of the abdominal part, the fluid was harvested. This procedure was repeated again. The fluids harvested were pooled and it was centrifuged at 1,000 rpm for 10 min. The sedimenting cells were resuspended in a few drops of HBSS and a drop of this suspension was smeared. After Giemsa's staining, cells were counted and classified into three types (macrophages, lymphocytes and polymorphs) according to the criteria described by Nelson *et al.* (1962). The differential cell count was expressed in percentage.

RESULTS

Suppression of Growth of Various Tumors at Sites of Intradermal Inoculation with Living BCG-Tumor Cell Mixtures and Rejection of Challenge Tumor Grafts

Studies were made with the various murine tumors to find whether BCG-tumor cell mixture could prevent growth of the tumors and whether the animals suppressing tumor growth at the primary injection sites would subsequently reject challenge grafts of the tumors. The routine procedures were as follows: mice sensitized or unsensitized to BCG were given intradermal injections with either a mixture of approximately 10⁶ viable tumor cells and 10⁷ colony-forming units of BCG or 10⁶ tumor cells alone. Each of the animals, bearing no visible tumor at the injection sites, was challenged, usually in 4 weeks by intradermal injection with 10⁶ viable tumor cells at the sites contralateral to the primary injection.

The results with S180 tumor system are illustrated in Fig. 1. Fourty male ddY

Fig. 1. Intradermal growth of S180 tumor cells in ddY mice.

- : Tumor cells alone in normal mice.
- : Tumor cells alone in BCG-sensitized mice.
- ▲—▲: BCG-tumor cell mixture in normal mice.
- △—△: BCG-tumor cell mixture in BCG-sensitized mice.
- : Tumor cells alone in normal mice.
- : Tumor cells alone in mice injected with BCG-tumor cell mixture 60 days beforehand.

mice were divided into four groups; two were sensitized to BCG and other two nonsensitized. One of the sensitized and of the nonsensitized groups were given with the BCG-S180 mixture and the remainders with S180 alone. In the group inoculated with S180 alone, the tumor grew progressively in both sensitized and nonsensitized mice, killing them ultimately. However, the growth rate of the tumor was significantly lower in the sensitized group than in the nonsensitized one. Intradermal injections with the BCG-S180 mixture into both groups produced nodules at the injection sites but those regressed within 20 days after the inoculation. These animals, having shown the regression of the tumor, were challenged intradermally each with 10^6 viable S180 cells at the contralateral site 60 days after the primary grafting. All the animals suppressed the growth of the challenge grafts, while the tumors grew progressively in the control mice.

Such rejections of both primary and secondary tumor-grafts as observed with S180 were not always the cases in other tumor systems as seen in Table II. Among syngeneic tumors, MCA-2, K1 and K3 showed results similar to those with S180; most mice having received BCG-tumor cell mixtures rejected the tumor cells and resisted the challenge tumor grafts. On the other hand, MCA-1, K5, M3 and

TABLE II

Incidences of tumor growth at the injection sites of BCG-tumor cell mixtures and incidences of growth of challenge tumor grafts

Tumor line	Mouse	Sensitization to BCG*	Injection of BCG with tumor cells**	Tumor incidence of***	
				primary grafts	challenge grafts****
MCA-1	SWM/Ms	—	—	10/10	
		—	+	6/38	25/32
		+	—	20/20	
		+	+	0/35	25/34
MCA-2	SWM/Ms	+	—	9/9	
		+	+	1/10	0/9
K1	C3H/He	—	—	9/9	
		—	+	1/9	0/8
K2	C3H/He	—	—	10/11	
		—	+	13/24	
		+	—	15/29	
		+	+	0/67	2/64
K3	SWM/Ms	—	—	20/20	
		—	+	3/25	4/22
		+	—	10/10	
		+	+	2/59	3/57
K5	SWM/Ms	—	—	10/10	
		—	+	10/10	
		+	—	10/10	
		+	+	6/10	2/4
M3	SWM/Ms	—	—	5/5	
		—	+	3/10	7/7
4NQO-1	SWM/Ms	—	—	5/5	
		—	+	5/5	
		+	—	5/5	
		+	+	4/5	1/1
Ehrlich	ddY	—	—	10/10	
		—	+	4/14	0/10
		+	—	10/10	
		+	+	1/15	
	CF#1	+	—	9/9	
		+	+	2/15	0/13
	DBA×Cd (F1)	—	—	8/8	
		—	+	7/7	
		+	—	9/9	
		+	+	10/10	

* Mice were sensitized or unsensitized to BCG about three weeks before tumor injections.

** Mice were injected with approximately 10^6 viable tumor cells with or without 10^7 BCG.

*** Number of mice with tumor/number of mice injected.

**** Mice having rejected primary tumor grafts were challenged intradermally with 10^6 viable tumor cells usually four weeks after the inoculation of primary grafts.

4NQO-1 were different from those tumors. With the former three tumors, most mice showed regression of the primary tumor growth when BCG was mixed before the injections, but few of them developed resistance against the secondary tumor grafts. In the case of 4NQO-1, only few mice rejected the primary tumor graft; even those having been sensitized to BCG and grafted with cells mixed with BCG before injections did not reject it. Of allogeneic tumors, Ehrlich gave results similar to those with S180 and L1210 behaved like 4NQO-1.

Factors Influencing the Effects of BCG-Tumor Cell Mixtures

In addition to the different effects of BCG-tumor cell mixtures seen among different tumor systems, some other factors also influenced the effects:

a) Prior sensitization to BCG. Prior sensitization to BCG did not significantly impair the growth of syngeneic tumors. However, as seen in Table II, this factor was important for suppression of tumor growth at the injection sites of BCG-tumor cell mixtures, particularly in the cases of MCA-1, K2, K5 and M3. In 4NQO-1, the effect of prior sensitization was not remarkable when BCG was injected together with 10^6 tumor cells (Table II). When a BCG-tumor cell mixture containing 10^5 tumor cells was employed, the effect of prior sensitization was remarkable; three of five sensitized mice rejected the primary grafts, while no unsensitized mice did so (Table III).

Intradermal injection with L1210 cells mixed with BCG killed all the animals, even when such a small number as 10^4 of the tumor cells were injected into BCG-sensitized mice. Survival time of those mice was prolonged significantly comparing to that of BCG-sensitized and nonsensitized mice injected with L1210 cells alone

TABLE III

Influence of different sizes of inocula of tumor cells (4NQO-1) mixed with BCG on suppression of tumor growth at the injection sites

Sensitization with BCG	No. of tumor cells injected	BCG injection with tumor cells*	Tumor incidence**
—	10^5	—	5/5
—		+	5/5
+		—	5/5
+		+	2/5
—	5×10^4	—	5/5
—		+	1/5
—		—	5/5
—		+	0/5
—	10^4	—	5/5
—		+	0/5
+		—	5/5
+		+	0/5

* Viable BCG cells injected with given numbers of tumor cells were 10^7 per mouse.

** Number of mice with tumor/number of mice injected.

TABLE IV
Effect of BCG on L1210 leukemia

No. of mice used	Prior sensitization with BCG	BCG injection with tumor cells	Day of death (mean S.D.)	P
13	—	—	8.20±0.41	<0.05
12	—	+	11.32±0.29	
15	+	—	8.71±0.56	
30	+	+	11.76±0.40	

Number of L1210 cells injected was 10^4 per mouse and that of BCG 10^7 .

(Table IV).

There seemed to be no difference in the potency of inducing tumor-specific immunity exhibited by resistance to secondary tumor grafts between BCG-sensitized and nonsensitized animals (Table II).

b) The number of tumor cells contained in the BCG-tumor cell mixture. The number of tumor cells contained in BCG-tumor cell mixtures was also an important factor affecting suppression of tumor growth at the sites of injection. Table III shows the results with 4NQO-1 tumor cells. The BCG-sensitized mice given with 10^5 cells mixed with BCG, developed tumors in 40 % while none developed tumors by injection with 10^4 to 5×10^4 cells. All developed tumors by injection with 10^6 cells mixed with BCG (Table II).

c) Routes of injection with BCG-tumor cell mixture and direct contact of BCG and tumor cells. Route of injection with the BCG-tumor cell mixture was also important for rejection of the primary tumor grafts. Incidences of tumor death in the mice injected by various routes with a mixture of BCG and Ehrlich ascites cells are shown in Table V. Tumor incidence was about 22 % when a mixture was given intradermally, 40 % when given subcutaneously, and 100 % when given intraperitoneally.

TABLE V
Effects of routes of injection with BCG-tumor cell mixtures on suppression of tumor growth

Tumor	Route of injection	Tumor incidence*
Ehrlich**	intradermal	2/9
	intraperitoneal	10/10
	subcutaneous	4/10
K3***	i.d. (same site)	3/15
	i.d. (separate sites)	10/10

* Number of mice died/number of mice injected.

** Mice were given approximately 10^6 viable tumor cells and 10^7 living BCG by different routes.

*** Mice were given intradermal injections of about 10^6 viable tumor cells and 10^7 living BCG at the same site or at separate, contralateral sites.

To know whether contact between BCG and tumor cells is necessary for suppression of tumor growth at the primary injection sites, mice were given intradermal injections with BCG and tumor cells at the same site or at separate contralateral sites. The results with K3 tumor are shown in Table V. Only the mice injected simultaneously with BCG at the same site suppressed the tumor growth. Similar results were obtained with MCA-1, indicating that contact between BCG and tumor cells is required for suppression of primary tumor grafts.

*Specificity of Tumor Immunity Induced with BCG-Tumor Cell Mixtures
as Measured by Suppression of Challenge Tumor Grafts*

To know whether or not tumor immunity induced by intradermal injection with BCG-tumor cell mixture is tumor-specific or not, SWM/Ms mice immunized with a mixture of K3 or K5 with BCG were challenged by intradermal injections with K3 and K5 tumor cells separately at contralateral sites. The results obtained are shown in Fig. 2 and Table VI. The challenged K3 tumor cells grew progressively in the K5-immunized and the untreated control mice at almost the equal growth rates, but they were rejected in most of the K3-immunized mice. On the other hand, K5 tumor cells challenged into the K3- or K5-immunized mice grew progressively but at a lower

Fig. 2. Growth of K3 and K5 tumor cells in normal, K3-immunized and K5-immunized mice.

- : Normal mice
- : K3-immunized mice
- △---△: K5-immunized mice

TABLE VI

Incidences of challenge tumor growth of K3 and K5 in mice immunized with K3 or K5, and of MCA-1 and MCA-2 in mice immunized with MCA-1 or MCA-2

Exp.	Mice	Tumor incidence**	
		Challenged with K3	K5
1	Unimmunized	5/5	5/5
	Immunized with K3*	3/16	16/16
	Immunized with K5*	11/12	8/12
		Challenged with MCA-1	MCA-2
2	Unimmunized	5/5	5/5
	Immunized with MCA-1*	5/6	5/6
	Immunized with MCA-2*	7/7	0/7

* Each of mice sensitized with BCG was immunized by an intradermal injection with 6×10^5 tumor cells and 2×10^7 living BCG. After four weeks, the mice having rejected the tumor growth were employed for the challenge experiments.

** Each mouse was injected with 1×10^6 cells of K3 and K5 at separate, contralateral sites. Tumor incidence was expressed in number of tumor-bearing mice after 5 weeks/number of mice employed.

growth rate than the control (Fig. 2). Tumor incidence of K5 in the K5-immunized mice was 8/12, while that of K3 in the same group was 11/12. The results suggest possibilities that the antigenicity of K5 may be relatively weak and shared by K3. A similar experiment was carried out with the systems of MCA-1 and MCA-2; mice immunized with MCA-1 or MCA-2 cells were challenged with MCA-1 and MCA-2 cells separately at contralateral sites. Incidences of the challenged tumors are shown in Table VI. The mice immunized with MCA-2 completely suppressed the growth of MCA-2 but not that of MCA-1. In the MCA-1-immunized mice, on the other hand, incidence of MCA-1 and that of MCA-2 tumors were almost the equal. Tumor size of MCA-1 in mean diameter squared (mm^2) $\pm$ S. D. was 176.4 ± 49.8 in the MCA-1 immunized mice and 377.2 ± 26.1 in the MCA-2 immunized mice in 4 weeks after the challenge, while 352.1 ± 35.7 in the untreated control. That of MCA-2 was 43.6 ± 8.8 in the MCA-1-immunized and almost nil in the MCA-2 immunized, while 186.1 ± 21.6 in the control. In the mice immunized with BCG-Ehrlich mixtures, intradermal growth of 10^6 cells of challenged S180 was inhibited showing cross-resistance.

Macrophage Migration Inhibition

Peritoneal exudate cells were obtained from SWM/Ms mice immunized with BCG alone, BCG plus K3 cells or BCG plus MCA-2 cells. The results of the migration inhibition tests of the peritoneal cells conducted with the antigenic suspensions of K3, K5 or MCA-2 are shown in Table VII. Migration of the cells was inhibited only with the antigen employed for immunization, suggesting that the rejections of challenged tumor grafts may have been caused by cell-mediated immunity.

TABLE VII

Migration inhibition of peritoneal exudate cells from mice immunized with K3 or MCA-2 tumor cells

Animals immunized with:	Tumor antigen	Migration	
		Distance* (mm)	Index (%)
BCG	—	0.93±0.03	100.00
	K3	0.83±0.04	89.24
K3+BCG	—	1.25±0.32	100.00
	K3	0.06±0.24	48.00
	K5	0.92±0.31	73.60
MCA-2+BCG	—	0.88±0.18	94.62
	MCA-2	0.03±0.02	3.23

* Mean distance± standard deviation of the mean for six capillary tubes.

Peritoneal Macrophage Disappearance

Tests for peritoneal macrophage disappearance were performed in mice immunized with BCG alone, BCG plus K3 or BCG plus K5 tumor cells. These animals were injected with PPD or a suspension of K3 or K5 antigen. Percentage of macrophages to the total cell population was calculated in each mouse and plotted in Fig. 3. An injection with K3 or K5 antigen caused some fall in the macrophage content of the exudate of normal mice. The macrophage contents of the mice immunized with BCG plus K3 or K5 were lower than those of the control mice when the corresponding

Fig. 3. Macrophage contents (%) in peritoneal exsudates of normal, BCG-, K3- and K5-immunized mice.

antigenic suspension was injected, also suggesting the presence of cell-mediated tumor immunity.

DISCUSSION

Active immunotherapy of malignant tumors with living BCG has extensively been investigated. From the mode of administration with BCG, immunotherapy can be divided into the following three categories: (1) Dermal scarification or intradermal injection with BCG into the normal skin of tumor-bearing patients or animals; (2) Injection with BCG directly into established, solid tumors; (3) Intradermal injection with a mixture of BCG and tumor cells. Remarkable effects of the first technique on acute lymphoblastic leukaemia was first described by Mathé *et al.* (1969) and subsequently many investigators used this method and reported their clinical experiences (Mathé, 1971, 1972; Leukaemic Committee and Working Party on Leukaemia in Childhood, 1971). Marked effects of the second method, intralesional injections with living BCG, was described by Mortorn *et al.* (1970) in human melanoma; the technique has been used by many clinicians (Morton *et al.*, 1971; Bluming *et al.*, 1972; Pinsky *et al.*, 1972; Sulit *et al.*, 1972). In experimental tumors, this method was first employed by Zbar and Tanaka (1971 a) for a syngeneic guinea pig tumor, and then by Bartlett *et al.* (1972 a) for a syngeneic mouse tumor. The present authors attempted at treating autochthonous mouse tumors by this method; more than 100 SMW/Ms mice bearing tumors induced by MCA were injected intralesionally once with living BCG (Tokunaga, Yamamoto, Kataoka and Nakamura, unpublished data). In approximately 20 % of the mice injected with BCG, regression of the established autochthonous tumors was observed; most of them, however, relapsed at the same or different sites within several weeks after disappearance. The last method, intradermal injection with a BCG-tumor cell mixture, was employed by Zbar *et al.* (1970 a) for the syngeneic guinea pig tumor system and has extensively been investigated (Zbar *et al.*, 1971 b; Bartlett *et al.*, 1972 b; Simmons *et al.*, 1971; Schwarz *et al.*, 1971). Living BCG mixed with tumor cells (line-10 of strain 2 guinea pigs) in the inoculum suppressed the growth of the tumor cells and injection of the mixture induced tumor-specific immunity manifested by delayed cutaneous hypersensitivity and rejection of inoculated tumor cells (Zbar *et al.*, 1971 b). A tumor-specific vaccine (Bartlett *et al.*, 1972 b) was studied for the safety and therapeutic potency. Tanaka and Tokunaga (1971) reported preliminarily that these were also true in the syngeneic mouse systems. Some clinical trials were also performed by this technique (Morton *et al.*, 1971; Sokal *et al.*, 1972). This method, if effective generally in inducing tumor-specific immunity, can be used clinically to prevent metastasis and relapse. Bartlett *et al.* (1972), however, raised a question about the general efficacy of this method. They observed that a mixture of tumor cells (line 1038) and BCG did not effectively induce tumor specific immunity in syngeneic mice, although they gave no answer to the question whether this was a common feature of the mouse tumor system or it simply reflected a peculiar behavior of this tumor line.

In the present study, various syngeneic and allogeneic mouse tumors were examined to see if the effect of BCG-tumor cell mixtures on the growth of primary and secondary tumor grafts could be generalized. The results demonstrated clearly that the effects of BCG-tumor cell mixture depend upon the tumor lines and also

upon the experimental conditions employed.

The tumors employed seemed to fall into the following three types from the modes of effects of BCG-tumor cell mixtures as shown in Table II: (1) Such tumor cells that were rejected at the site of injection with a BCG-tumor cell mixture and developed subsequent transplant resistance to a challenged tumor graft in the animal, similar to the one reported in the guinea pig system (Zbar et al., 1971 b); (2) Such tumor cells whose intradermal growth was rejected at the injection site of a BCG-tumor cell mixture, but induced no subsequent tumor immunity, resembling the line 1038 mouse tumor (Bartlett et al., 1972 a); (3) Such tumor cells that were not rejected nor induced detectable tumor immunity.

Syngeneic tumors of K1, K2, K3 and MCA-2 and allogeneic tumors of S180 and Ehrlich seemed to belong to the first type; syngeneic tumors of K5, MCA-1 and M3 seemed to belong to the second type; and both syngeneic 4NQO-1 and allogeneic L1210 belonged to the third type. Difference among these types, however, is not essential, because the effects of BCG-tumor cell mixtures were influenced by other factors; for instance, tumor 4NQO-1 could have been included into the second type if a mixture containing 10^4 tumor cells, instead of 10^6 cells, had been given.

When a large number of cells of K3, K5 or 4NQO-1 were irradiated with 15,000 rad from Cobalt-60 and injected into mice without BCG, the animals developed various levels of resistance against challenged tumor grafts; irradiated K3 cells induced a relatively strong resistance against transplant with K3, irradiated K5 cells evoked a significant but weak resistance against challenged K5 cells, but irradiated 4NQO-1 cells induced no detectable immunity (Nakamura, Kataoka, Yamamoto and Tokunaka, unpublished data). These results may suggest that among the three tumors, the antigenicity of K3 cells is the highest, that of K5 intermediate and that of 4NQO-1 the lowest. It was also noticed that the growth rates of these tumors are different; K5 cells grow much faster than K3 cells and 4NQO-1 cells grow still faster than K5 cells. These data suggest that the antigenicities and growth rates of the tumors seem to play important roles in exhibiting the effects of the BCG-tumor cell mixtures.

The role of BCG contained in the BCG-tumor cell mixtures may consist of two different mechanisms. First, BCG triggers off provoking BCG-specific, cellular reactions at the injection sites. The resulting inflammatory reactions may destroy the tumor cells at the reaction sites nonspecifically. In the animals sensitized to BCG, the reactions arise much more rapidly and vigorously than in nonsensitized animals. Zbar and his co-workers presented enough evidences to propose this two-step mechanism, nonspecific destruction of tumor cells with BCG-specific reactions (Zbar et al., 1970 b, c, 1971 b; Hanna, Zbar and Rapp, 1972 a, b; Bartlett et al., 1972 a, b). The notion of nonspecific inhibition is supported by the facts that prior sensitization to BCG is required to ensure the suppression of tumor growth at the primary injection sites and that mice can reject tumor cells but develop no tumor immunity with K5, MCA-1 and M3. Histopathological findings in the time-course experiments with the BCG-K3 tumor system also supported the notion of nonspecific destruction of the tumor cells (Takahashi, Kataoka, Yamamoto, Nakamura and Tokunaga, unpublished data).

Secondly, BCG generally acts as an adjuvant enhancing both cellular and humoral immunity. In addition, it is known that a delayed-type inflammatory reaction itself could exhibit adjuvant activity inducing delayed hypersensitivity under certain condi-

tions; for instance, an intradermal injection with a mixture of PPD and BSA into a BCG-sensitized guinea pig induced delayed cutaneous hypersensitivity to BSA after four weeks (Bloom, 1969; Kataoka, Tokunaga and Yamamoto, unpublished data). In the BCG-tumor cell systems, the tumor-specific antigen at the reaction sites evoked by BCG could be recognized effectively by the host. Consequently, cell-mediated tumor immunity is induced and manifested by the rejection of challenged tumor grafts and by the *in vitro* tests.

If the antigenicity of the tumor is too weak to be recognized, if the tumor antigen is destroyed too early, or if antigenic competition occurs between tumor cells and BCG, the tumor specific immunity would not be induced. Although more prompt and vigorous reaction is seen in BCG-sensitized animals at the site of injection with a BCG-tumor cell mixture than in nonsensitized ones and the reaction suppresses the tumor growth effectively, it has not been clarified whether this reaction effectively induces the tumor-specific immunity. Further studies on the development of tumor-specific, cellular and humoral immunity in the host animals injected with BCG-tumor cell mixtures are in progress.

ACKNOWLEDGEMENT

We are deeply indebted to Dr. T. Murohashi, Director, Department of Tuberculosis, National Institute of Health, Japan, for his interest in this work and encouragement and his critical reviewing of this manuscript. We thank Mrs. K. Takahashi, Miss Y. Ohta and Mrs. M. Imai for their technical assistance. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare (the principal investigator: Prof. D. Mizuno, Faculty of Pharmacological Science, Tokyo University).

REFERENCES

- BARTLETT, G. L., ZBAR, B. AND RAPP, H. J. (1972 a): Suppression of murine tumor growth by immune reaction to the Bacillus Calmette-Guérin strain of *Mycobacterium bovis*. J. Nat. Cancer Inst., 48, 245-257.
- BARTLETT, G. L. AND ZBAR, B. (1972 b): Tumor-specific vaccine containing *Mycobacterium bovis* and tumor cells: safety and efficacy. J. Nat. Cancer Inst., 48, 1709-1726.
- BLOOM, B. R. (1969): Elaboration of effector molecules by activated lymphocytes. p. 247-319. In H. S. Lawrence and M. Landy (ed.) Mediators of cellular immunity. Academic Press, N. Y., London.
- BLUMING, A. Z., VOGEL, C. L., ZIEGLER, J. L., MODY, N. AND KAMYA, G. (1972): Immunological effects of BCG in malignant melanoma: two modes of administration compared. Ann. Internal Medicine, 76, 405-411.
- HANNA, M. G., ZBAR, B. AND RAPP, H. J. (1972 a): Histopathology of tumor regression after intralesional injection of *Mycobacterium bovis*. I. Tumor growth and metastasis. J. Nat. Cancer Inst., 48, 1441-1455.
- HANNA, M. G., ZBAR, B. AND RAPP, H. J. (1972 b): Histopathology of tumor regression after intralesional injection of *Mycobacterium bovis*. II. Comparative effects of vaccinia virus, oxazolone, and turpentine. J. Nat. Cancer Inst., 48, 1697-1707.
- LEUKAEMIA COMMITTEE AND WORKING PARTY ON LEUKAEMIA IN CHILDHOOD (1971): Treatment of acute lymphoblastic leukaemia. Brit. Med. J., 23, 189-194.
- MATHÉ, G., AMIEL, J. L., SCHWARZENBERG, L., SCHNEIDER, M., CATTAN, A., SCHLUMBERGER, J. R., HYYAT, M. AND VASSAL, F. (1969): Active immunotherapy for acute lymphoblastic leukemia. Lancet, 1, 697-699.

- MATHÉ, G. (1971): Active immunotherapy. *Adv. Cancer Res.*, **14**, 1-36.
- MATHÉ, G. (1972): Immunological approaches of leukaemia treatment. *Ann. Inst. Pasteur*, **122**, 855-881.
- MORTON, D. L., EILBER, F. R., MALMGREN, R. A. AND WOOD, W. C. (1970): Immunological factors which influence response to immunotherapy in malignant melanoma. *Surgery*, **68**, 158-164.
- MORTON, D. L., HOLMES, E. C., EILBER, F. R. AND WOOD, W. C. (1971): Immunological aspects of neoplasia: a rational basis for immunotherapy. *Ann. Internal Med.*, **74**, 587-607.
- NELSON, D. S. AND BOYDEN, S. V. (1963): The loss of macrophages from peritoneal exudates following the injection of antigens into guinea-pigs with delayed-type hypersensitivity. *Immunology*, **6**, 264-275.
- PINSKY, C., HIRSHAUT, Y. AND OETTGEN, H. (1972): Treatment of malignant melanoma by intratumoral injection of BCG. *Proc. Amer. Assoc. Cancer Res.*, **13**, 21.
- RAPP, H. J., CHURCHILL, W. H., KRONMAN, B., ROLLEY, R. T., HAMMOND, W. G. AND BORSOS, T. (1968): Antigenicity of a new diethylnitrosamine-induced transplantable guinea pig hepatoma: Pathology and formation of ascites variant. *J. Nat. Cancer Inst.*, **41**, 1-11.
- SCHWARTZ, D. B., ZBAR, B., GIBSON, W. T. AND CHIRIGOS, M. A. (1971): Inhibition of murine sarcoma virus oncogenesis with living BCG. *Int. J. Cancer*, **8**, 320-325.
- SIMMONS, R. L. AND RIOS, A. (1971): Immunotherapy of cancer: Immunospecific rejection of tumors in recipients of neuraminidase-treated tumor cells plus BCG. *Science*, **5**, 591-593.
- SOKAL, J. E., AUNGS, W. AND HAN, T. (1972): Use of Bacillus Calmette-Guérin as adjuvant in human cell vaccine. *Cancer Res.*, **32**, 1584-1589.
- SULIT, H., CHEE, D., MASTRANGELO, M., BORNSTEN, R., PREHN, R. T. AND YARBRO, J. (1972): In vitro assays of cellular and humoral immunity during a clinical trial of immunotherapy in human melanoma. *Proc. Amer. Assoc. Cancer Res.*, **13**, 74.
- TANAKA, T. AND TOKUNAGA, T. (1971): Suppression of tumor growth and induction of specific tumor immunity by intradermal inoculation of a mixture of living tumor cells and live *Mycobacterium bovis* in syngeneic mice. *Gann*, **62**, 433-434.
- ZBAR, B., BERNSTERIN, I., TANAKA, T. AND RAPP, H. J. (1970 a): Tumor immunity produced by the intradermal inoculation of living tumor cells and living *Mycobacterium bovis* (strain BCG). *Science*, **170**, 1217-1218.
- ZBAR, B., WEPSTIC, H. T., BORSOS, T. AND RAPP, H. J. (1970 b): Tumor-graft rejection in syngeneic guinea pigs: evidence for a two-step mechanism. *J. Nat. Cancer Inst.*, **44**, 473-481.
- ZBAR, B., WEPSTIC, H. T., RAPP, H. J., STEWART, L. C. AND BORSOS, T. (1970 c): Two step mechanism of tumor graft rejection in syngeneic guinea pigs. II. Initiation of reaction by a cell fraction containing lymphocytes and neutrophils. *J. Nat. Cancer Inst.*, **44**, 701-717.
- ZBAR, B. AND TANAKA, T. (1971 a): Immunotherapy of cancer: regression of tumors after intralesional injection of living *Mycobacterium bovis*. *Science*, **172**, 271-273.
- ZBAR, B., BERNSTEIN, I. D. AND RAPP, H. J. (1971 b): Suppression of tumor growth at the site of infection with living Bacillus Calmette-Guérin. *J. Nat. Cancer Inst.*, **46**, 831-839.