

## Antitumor Activity of Lipopolysaccharide in Tumor-Bearing Mice Pretreated with BCG

HIROSHI SATO, MASAYUKI ITO, TATSUYA ABE, NOBUKO  
KUMANO, MASAKICHI MOTOMIYA and KIYOSHI KONNO

*Department of Internal Medicine, the Research Institute for  
Tuberculosis and Cancer, Tohoku University, Sendai 980*

SATO, H., ITO, M., ABE, T., KUMANO, N., MOTOMIYA, M. and KONNO, K.  
*Antitumor Activity of Lipopolysaccharide in Tumor-Bearing Mice Pretreated with  
BCG.* Tohoku J. exp. Med., 1986, **150** (4), 391-399 — The antitumor activity of  
lipopolysaccharide (LPS) was investigated in BCG-treated mice. C3H/He mice  
and CDF<sub>1</sub> mice were injected with BCG and then were inoculated with syngeneic  
mouse hepatoma MH134 and mastocytoma P815 respectively. Hemorrhagic ne-  
crosis and retarded growth of tumor were observed after an intravenous (i.v.)  
injection of LPS, when tumor cells had been inoculated subcutaneously (s.c.).  
However an intraperitoneal (i.p.) injection of BCG plus LPS did not increase the  
mean survival time of mice that had been inoculated with tumor cells i.p. Sera  
from mice that had been treated with BCG plus LPS i.v. were cytotoxic for  
cultured tumor cells. These results seemed to indicate that growth-inhibitory  
effects of LPS on tumors inoculated s.c. were mediated by a humoral factor.  
——— LPS; BCG; antitumor effect

The antitumor effect of *Mycobacterium bovis*, strain BCG in experimental  
neoplasms has been well documented. Also it is accepted that the bacterial  
lipopolysaccharide (LPS) causes necrosis of tumors in vivo. Recently Carswell et  
al. (1975) have demonstrated that necrosis of tumors occurred by an injection of  
sera from the animals pretreated with BCG and LPS.

However there have been only a few reports dealing with the antitumor  
activity of LPS in tumor-bearing animals which had been injected with BCG.  
No publications are available concerning the effects of LPS in BCG-treated mice  
which had been inoculated with tumor cells i.p.

In the present study, BCG-treated CDF<sub>1</sub> mice and C3H/He mice received s.c.  
and i.p. inoculations of syngeneic tumor cells and the antitumor effect of LPS was  
investigated.

### MATERIALS AND METHODS

*Mice.* Male C3H/He mice and hybride CDF<sub>1</sub> (BALB/C×DBA/2) mice were pur-  
chased from Shizuoka Experimental Animal Farm (Shizuoka). Mice were 6 to 8 weeks old

---

Received May 10, 1986; accepted for publication November 5, 1986.

at the start of experiments.

*Tumor cells.* Mouse hepatoma MH134, originally induced with carbon tetrachloride in C3H mice and converted into ascites form (Sato et al. 1956) has been maintained in C3H/He mice by i.p. inoculations. P815 (mastocytoma), induced by Dunn and Potter (1957) in DBA/2 mice, has been maintained in ascites by i.p. transplantations in histocompatible CDF<sub>1</sub> mice. The tumor cells were harvested from the abdominal cavities and washed with saline immediately before inoculations.

*BCG.* Lyophilized *Mycobacterium bovis*, strain BCG (Japanese strain Tokyo No. 172 supplied by the National Institute of Health, Tokyo) were used throughout this series of experiments.

*LPS.* Lipopolysaccharide (LPS) derived from *Escherichia coli* was obtained from Difco (Detroit, MI, USA).

#### *Preparation of sera for cytotoxicity test*

Mice were injected with 4 mg ( $2 \times 10^7$ ) viable BCG organisms. After 14 days, 25  $\mu$ g of LPS was injected i.v. Blood was taken 2 hr after injection of LPS. The blood from the BCG-treated mice was taken 14 days after injection of BCG and that from LPS-treated mice 2 hr after injection of LPS.

#### *Assay in tumor-bearing mice*

##### *Effects on tumors inoculated s.c.*

Male C3H/He mice and CDF<sub>1</sub> mice were injected i.v. with 1 mg ( $5 \times 10^6$ ) viable BCG organisms. After 4 days, they were inoculated s.c. with  $1 \times 10^6$  cells of MH134 and  $2 \times 10^5$  cells of P815 respectively. Ten days thereafter 15  $\mu$ g of LPS was injected i.v. Tumor size was expressed as the mean of two perpendicular diameter. In one experiment CDF<sub>1</sub> mice injected with BCG one day after inoculation of P815 cells.

##### *Effects on tumors inoculated i.p.*

Mice were injected i.p. with 1 mg ( $5 \times 10^6$ ) viable BCG organisms. After 14 days, they received an i.p. injection of 20  $\mu$ g of LPS. Male C3H/He mice were inoculated i.p. with  $1 \times 10^5$  cells of MH134 one day after injection of BCG. Male CDF<sub>1</sub> mice received an i.p. inoculation of  $2 \times 10^4$  P815 cells 4 days after injection of BCG. In one experiment, CDF<sub>1</sub> mice were inoculated with  $3 \times 10^3$  cells 7 days after injection of BCG. Survival days of each group were compared.

##### *Effects of sera on cultured P815 cells*

P815 cells were cultured in RPMI 1640 medium (Nissui Co., Tokyo) supplemented with 10% heat-inactivated fetal calf serum. Sera to be tested from CDF<sub>1</sub> mice, heated at 56°C 30 min, were added and the cell suspension was incubated in 5% CO<sub>2</sub> in air at 37°C for 48 hr.

Viable cells were counted using trypan blue dye exclusion method. Cytotoxicity was expressed by the formula:

$$\text{Cytotoxicity (\%)} = (1 - a/b) \times 100$$

where "a" is the number of viable cells in culture with test serum and "b" is the number of viable cells in culture without serum.

Values are given in terms of mean  $\pm$  S.D. Student's *t*-test was used in all statistical evaluations. Any *p* value less than 0.05 was considered significant.

## RESULTS

### *Effects on tumors inoculated s.c.*

Figs. 1 and 2 show the results of experiments in which BCG was injected before inoculation of tumor cells.

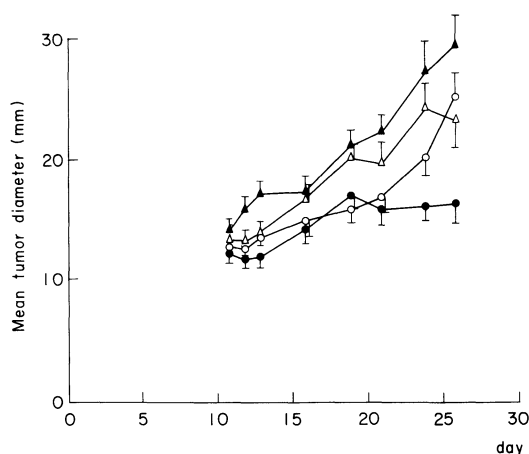


Fig. 1. Antitumor activity on P815.

Male CDF<sub>1</sub> mice were inoculated s.c. with  $2 \times 10^5$  cells of P815. Groups 1 and 2 received an i.v. injection of 1 mg of BCG 4 days before the inoculation of tumor cells. Groups 2 and 3 were injected i.v. with  $15 \mu\text{g}$  of LPS 10 days after the inoculation of tumor cells. Group 4 served as a control group. Mean tumor diameter of each group was compared. Values are means  $\pm$  s.d. group 1 (○—○); group 2 (●—●); group 3 (△—△); group 4 (▲—▲).

As shown in Fig. 1, when P815 cells had been inoculated s.c. in CDF<sub>1</sub> mice, an injection of LPS caused hemorrhagic necrosis of tumor and reduction of tumor size (groups 2 and 3). In mice pretreated with BCG plus LPS (group 2) regrowth of tumor was retarded. The mean survival days of the BCG-treated group (group 1,  $28.9 \pm 4.2$ ) and those of the group treated with BCG plus LPS (group 2,  $29.9 \pm 5.2$ ) increased significantly as compared with those of the untreated group (group 4,  $24.0 \pm 1.8$ ). However LPS did not prolong the mean survival days significantly; no statistical difference was observed between groups 1 and 2 and the mean survival days of the LPS-treated group (group 3,  $26.2 \pm 4.6$ ) were not increased significantly than those of the untreated group. In this experiment all the mice were dead at 41st day after inoculation of tumor cells.

As shown in Fig. 2, in C3H/He mice which had been inoculated with cells of MH134, an injection of LPS caused necrosis of tumors and reduction of tumor size (groups 2 and 3). However, unlike CDF<sub>1</sub> mice, the mean tumor diameter in the group treated with BCG plus LPS (group 2) remained larger than that of LPS-treated group (group 3) after the 8th day following injection of LPS. The prolongation of survival days was statistically significant for the groups treated with BCG (group 1,  $30.5 \pm 4.2$ ), BCG plus LPS (group 2,  $29.3 \pm 2.6$ ) or LPS (group 3,  $32.4 \pm 6.3$ ) as compared with those of the untreated group (group 4,  $20.9 \pm 2.0$ ). However there was no significant difference in survival time among the three experimental groups. There was no survivor in experimental groups at 52nd day after inoculation of tumor cells.

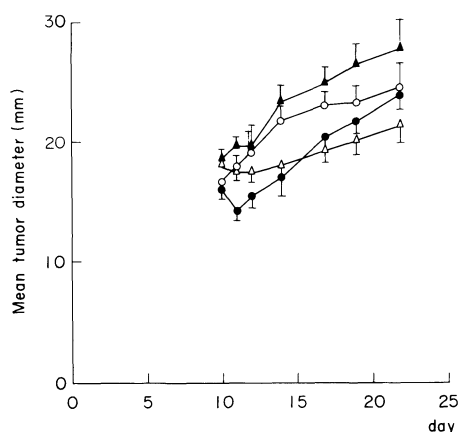


Fig. 2. Antitumor activity on MH134.

Male C3H/He mice were inoculated s.c. with  $1 \times 10^6$  cells of MH134. Groups 1 and 2 received an i.v. injection of 1 mg of BCG, 4 days before the inoculation of tumor cells. Groups 2 and 3 were injected i.v. with  $15 \mu\text{g}$  of LPS, 10 days after the inoculation of tumor cells. Group 4 served as a control group. Mean tumor diameter of each group was compared.

group 1 (○—○); group 2 (●—●); group 3 (△—△); group 4 (▲—▲).

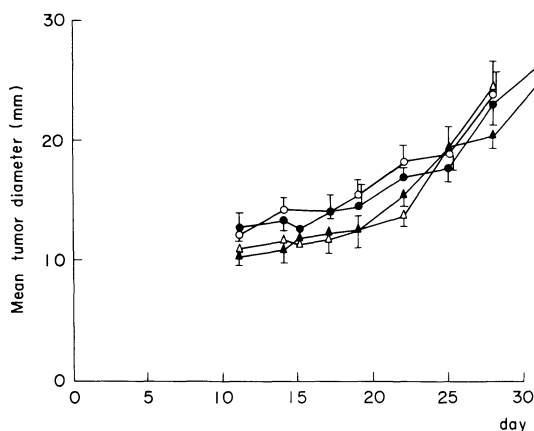


Fig. 3. Antitumor activity on P815.

Male CDF<sub>1</sub> mice were inoculated s.c. with  $2 \times 10^5$  cells of P815. Groups 1 and 2 received an i.v. injection of 1 mg of BCG one day after the inoculation of tumor cells. Groups 2 and 3 were injected i.v. with  $15 \mu\text{g}$  of LPS 14 days after the inoculation of tumor cells. Group 4 served as a control group.

group 1 (○—○); group 2 (●—●); group 3 (△—△); group 4 (▲—▲).

Fig. 3 shows the result of an experiment in which BCG was injected in CDF<sub>1</sub> mice one day after inoculation of P815 cells. The growth of tumor was accelerated in the BCG-injected groups (groups 1 and 2) and the growth of tumor was not retarded after injection of LPS. The mean survival days of BCG-treated group (group 1), the group treated with BCG plus LPS (group 2), LPS-treated group

(group 3) and the untreated group (group 4) were  $25.9 \pm 6.2$ ,  $29.1 \pm 6.0$ ,  $29.6 \pm 4.0$  and  $28.0 \pm 4.5$ , respectively. No significant difference in mean survival time was found among the three experimental groups and the untreated group.

*Effects on tumors inoculated i.p.*

As indicated in Table 1, when  $2 \times 10^4$  P815 cells were inoculated i.p. the mean survival time of the control group was  $14.0 \pm 1.0$  days. Treatment with BCG, LPS or BCG plus LPS was ineffective. No prolongation of survival days was observed.

Table 2 summarizes the results of an experiment in which  $1 \times 10^5$  cells of MH134 were inoculated into C3H/He mice i.p. The mean survival time of the control group was  $30.1 \pm 6.5$  days. The survival time in the BCG-treated group, or LPS-treated group was shortened rather than prolonged as compared with that of the control group. In this case also, treatment with BCG plus LPS did not prolong survival days. However no significant difference in mean survival time was found among the three experimental groups and the control group.

TABLE 1. *Antitumor activity of BCG and/or LPS on P815*

Group	Number of mice	Treated with	Survival days (mean $\pm$ s.d.)
1	8	BCG + LPS	$15.9 \pm 4.2^*$
2	8	BCG	$14.8 \pm 2.1$
3	8	LPS	$14.8 \pm 1.2$
4	8	Saline	$14.1 \pm 1.0$

\* not significant compared with group 4.

Male CDF<sub>1</sub> mice were inoculated i.p. with  $2 \times 10^4$  cells of P815. Groups 1 and 2 were injected i.p. with 1 mg of BCG, 4 days before the inoculation of tumor cells. Groups 1 and 3 received the i.p. injection of 20  $\mu$ g of LPS, 10 days after the inoculation of tumor cells. Group 4 served as a control group. Survival time in days of each group was compared.

TABLE 2. *Antitumor activity of BCG and/or LPS on MH134*

Group	Number of mice	Treated with	Survival days (mean $\pm$ s.d.)
1	8	BCG + LPS	$22.1 \pm 11.2^*$
2	8	BCG	$23.6 \pm 4.3^*$
3	8	LPS	$26.1 \pm 8.6^*$
4	8	Saline	$30.1 \pm 6.5$

\* not significant compared with group 4.

Male C3H/He mice were inoculated i.p. with  $1 \times 10^5$  cells of MH134. Groups 1 and 2 were injected i.p. with 1 mg of BCG, one day before the inoculation of tumor cells. Groups 1 and 3 received the i.p. injection of 20  $\mu$ g of LPS, 13 days after the inoculation of tumor cells. Group 4 served as a control group. Survival time in days of each group was compared.

As shown in Table 3, when CDF<sub>1</sub> mice received an i.p. inoculation of as few as  $3 \times 10^3$  cells of P815, the mean survival time of the control group was  $15.6 \pm 0.9$  days. Prolongation of the survival time in BCG-injected groups (groups 1 and 2) was statistically significant as compared with that of the control group. However no significant difference in survival days was observed between the BCG-treated group (group 1) and the group treated with BCG plus LPS (group 2). The mean survival time in LPS-treated group (group 3) was not different from that of the control group statistically.

#### *Effects of sera on cultured tumor cells*

Neither the serum from LPS-treated mice, nor that from BCG-treated mice was cytotoxic for cultured P815 cells. However the serum from mice treated with BCG plus LPS was highly cytotoxic, suggesting the presence of a cytotoxic factor

TABLE 3. *Antitumor activity of BCG and/or LPS on P815*

Group	Number of mice	Treated with	Survival days (mean $\pm$ s.d.)
1	10	BCG + LPS	$20.6 \pm 1.0^*$
2	10	BCG	$19.1 \pm 1.2^*$
3	10	LPS	$14.3 \pm 0.9$
4	10	Saline	$15.6 \pm 0.9$

\* significant compared with group 4.

Male CDF<sub>1</sub> mice were inoculated i.p. with  $3 \times 10^3$  cells of P815. Groups 1 and 2 were injected i.p. with 1 mg of BCG, 7 days before the inoculation of tumor cells. Groups 1 and 3 received the i.p. injection of 20  $\mu$ g of LPS, 7 days after the inoculation of tumor cells. Group 4 served as a control group. Survival time in days of each group was compared.

TABLE 4. *Cytotoxicity of sera on cultured tumor cells*

Group	Serum donor, treated with	Cytotoxicity (%) (mean $\pm$ s.d.)
A	BCG + LPS	$82.5 \pm 0.3^*$
B	BCG	$4.2 \pm 1.2^\dagger$
C	LPS	$3.5 \pm 0.3$
D	Saline	$3.0 \pm 0.4$

\* significant compared with groups B, C and D ;

† not significant compared with group D.

P815 cells ( $2 \times 10^5$ ) were suspended in 0.2 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Sera to be tested (0.02 ml) from CDF<sub>1</sub> mice, heated at 56°C 30 min, were added. Cell suspension was incubated in 5% CO<sub>2</sub> in air at 37°C in round bottom micro wells. After 48 hr, the number of viable cells was counted with a hemocytometer by trypan blue dye exclusion method. Each serum was assayed in triplicate cultures.

in this serum.

## DISCUSSION

Hemorrhagic necrosis of tumors by an injection of bacterial lipopolysaccharide (LPS) has been reported by Shear (1944). Such necrosis has been observed not only by injection of LPS but also by that of the serum from LPS-treated mice (O'Malley et al. 1962). Since the publication of reports of Halpern et al. (1958) and Suter et al. (1958) it has widely been known that mice inoculated with BCG become hyperreactive to LPS. Carswell et al. (1975) reported that an injection of the serum from animals treated with BCG plus LPS i.v. caused hemorrhagic necrosis of transplanted tumors. A factor responsible for the antitumor activity of the serum has been designated tumor necrosis factor (TNF), which is believed to be a lysosomal enzyme released from activated macrophages (Green et al. 1976; Matthews and Watkins 1978).

So far only a few publications are available concerning the *in vivo* treatment with BCG and substances of bacterial origin in tumor-bearing animals. Kiger et al. (1980) observed the antitumor effect of *Pseudomonas* instead of LPS in the assay using tumor-bearing mice pretreated with BCG. Tamura and Ishida (1984) observed complete regression of tumor in BALB/C mice by treatment with BCG plus LPS. In the present investigation regression of tumor was incomplete and all mice succumbed to tumors.

An injection of LPS (15  $\mu$ g i.v. or 20  $\mu$ g i.p.) was well tolerated even by BCG-treated mice. Apart from a transient loss of body weight, the mice showed no sign of toxicity.

The antitumor activity of BCG has already been confirmed in experimental animal models. On the other hand, the timing of BCG injection relative to the inoculation of tumor cells has been shown to be very important (Amiel and Berardet 1972; Lavrin et al. 1973; Tokunaga et al. 1974; Proctor et al. 1976; Nedvedova et al. 1978). It has also been demonstrated that BCG enhances tumor growth in certain situations (Piessens et al. 1970; Baldwin and Pimm 1973; Sparks and Breeding 1974; Ishibashi et al. 1978). In this study, when BCG was injected after inoculation of tumor cells the mean tumor size was increased in the BCG-treated mice and inhibition by injection of LPS of tumor growth was not observed.

In the treatment with BCG of experimental neoplasms, activated macrophages have been found to play an important role. It is well known also that LPS stimulates macrophages to the release of interferon. However LPS-induced interferon has been shown to be inactivated by heating at 56°C 30 min. Since the serum from the mice treated with BCG and LPS has remained cytotoxic even after heating in the present experiments, it is unlikely that the cytotoxicity is due to the presence of interferon. Therefore the results of cytotoxicity test *in vitro* seem to indicate the presence of an other-than-interferon serum factor which mediates

antitumor activity in vivo. However further investigations are required to find out whether this factor is identical with TNF. Furthermore in the treatment of neoplasms using LPS, the possibility that interleukin 1 mediates antitumor activity should be taken into account (Lovett et al. 1986).

In the present study, an injection of LPS caused hemorrhagic necrosis and retardation of tumor growth in the BCG-treated mice when tumor cells had been inoculated s.c. The reason remains as yet obscure, however, why an injection of BCG and LPS caused no inhibitory effects on tumor cells inoculated i.p. It would be of importance to investigate more closely effects of LPS on peritoneal macrophages. Experiments are now being carried out along this line.

### References

- 1) Amiel, J.L. & Berardet, M. (1972) BCG et leucémies expérimentales. *Ann. Inst. Pasteur*, **122**, 701-714.
- 2) Baldwin, R.W. & Pimm, M.V. (1973) Immunotherapy of rat tumors of defined immunogenicity. *Nat. Cancer Inst. Monogr.*, **39**, 11-19.
- 3) Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. & Williamson, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumor. *Proc. nat. Acad. Sci. USA*, **72**, 3666-3670.
- 4) Dunn, T.B. & Potter, M. (1957) A transplantable mast-cell neoplasm in the mouse. *J. nat. Cancer Inst.*, **18**, 587-595.
- 5) Green, S., Dobrjansky, A., Carswell, E.A., Kassel, R.L., Old, L.J., Fiore, N. & Schwarz, M.K. (1976) Partial purification of a serum factor that causes necrosis of tumors. *Proc. nat. Acad. Sci. USA*, **73**, 381-385.
- 6) Halpern, B.N., Biozzi, G., Howard, J., Stiffel, C. & Mouton, D. (1958) Exaltation du pouvoir toxique d'*Eberthella typhosa* tuée chez la souris inoculée avec le B.C.G. vivant. Relation entre cette augmentation de la susceptibilité et l'état fonctionnel du système réticulo-endothélial. *C.R. Soc. Biol. (Paris)*, **152**, 899-902.
- 7) Ishibashi, T., Yamada, H., Harada, S., Harada, Y., Takamoto, M. & Sugiyama, K. (1978) Inhibition and promotion of tumor growth by BCG: Evidence for stimulation of humoral enhancing factors by BCG. *Int. J. Cancer*, **21**, 67-71.
- 8) Kiger, N., Khalil, A. & Mathé, G. (1980) Tumor-necrotizing serum production by administration of BCG + *Pseudomonas*: Its application in treatment of fibrosarcoma in mice. *Recent Results. Cancer Res. (Berl.)*, **75**, 220-225.
- 9) Lavrin, D.H., Rosemberg, S.A., Connor, R.J. & Terry, W.D. (1973) Immunoprophylaxis of methylcholanthrene-induced tumors in mice with *Bacillus Calmette-Guérin* and methanol-extracted residue. *Cancer Res.*, **33**, 472-477.
- 10) Lovett, D., Kozan, B., Hadam, M., Resch, K. & Gems, D. (1986) Macrophage cytotoxicity: Interleukin 1 as a mediator of tumor cytostasis. *J. Immunol.*, **136**, 340-347.
- 11) Matthews, N. & Watkins, J.F. (1978) Tumor necrosis factor from the rabbit. I. Mode of action, specificity and physico-chemical properties. *Brit. J. Cancer*, **38**, 302-309.
- 12) Nedvedova, V., Sir, Z., Hyniova, V. & Buresova, M. (1978) Effect of *M. bovis* BCG and *M. microti* on Sarcoma 180 growth in mice. *Neoplasma*, **25**, 685-690.
- 13) O'Malley, W.E., Achinstein, B. & Shear, M.J. (1962) Action of bacterial polysaccharide on tumors. II. Damage of Sarcoma 37 by serum of mice treated with *Serratia marcescens* polysaccharide and induced tolerance. *J. nat. Cancer Inst.*, **29**, 1169-1175.



- 14) Piessens, W.F., Lachapelle, F.C., Legros, N. & Henson, J.C. (1970) Facilitation of rat mammary tumor growth by BCG. *Nature (Lond.)*, **288**, 1210-1211.
  - 15) Proctor, J.W., Auclair, B.G. & Lewis, M.G. (1976) The effect of BCG on B16 mouse melanoma: A comparison of routes of administration on tumor growth at different anatomical sites. *Europ. J. Cancer*, **12**, 203-210.
  - 16) Sato, H., Belkin, N. & Essner, E. (1956) Experiments on an ascites hepatoma. III. The conversion of mouse hepatoma into the ascites form. *J. nat. Cancer Inst.*, **17**, 1-17.
  - 17) Shear, M.J. (1944) Chemical treatment of tumors. IX. Reactions of mice with primary subcutaneous tumors to injection of a hemorrhage-producing bacterial polysaccharide. *J. nat. Cancer Inst.*, **4**, 461-476.
  - 18) Sparks, F.C. & Breeding, J.H. (1974) Tumor regression and enhancement resulting from immunotherapy with *Bacillus Calmette-Guérin* and neuraminidase. *Cancer Res.*, **34**, 3262-3269.
  - 19) Suter, E., Ullman, G.E. & Hoffman, R.G. (1958) Sensitivity of mice to endotoxin after vaccination with BCG (*Bacille Calmette-Guérin*). *Proc. Soc. exp. Biol. (N.Y.)*, **99**, 167-169.
  - 20) Tamura, K. & Ishida, N. (1984) Induction of tumor necrosis factor (TNF) in tumor-bearing mice. *Jap. J. Cancer Chemother.*, **11**, 1369-1378.
  - 21) Tokunaga, T., Yamamoto, S., Nakamura, R. & Kataoka, T. (1974) Immunotherapeutic and immunoprophylactic effects of BCG on 3-methylcholanthrene-induced autochthonous tumors in Swiss mice. *J. nat. Cancer Inst.*, **53**, 459-463.
-