Short Communication

EFFECTS OF BCG AND 1-(4-AMINO-2-METHYL-5-PYRIMIDINYL)-
METHYL-3-(2-CHLOROETHYL)-3-NITROSOUREA HYDROCHLORIDE
(ACNU) ON THE CYTOSTATIC ACTIVITY OF MACROPHAGES
IN NORMAL AND TUMOUR-BEARING RATS

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Numerous studies have revealed that bacterial adjuvants such as BCG and
BCG cell-wall skeletons have strong antitumour activity for animal tumours and
also for human malignancies (Ogura et al., 1978; Sarna et al., 1978; Yamamura et al.,
1979). These bacterial adjuvants are presumed to activate mainly macrophages
and lymphocytes, and render them toxic to tumour cells (Keller, 1974; Currie &
Basham, 1975; Mansfield & Wallace, 1977). We have reported that the cytostatic
activity of macrophages in tumour-bearing rats is decreased in the advanced
stage, and that the decreased cytostatic activity of macrophages in tumour-bearing
rats can be restored by local (i.p.) administration of BCG (Chikama et al., 1979).
We have also investigated the effect of immunopotentiators in the clinically
relevant model of immunochemotherapy for advanced solid tumours (Saijo et al.,
1979b). In the present study, we first examined the optimum dose and the
route and schedule of administration of BCG, focusing our attention on the
systemic effect of BCG. Secondly, we examined the effect of the anticancer
agent (ACNU) alone or with BCG on the cytostatic activity of macrophages. We
also analysed the correlation between this cytostatic activity and the tumour
growth in rats receiving immunochemotherapy.

Male Donryu strain rats weighing 120–200 g were obtained from Nihon Rat Co.
(Urawa, Japan). These animals had been inbred for 25 generations and thereafter
bred randomly. The tumour studied was Sato lung carcinoma (SLC) which had been
maintained by s.c. transplantation in our laboratory for more than 100 generations.
This tumour was 100% transplantable to Donryu strain rats and killed the host
within 10–15 or 15–40 days after i.v. (10^6 cells) or i.m. (10^7 cells) transplantation
respectively. The SLC is weakly immunogenic (Saijo et al., 1978, 1979)
immunized rats rejecting >10^7 viable tumour cells, whilst the minimum inoculum
for growth in unimmunized rats is 10^4 cells. In the experiments described
here, tumours used as the source of target cells for cytostatic assay were taken 7 days
after s.c. injection of 10^7 tumour cells into normal syngeneic rats. Solid SLC
was dissected free of fibrous tissue and cut into small pieces, which were incubated
with 0.25% trypsin for 10 min at 37°C with gentle stirring. The cells were collected by
centrifugation (400 g, 10 min) and washed twice with Hanks' balanced salt solution
(HBSS). The cell suspension contained about 15% macrophages.

SLC cells (10^7) suspended in HBSS at a concentration of 2×10^7/ml were inoculated
i.m. into the left thighs of Donryu strain rats, and the thickness of both
thighs was measured every other day. The difference in thickness between the left
and right thighs was taken as the diameter of the tumour. In the cytostatic assay, the SLC cell suspension was adjusted to $10^6$/ml of RPMI 1640 containing 10% heat-inactivated foetal calf serum (RPMI–FCS). To remove contaminating macrophages, 10 ml of this SLC suspension was incubated in a Terumo plastic dish (90 mm in diameter) (Terumo Co., Tokyo, Japan) in an atmosphere of humid 5% CO$_2$ 95% air, at 37°C for 1 h. Non-adherent cells were then collected by repeated intensive washing with HBSS. More than 95% of non-adherent cells were identified as SLC cells by Giemsa stain.

Living BCG (Nihon BCG Laboratory, Tokyo, Japan) (1–10 mg) was used as immunopotentiator. BCG suspended in physiological saline was injected (i.p. or i.v.) into Donryu strain rats. Five mg of BCG contained 3–4 x $10^8$ living cells.

1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) (Sankyo Pharmaceutical Co.) was dissolved in physiological saline and injected i.v. into normal and tumour-bearing rats. ACNU, a water soluble nitrosourea, is effective for animal tumours and for human malignancies in large intermittent doses (Saijo & Niitani, 1980).

Peritoneal cells were collected by washing the peritoneal cavity of Donryu strain rats with 40 ml of Eagles' MEM containing heparin 5 u/ml, streptomycin 100 μg/ml, penicillin 100 u/ml. The cells were washed twice with the same solution and finally suspended in RPMI–FCS at a concentration of $5 \times 10^5$/ml. One-ml quantities of nucleated-cell suspension were plated in flat-bottomed glass culture tubes (15 mm in diameter) and incubated in an atmosphere of humid 5% CO$_2$ 95% air, at 37°C for 1 h. Each culture was then washed x 3 with PRMI–FCS. The recovery of adherent cells in each experimental group ranged from 41.2–60.8% of plated peritoneal cells. More than 95% of these adherent cells were identified as macrophages on the basis of cytoplasm and nuclear shape shown by Giemsa stain, and by their phagocytic activity for Indian-ink particles.

To detect in vivo antitumour activity of activated peritoneal-exudate cells and macrophages, the Winn type of neutralization test was performed. Peritoneal cells were harvested from normal rats and from rats inoculated i.v. with 5 mg of BCG 21 days before by washing their peritoneal cavities. They were washed twice with MEM and finally suspended in RPMI–FCS at $2 \times 10^6$/ml. These cells were plated in the Terumo plastic dishes, and incubated for 1 h at 37°C in an atmosphere of humid 5% CO$_2$ 95% air. After non-adherent cells were removed by repeated intensive washing with MEM, macrophages adhering to the dishes were scraped with a rubber policeman and washed twice with RPMI–FCS. They were then suspended in RPMI–FCS and checked for viability. SLC cells ($5 \times 10^4$/0.25 ml) and peritoneal cells or macrophages ($5 \times 10^6$/0.25 ml) were mixed and incubated at 37°C for 1 h. The mixture of these cells was inoculated i.m. into the left thigh of normal Donryu strain rats, and the tumour size was measured every other day.

The cytostatic activity was determined by the modification of Germain's method (Germain et al., 1975; Williams et al., 1975). Flat-bottomed glass tubes (15 mm in diameter) were used for the cytostatic assay. SLC cells (2.5 x $10^5$ cells/0.25 ml of RPMI–FCS) and 0.5 ml of RPMI–FCS were placed on the monolayer of the macrophages obtained as described above. The cytostatic activity of macrophages from normal rats was the control. The cells were incubated in 5% CO$_2$, at 37°C for 3 h, and 0.5 μCi of $^3$H-thymidine (Radiochemical Centre, Amersham, U.K.) in 0.25 ml of RPMI–FCS was added. After further incubation for 3 h, the cells were harvested on a Whatman GF/C filter (W and R Balston Co., U.K.) and washed with 5 ml of 5% cold trichloroacetic acid x 3 and dried with methanol. The filters containing the cells were transferred to glass vials containing toluene-based
scintillation fluid, and their radioactivity was counted with a liquid scintillation counter (Packard, U.S.A.). Under these conditions, the average radioactivity in the culture of tumour cells alone or macrophages alone was 36,788 ± 2645 ct/min, and 887 ± 182 ct/min respectively. The cytostatic activity was expressed as the percentage of inhibition of DNA synthesis of SLC cells calculated from the following formula:

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\text{Inhibition of DNA synthesis} (\%) = \left( 1 - \frac{\text{ct/min (effector cell + target cell)} - \text{ct/min (effector cell)}}{\text{ct/min (target cell)} - \text{ct/min (medium alone)}} \right) \times 100
\]

The cytostatic activity of normal macrophages was 44.3, 49.4, 55.0 and 62.0% when the ratio of macrophages to SLC cells was 0.5:1, 1:1, 1.5:1 and 2:1 respectively. Based on these data, the effector to target cell (E:T) ratio of 1:1 was used in all the experiments.

The results in Fig. 1 represent the effect of i.v. BCG on the number of peritoneal cells and on the cytostatic activity of peritoneal macrophages. The respective values in normal rats were 22.0 ± 0.8 x 10^6 and 49.4 ± 2.6%. The values gradually increased after i.v. BCG and reached a maximum level in 21 days (Fig. 1A). The number of peritoneal cells increased in rats receiving 5 mg or more of BCG. On the other hand, the cytostatic activity of peritoneal macrophages increased with each dose, in rats administered BCG i.v. from 1–10 mg (Fig. 1B). About 20% of the rats receiving 10 mg of BCG died of toxicity. Based on these data, it seemed to be reasonable to give 5 mg of BCG i.v. 21 days before the experiments in order to obtain the maximum effect.

To clarify the mechanism of in vitro cytostatic activity of immunopotentiator-activated macrophages for SLC cells, a neutralization test was performed. The tumour grew progressively in all the 8 rats or 5/6 rats inoculated i.m. with SLC cells mixed with normal peritoneal cells or macrophages, respectively. On the other hand, we could detect tumour growth in only 2/8 rats or 1/6 rats receiving SLC cells mixed with peritoneal exudate cells or macrophages from rats inoculated i.v. with BCG, respectively.

The results in Fig. 2 demonstrate the effect of ACNU alone or ACNU+BCG on the number of peritoneal cells and the cytostatic activity of peritoneal macro-

![Fig. 1](image-url)

**Fig. 1.**—Effect of BCG (i.v.) on the number (○—○) of peritoneal cells and cytostatic activity (●—●) of peritoneal macrophages. Normal range (mean ± s.e.) of cytostatic activity ■; normal range (mean ± s.e.) of the number of peritoneal cells ——.
phages in normal rats. The maximum tolerated dose of ACNU (30 mg/kg) was selected for this experiment. One week after the administration of ACNU, the number of peritoneal cells and the cytostatic activity of the macrophages decreased significantly. The cytostatic activity was 21.7 ± 4.9% in animals treated with ACNU, and returned to normal level 2 weeks later. The number of peritoneal cells and the cytostatic activity of macrophages were not decreased in rats receiving less than 20 mg of ACNU per kg. The cytostatic activity of macrophages was increased to 63.1 ± 5.1% in rats administered both ACNU and BCG.

In order to evaluate the effect of the anticancer agent and/or the immunopotentiator on the number of peritoneal cells and the cytostatic activity of macrophages, as well as on tumour growth, 10^7 SLC cells were inoculated i.m. into the left thigh of Donryu strain rats 21 days before the assays. ACNU (30 mg/kg) and BCG (5 mg) were administered i.v. 7 and 21 days before the assays, respectively. The tumour size in untreated rats was 28.7 ± 1.0 mm 21 days after i.m. inoculation. The number of peritoneal cells and their cytostatic activity were significantly decreased in this group of tumour-bearing rats (Fig. 3). In rats receiving 5 mg i.v. BCG at the time of tumour inoculation, the tumour size was 27.7 ± 1.3 mm. However, the number of peritoneal cells and the cytostatic activity of macrophages returned to the normal level (Fig. 3). In rats treated with 30 mg/kg of ACNU i.v., the tumour size decreased to 23.9 ± 1.0 mm; though the number of peritoneal cells and the cytostatic activity of macrophages remained at low levels (Fig. 3). On the other hand, in rats treated with the combination of ACNU and BCG, the tumour size decreased to 16.3 ± 3.4 mm, and the number of peritoneal cells, and the cytostatic activity of macrophages, were 20.8 ± 7.1 × 10^6 and 73.9 ± 7.4% respectively (Fig. 3).

The vital role of macrophages in control of tumour growth and dissemination is well recognized, and many studies have reported nonspecific activation of macrophages by a variety of immunopotentiators, to become inhibitory for tumour cells in vivo and in vitro (Schultz et al., 1977). However, there are few available data on the cytostatic activity of macrophages in rats (normal or tumour-bearing) administered systemically with anticancer agents and immunopotentiators.
In an earlier study, we detected a strong cytostatic activity of macrophages in Donryu strain rats inoculated i.p. with 1 mg of BCG (Chikama et al., 1979). In the present study, we showed that it was necessary to give 5 mg or more of BCG by i.v. administration in order to increase the cytostatic activity of macrophages, and that the maximum cytostatic activity against SLC cells appeared 3 weeks later. These results suggest the activation of peritoneal macrophages by systemic administration of BCG, the effect varying according to dose, and to the route and schedule of administration. In the Winn type of neutralization test, the macrophages of normal rats failed to suppress tumour growth which was strongly suppressed by BCG-activated macrophages. In other words, the increased cytostatic activity of macrophages correlated well with the in vivo suppression of tumour growth. In our study of the effect of ACNU on the cytostatic activity of macrophages in normal rats, we found that this was decreased in rats receiving the maximum tolerated dose of ACNU; but it was easily restored or even elevated by the combination of ACNU and 5 mg of i.v. BCG. Thus the decrease in cytostatic activity of macrophages induced by ACNU can be prevented by the systemic administration of BCG. The cytostatic activity of macrophages was significantly decreased in tumour-bearing rats, but was restored to the normal level by i.v. BCG, though the tumour growth was not influenced by BCG administration. Tumour growth was significantly suppressed in animals receiving ACNU. In animals treated with ACNU and BCG, tumour growth was more effectively suppressed than when treated with ACNU alone. The cytostatic activity of macrophages in these animals was also apparently higher than in those treated with BCG or ACNU alone. It is suggested, therefore, that the systemic administration of BCG can potentiate the effect of the antinecancer agent, and that raised cytostatic activity of macrophages contributes to the suppression of tumour growth. There have been numerous clinical studies on the effect of immunopotentiators such as BCG and BCG-CWS on malignant melanoma, lung cancer, and leukaemia (Holoye et al., 1978; Sarna et al., 1978; Yamamura et al., 1979). However, the positive role of immunochemotherapy for advanced solid tumours is still under discussion. Hosokawa et al. (1971) reported that the optimal dose, route and timing of immunopotentiators have a very limited range. In our study the dose, route and timing of BCG administration were determined from the cytostatic activity of macrophages which inhibited the regrowth of tumours treated by chemotherapy. In conclusion, it is argued that the preferred administration of immunopotentiators should be determined from the analysis of nonspecific and specific immune responses, such as macrophage-mediated cytostatic and cytolytic activity, natural killer activity (Saijo et al., 1980) and antibody-dependent cellular cytotoxicity, as well as specific cytotoxicity of T-cell and mixed lymphocyte target interaction, which are directed at the tumour itself.

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