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Shigeo Hayashi*

*Okayama University,

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Abstract

In order to approach human cancer immunotherapy, the author carried out the immunotherapy with BCG on mice having homotransplanted cancer, observed the posttransplantation results with lapse of time, conduced daily macrophage inhibition test (MI test) and found the immunotherapy to be effective. At the same time the MI test proved to be a useful criterion in determining the course of cancer progress and effectiveness of the immunotherapy.

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EXPERIMENTAL STUDIES OF TUMOR IMMUNOTHERAPY II. TUMOR IMMUNOTHERAPY FOLLOWING TUMOR EXTIRPATION

Shigeo HAYASHI

Department of Surgery, Okayama University Medical School, Okayama 700, Japan (Director: Prof. S. Tanaka) Received for publication, January 21, 1976

Abstracts. In order to approach human cancer immunotherapy, the author carried out the immunotherapy with BCG on mice having homotransplanted cancer, observed the posttransplantation results with lapse of time, conducted daily macrophage inhibition test (MI test) and found the immunotherapy to be effective. At the same time the MI test proved to be a useful criterion in determining the course of cancer progress and effectiveness of the immunotherapy.

The immunotherapy is the fourth therapeutic method following the surgical treatment, radiotherapy, and chemical treatment, and it has recently attracted a great interest. In order to attain effective results by immunotherapy it is necessary to demonstrate the tumor specific antigen (TSA), i.e. cancer cell antigen that elicits an immune reaction in vivo (1). Ever since Foley's (2) discovery (1953) of tumor antigen in the autogenous cancer of animal induced by methylcholanthrene, Habel (3) has demonstrated tumor antigen in virus-induced tumor, Klein (4) in human lymphoma cells, and Hellström et al. in colon and kidney cancers (5, 6). However, even if there exists cancer specific antigen, the immune reaction does not occur unless the host recognizes it. And as the induction methods of cancer specific immunity, there have been reported such methods as transplantation and removal (i.e., extirpation or ligation) of primary or autogenous cancer and of homogenic cancer, transplantation of modified auto-homogenic cancer, and transplantation of modified auto-homogenous cancer cells previously treated with radiation or anticancer agents.

On the other hand, the nonspecific immunologic stimulus represented by BCG is thought to enhance general immune reactivity of cancer-bearing body and also to induce ultimately a cancer specific immunity. For the past half a century BCG has been essentially considered as a vaccine for tuberculosis treatment, and widely used in the world. Since reports of Halpern (7) and Lemond (8) stating that, after the BCG inoculation, the reticuloendothelial system can be stimulated due to the proliferation of transplanted tumor, Mathé (9) yielded

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more effective results by smearing fresh BCG suspension over the scratch made on the skin, as compared with untreated control group. Morton (10) injected BCG into melanoma-bearing patients who were not indicated for surgical treatment, and observed the shrinkage of the half number of tumors so treated. In addition Zbar (11) et al. found the antitumor effect of BCG on the syngeneic hepatocarcinoma induced artificially in guinea pigs by administering diethyl nitrosamin. Tanaka (12) and Tokunaga (13) also reported the results obtained with mice and guinea pigs.

Up to date, concerning the methods of using BCG in the immunotherapy for human and animal cancers, there have been (1) its inoculation into the skin of cancer-bearing body, (2) its injection into solid tumor, (3) the intradermal injection of cancer cells with BCG, and (4) oral administration. Regarding the method and time of administration or effective duration of such injection, however, opinions are varied and also sufficient investigations remain to be carried out.

In the present study the author carried out the immunotherapy with BCG on mice having homotransplanted cancer, observed the post-transplantation results with lapse of time, conducted daily macrophage inhibition test (MI test) and found the immunotherapy to be effective. At the same time the MI test proved to be a useful criterion in determining the course of cancer progress and effectiveness of the immunotherapy.

MATERIALS AND METHODS

a. Animals: Animals used were C3H/He mice weighing about 20g and purchased from the Fujii Medico-chemical Test Animal Dealers. They were fed on solid Oriental food, and tap water was given ad libitum. Guinea pigs weighing 200-300 g were also purchased from the same dealers and were fed on rice bran mixed with solid Oriental food.

b. Test tumor: Tumor used was ascites hepatoma 134 (MH 134); this has been derived from C3H mouse obtained from Tokyo Medical Laboratory, and maintained successively in the peritoneal cavity of C3H mice as ascites until use.

c. BCG: BCG used was obtained from Japan BCG Manufacturing Company in a frozen-dried state, which was dissolved in saline solution before use.

d. The preparation of lymphocytes of mouse: Mice were sacrificed in an ether bottle. Axillay lymph nodes from both sides were taken out asceptically, which were washed with saline solution, and sliced into fine pieces with scissors. These pieces were immersed in 5ml physiological saline to make a lymphocyte suspension, which was left standing at room temperature for 30 min then passed through an 80-mesh filter, and centrifuged at 1000 r.p.m. for 10 min. After repeating the centrifugation, the sediment was suspended in TC-199 solution for the experiment.

e. Preparation of macrophages: Twenty to thirty ml of fluid paraffin sterilized

by high pressure is injected into the peritoneal cavity of guinea pigs 4-5 days prior to the preparation of macrophages, and the animals are sacrificed by severing the cervical artery. Then the peritoneal cavity is opened, the cavity is washed with 100-150 ml saline solution, and the cell-containing abdominal fluid is collected in a centrifuge tube. This fluid is centrifuged at 1000 r.p.m. for 5 min, and then paraffin of the upper layer is removed by sucking. The residue is washed again with saline solution, centrifuged once more at 1000 r.p.m. for 5 min, and suspended in TC-199 solution for the experiment.

f. Preparation of antigen solution: MH 134 is suspended in TC-199 medium, adjusting the cell concentration to 10⁷ cells/ml, supersonicated at 150 mA for 5 min, and then centrifuged at 3000 r.p.m. for 20 min. The supernatant is taken as the antigen solution; this is sealed into ampules, each of which is made to contain 0.5ml antigen solution, and such ampules are kept at -20° C before use. This solution contains protein in the concentration of 2mg/ml, which is diluted 20-fold before use. Namely, the protein concentration of antigen solution in the MI test is adjusted to 100 μ g/ml. At this concentration there occurs no non-specific migration inhibition of macrophages.

g. Measuring of MI activity: As already described in Part I, the MI activity measurements are taken by the capillary tube direct method, where lymphocytes and macrophages are mixed in the ratio of 1:5, and centrifuged at 1000 r.p.m. for 5min. The sediment so obtained is put into capillary tubes, one end of which is sealed with open end up. These tubes are centrifuged at 800 r.p.m. for 3min, and are cut with an ampule cutter at the boundary line between the supernatant and the cell layer. The tubes containing the cell layer are fixed with silicon glass onto a small Petri dish, approximately one cm in diameter, the bottom of which is covered with a piece of round coverglass. Such small Petri dishes are divided into antigen-containing and nonantigen-containing groups, then covered with coverglasses, and incubated in an incubator containing 5% CO₂ gas at 37°C for 24 hr, and then the migration areas are measured. The long diameter × short diameter of the cell area, in which the cells have migrated from the cut-end of the capillary tube, is taken as the migration area, and the ratio of the antigen containing group to that of non-antigen group is taken as the migration index (MI). And those that gave the ratio below 85% are taken as positive.

Experiment 1. Two weeks after subcutaneous injection of 2×10^4 MH-134 cells on the back of C3H mice, these animals were divided into 4 groups; the number of mice in each group was 6: (1) receiving the injection of 0.1 ml saline solution; (2) receiving 0.04 mg/0.1 ml BCG; (3) receiving 0.4 mg/0.1 ml BCG: and (4) receiving 4 mg/0.1 BCG. Saline solution or BCG was injected directly into the tumors. Survival time and tumor growth were observed on each group. In addition, on groups (1) and (4) the MI activity of regional lymph-node cells was measured with lapse of days.

Experiment 2. C3H mice were transplanted with 2×10^4 MH-134 cells subcutaneously on the back, and these were divided into 5 groups; the number of mice in each group was 10: (1) non-treated control group; (2) group receiving

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4 mg BCG and 2×10^4 MH-134 cells one week later on the loin away from tumor; (3) group receiving an additional 4 mg BCG plus 2×10^4 MH-134 cells one week after (2); (4) group receiving 4 mg BCG and 2×10^4 MH-134 cells two weeks later on the loin away from the tumor; and (5) group receiving an additional 4 mg BCG plus 2×10^4 MH-134 cells two weeks after (4). The survival time and the tumor growth were observed on these five groups, and also MI activity was measured daily.

Experiment 3. C3H mice were transplanted subcutaneously on the back with 2×10^4 MH-134 cells, and were divided into 5 groups; the number of mice in each group consisted of 10: (1) non-treated group; (2) the group of which tumor was extirpated on the second week; (3) the group in which tumor was removed on the second week and 4mg BCG was injected at a site away from the tumor site; (4) the group in which tumor was removed on the second week and 2×10^4 MH-134 cells previously treated with mitomycin C was injected at a site away from the tumor site; and (5) the group in which tumor was removed on the second week and the combination of 4mg BCG plus 2×10^4 MH-134 cells previously treated with mitomycin C was injected at a site away from the tumor site. Tumor growth and survival time of these five groups were observed, and simultaneously MI activity of the axillary lymph node cells was measured with lapse of days. As for mitomycin C the cells were treated with $25 \mu g/ml$ for 30 min at room temperature.

RESULTS

Exp. 1. In the (1) control group tumor was palpable around one week and grew rapidly after two weeks, and about half the animals died by the 24th day, the average survival time being 26 days. In the (2) group the proliferation of tumor was slower than that of the (1) group, the average survival time being prolonged to 29.6 days. The proliferation curve of the (3) group was slower than that of the (2) group, the average survival time being 33.5 days. The (4) group showed the slowest growth as compared with other groups, the average survival time being 34.2 days. (Fig. 1).

Concerning the MI activity, the (1) group became positive by the third day, reached the maximum of 66% on the sixth day, decreased gradually thereafter, and became negative from the second week. The (4) group reached its peak on the sixth day and then decreased, whereas by BCG injection into the tumor, MI activity again reached the maximum of 60% on the 17th day (namely, on the fourth day after BCG injection), thereafter decreased gradually and disappeared (Fig. 2).

Exp. 2. The average survival time was 29 days in (1) group, 36.3 days in (2) group, 43.3 days in (3) group, 31.6 days in (4) group, and 34.5 days in (5) group, while the 50% survival time was 31 days in (1) group, 34.5 days in (2) group, 40 days in (3) group, 33 days in (4) group, and 36 days in (5) group.



...... control group

--- • 4 mg BCG injected group

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The group of which average survival time was longest was (3) group that received immunization at an interval of a week twice, and the average survival time in (5) group was less than that of (2) group.

As for the MI activity, it reached its maximum on the sixth day in (1) group, gradually decreased thereafter and disappeared by the second week. In (2) group, the activity once tended to decrease after the injection but reached again its peak of 59% by the 14th day (the seventh day after injection), decreased graduadually thereafter, and turned negative on the 21st day. Compared with the control group the positive period is 7 days longer. In (3) group which received its second injection on the 14th day, MI activity decreased more slowly than in (2) group and became negative on the 29th day, showing the positive period 8 days longer than (2) group (Fig. 3). In (4) group, the activity appeared to reach its peak of 60% on the 17th day (on the third day after injection), decreased gradually thereafter, and turned negative on the 23rd day. This is a prolongation of 9 days as compared with control group. In (5) group in





③ second immunotherapy treatment
……○…… control
……△…… response to first treatment

response to second treatment

which the second injection was done on the 28th day, MI activity did not became positive (Fig. 4).



Fig. 4. Effect of immunotherapy on MI activity and survival time of tumor bearing mice.

① first immunotherapy treatment
② second immunotherapy treatment
○ control
○ response to first treatment
○ response to second treatment

Exp. 3. In (1) control group, tumor began to be palpable as a solid node by about the 7th day and grew rapidly by about the second week. MI activity reached its highest by the sixth day, gradually diminished along with the tumor enlargement, and turned negative by about the second week. The 50%survival time of this group was 24 days. In the group of (2) where tumor had been extirpated in the second week, MI activity turned negative after the tumor removal, then returned positive again by about the 20th day, persisted for 3 to 4 days and finally turned negative. Tumor recurred one after another a few days later. In (3) group, MI activity did not grow stronger even after BCG injection, and the 50% survival time of this group was 47 days. In (5) group, MI activity had disappeared once in the second week, then slightly later than

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(2) group, i.e., around 20 days later, became positive for a long period of about 15 days. During this period no recurrence of tumor was seen, and after the MI activity had turned negative there was recurrence in two out of ten mice, although the proliferation was very slow. The 50% survival time of this group was 93 days, showing an extensive prolongation as compared with other groups (Fig 5).



Fig. 5. Effect of tumor extirpation and immunotherapy on MI activity and survival time of tumor bearing mice.

1		control group
2	$ \triangle$	tumor extirpation group
3		extirpation plus BCG injected group
4		extirpation plus MMC treated MH-134 injected group
(5)		extirpation plus BCG plus MMC treated MH-134 injected group

DISCUSSION

Inhibition of the cell migration by antigen *in vitro* was first studied by Rich and Lewis (14) on explants of lymphoid tissue maintained in culture. This and subsequent work have shown that inhibition of cell migration depends upon the existence of delayed hypersensitivity of host, and the effect is seen only with lymphoreticular tissue. The capillary tube technique developed by

George and Vaughan (15) permitted a more analytical approach, both quantitatively and in the identification of the cell types affected. With this technique, David *et al.* (16) have demonstrated reactivity to bacterial antigens, proteins and haptens which paralleled skin reactivity to these antigens *in vivo*.

Bloom and Bennett (17) and David (16) have independently found that, through interaction by specific antigens, lymphoid cells obtained from animals with delayed hypersensitivity release a substance that inhibits migration of macrophages from normal as well as from hypersensitive animals. David has also shown that inhibition does not occur if syntheses of protein or messenger ribonucleic acid are blocked by puromycin or actinomycin-D respectively. Now, in Exp. 1 when BCG is injected into tumor, specific inflammatory reaction of BCG enfolds tumor cells nonspecifically and destroys them, in other words, by the so-called enfolding effect, sensitized lymphocytes (T-cells) are reinforced as to vield an antitumor effect, which revives the MI activity once on the verge of disappearance. Even the average survival time is lengthened by 8 days, indicating BCG to be effective as antitumor action. In addition, when BCG is injected directly into the tumor, tumor-growth inhibitory effect and survival effect show BCG dose-response, and the administration of BCG in the doses of 0.04, 0.4 and 4 mg reinforces its effect in the order mentioned. Moreover, when the combined BCG and tumor cells are injected at a site away from the tumor focus (that is, when BCG adjuvant therapy is performed), though inferior to direct injection into tumor, a fairly good antitumor effect can be attained, and even in MI activity its positive period is lengthened by about one week. From the results of Exps. 1 and 2, the BCG adjuvant therapy, in which BCG is injected at a site a little distant from pathologic focus rather than directly into the tumor, enhances the cancer immunity of tumor-bearing mice, has been clarified to inhibit the proliferation of main pathologic focus, and also gives us a hope in the prevention of postoperative recurrence and the proliferation-inhibion of metastatic residual focus. The BCG adjuvant therapy reinforces this MI activity, in other words, it enchances specific cellular immunity. Regarding the optimal concentration of tumor antigen, the problem was already described in the previous paper. And MI activity was measured on regional lymph node cells of the mice which were previously treated with mitomycin C subcutaneously on the back to inhibit the proliferation of tumor cells; and then tumor cells were injected into the animals in step-wise doses. The group receiving 105 tumor cells showed the highest MI activity, while the groups receiving the dose of 103 and 108 tumor cells hardly showed positivity. This phenomenon seems to suggest that for the MI activity to be positive presence of the tumor cells in vivo have a certain latitude, beyond which or below which it turns negative. In order to apply this fact and the BCG adjuvant therapy

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after surgical operation, Exp. 3 was conducted. In (1) group, after tumor transplantation the MI activity of regional lymph-node cells was positive already on the third day, reached its maximum on the sixth day, thereafter decreased gradually and turned negative in the second week. The tumor proliferation rapidly gains its speed for past two weeks when MI activity turns negative. And when this result is compared with the previous results (Part 1), the positive period of MI activity corresponds to the period during which the number of tumor cells is limited to a certain number. And when the tumor cells proliferate beyond a certain limit, the MI activity also seems to disapppear. In (2) group where tumor was extirpated in the second week, the activity of MI once lost became positive again one week later and turned negative again 3-4 days The 'recurrence of tumor could be recognized macroscopically another 4 later. to 5 days later, and at least during the period when MI activity was positive, no recurrence of tumor could be observed. Therefore in order to check the recurrence or eliciting the proliferation inhibition most effectively, it would be necessary to maintain MI activity as long as possible; that is, to administer the tumor antigens of which cell division has been inhibited. However, as observed in the (3) and (4) groups by the subcutaneous injection of tumor antigens alone or BCG alone, no any marked effect could be observed on the survival time or on reinforcement of MI activity. It seems that by the coexistence of tumor antigen and BCG the antigenity is transmitted and strengthened in vivo, and also BCG, as a nonspecific stimulant, plays a role of the specific stimulant through contact with antigens. The author observed that MI activity can be reinforced and maintained and observed the inhibition of tumor recurrence as well as the prolongation of survival time in the mice extirpated of tumor, when the combination of BCG and MH-134 cells, of which proliferation had been inhibited by mitomycin-C treatment, was injected into the mice. This indicates the possibility in clinical practice that the immunotherapy would yield favorable results in the recurrence of cancer and the prognosis after the removal of tumor. For such a purpose it would be advisable to follow up the specific cellular immunity as represented by the MI-test.

Nevertheless, in the postoperative treatment of cases with malignant tumor there remain problems as regards the immunotherapy on every patient, and the BCG adjuvant therapy might not get expected good results on absolutely noncurative patients. In Exp. 2 the average survival time was prolonged in the groups of (3), (2), (5), (4) and (1), in that order. And the group receiving immune sensitization twice on the 14th and 28th days after tumor transplantation give the poorer results than the group sensitized once only. Even from the aspect of MI activity, (5) group sensitized twice does not acquire positive MI activity, indicating a limitation in immunotherapy in the cases of the terminal

stage of cancer. In the group sensitized in a relatively early stage of the first week, especially in (3) group sensitized consecutively twice, the survival time is prolonged as much as 14 days in comparison with (1) control group, indicating that early frequent immunization is effective. These findings seem to suggest that the immunotherapy is indicated for those cases after radical operation or those in the condition close to it; namely, in the state of the minimum residual tumor tissue.

Surgical biochemical, and radiation therapies are carried out for the purpose of eradicating or destroying cancer cells, but it is difficult to annihilate completely cancer cells to the very last one. Hence it is suggested that specific immunotherapy is a choice effective for those cases after surgical therapy. Furthermore, chemotherapy and radiation therapy so damage normal cells to a high percentage that even on this point alone the immunotherapy is superior. However, when applying it in clinics after surgical operation, depending on the histological type of cancer and also the difference in antigens, it is necessary to carry out further studies as to the method of immunotherapy te be employed and the number of times of the immunotherapy to be given.

Recently there are reports stating that, in the patients whose immunity is low, BCG live bacteria elicit infection-like symptoms or anaphylaxis-like shock. And for an improved method on overcoming these dangers, peptide glycolipids that constitute the cell wall (18) and skeleton of BCG are attracting attention. Such problems of bacterial components would be further studied for the future clinical application of this immunotherapy.

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