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Abstract

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EXPERIMENTAL STUDIES OF TUMOR IMMUNOTHERAPY. I. MACROPHAGE MIGRATION INHIBITORY ACTIVITY AS AN IMMUNOLOGICAL PARAMETER

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Abstract. The macrophage migration inhibition activity (MI activity) was stable in sensitized lymphocyte-to-macrophage ratios of 1 : 5 to 1 : 20 in mice. Antigen protein concentrations under 100 μg/ml did not induce nonspecific macrophage migration inhibition. Inhibition of tumor proliferation and survival was observed after a combined injection of BCG and MH-134 cells. After a single injection of MH-134 tumor cells, MI activity was reinforced and prolonged, demonstrating the clear effects of BCG as adjuvant. In DDS mice MI activity was weakened in the regional lymph node after a subcutaneous injection of just above or below 10^5 Ehrlich cancer cells previously treated with mitomycin C. This finding suggests the presence of an optimal tumor antigen concentration.

The macrophage migration inhibition test (MI test) is a method devised by Rich and Lewis (1) in 1932 to demonstrate the specific inhibition of cell migration when blood cells from guinea pig sensitized with tubercle bacillus are cultured in the presence of old tuberculin at an appropriate concentration. George and Vaughan (2) observed macrophage migration inhibition by antigen when peritoneal exudative cells from sensitized animals were placed into a glass micropipet. This method was further improved by David *et al.* (3) and Bloom *et al.* (4). This method is widely used as a model of *in vitro* delayed hypersensitivity. This phenomenon has been demonstrated to be due to the reaction of cellular antibody of the delayed allergic-sensitized body, i.e., sensitized lymphocytes and antigen, which results in the production of macrophage migration inhibition factor (MIF). In the present *in vivo* study of delayed allergic reaction, the cell immunity established at the time of organ or tissue transplantation and the cell immunity relative to tumor immunity were suggested as arising from the identical mechanism. The author observed MI changes in delayed allergy using mice sensitized with BCG and studied the alterations in transplantation immunity and tumor immunity after tumor transplantation.

MATERIALS AND METHODS

Animals. The animals used were C3H/He mice and DDS mice, weighing about 20g each. They were procured from the Fujii Medico-Chemical Test Animal Dealers. The animals were given solid Oriental Feed (Oriental Yeast Co.) and tap water. Guinea pigs weighing 200-300g purchased from the same dealer were fed on a mixture of rice bran and solid Oriental Feed (for guinea pigs).

Test tumors. The tumor used was ascites hepatoma 134 (MH 134) derived from C3H mouse, donated by the Tokyo Medical Research Laboratory; they were maintained successively in the peritoneal cavity of C3H mice until use. Ehrlich ascites cancer cells were obtained from the Cancer Research Institute of Okayama University Medical School; they were maintained successively in the peritoneal cavity of DDS mice until use.

BCG. BCG (bovine tuberculous bacillus) was purchased from the Japan BCG Mfg. Co. as a freeze-dried sample. It was dissolved in physiological saline solution before use.

PPD. The samples were purchased from the Japan BCG Mfg. Co. They were dissolved in solvent containing purified tuberculin for confirmation-diagnosis before use.

Preparation of mouse lymphocytes. The mouse was killed by ether, and both axillary lymph nodes were removed asceptically and washed with saline solution. They were sliced into thin pieces with anatomical scissors and the fine pieces were immersed in 5mg of saline solution to obtain the lymphocyte suspension. After 30min at room temperature, the suspension was filtered through a 80-mesh filter; the filtrate was centrifuged at 1,000rpm for 10min; and the supernatant discarded. The residue was resuspended in saline solution, centrifuged at 1,000rpm for 10min and the final residue was suspended in medium TC-199 before use.

Preparation of macrophages. Fluid paraffin at 20-30ml was inoculated into the peritonial cavity of guinea pigs. After 4 or 5 days the guinea pigs were anesthetized with ether, the cervical artery severed and the animals bled to death. The abdomen was opened; the abdominal cavity rinsed with 100-150ml saline solution and the abdominal cavity fluid (ascites) was collected in a centrifuge tube. The collection was centrifuged at 1,000rpm for 5min; the paraffin at the upper layer was discarded; the residue was washed with saline solution and centrifuged again at 1,000rpm for 5min. The final residue was suspended in medium TC-199. This suspension was used for the experiment.

Preparation of antigen solution. The Ehrlich ascites cancer cells and MH 134 cells were suspended in medium TC 199 at a concentration of 10^7/ml, supersonicated at 150mA for 5min to destroy the cells, centrifuged at 3,000rpm for 20min, and 0.5ml of each supernatant was put into separate ampules and kept at $-20°C$ until used. The protein concentration of such an antigen solution was 200mg/dl, and it was diluted 20-fold before use. Prior to the experiment it was confirmed that no unspecific migration inhibition would occur at this

concentration by testing for contamination of sensitized lymphocytes or macrophage migration.

Preliminary measurement of MI activity. Lymph-node cells and macrophages were mixed by the capillary tube-direct method. The mixture was centrifuged at 1,000 rpm for 5 min, placed into a capillary tube (micropipet, 75 mm × 1.5 mm, Terumo, Tokyo) with the bottom end sealed and top end opened and centrifuged at 800 rpm for 3 min. The capillary tube was cut with an ampule cutter at the boundary between the supernatant and the cell layer. The cell layer region was fixed on a cover-glass on a micro-Petri dish. Such Petri dishes were divided into a test group filled with antigen plus 20% calf serum plus TC-199 and a control group filled with 20% calf serum plus TC-199 without antigen. These micro-Petri dishes were each covered with a cover-glass, incubated with 5% CO_2 gas at 37°C, and after 24 hr-incubation, the area of cell migration was measured (Fig. 1.)

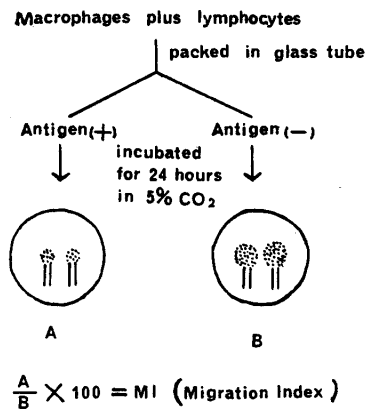

Fig. 1. Macrophage migration inhibition test (Direct method).

Optimal ratio between lymphocytes and macrophages. C3H mice were injected subcutaneously in the dorsal region with 2×10^4 MH 134 cells. At 3 days after injection, regional lymph node cells were collected. Macrophages and sensitized lympocytes were mixed in ratios of 1 : 1, 2 : 1, 5 : 1, 10 : 1, 100 : 1 or 200 : 1 and incubated. In MI tests conducted with antigen fixed at 100 μg/ml with ratios of 5% to 20% of sensitized lymphocytes against macrophages, constant values were obtained as shown in Fig. 1. Therefore, such ratios were used in the present experiment (Fig. 2).

Optimal concentration of antigen. MH 134 cells were suspended in 5 ml of TC-199 medium 10^7, and the suspension was subjected to ultrasonication at 150 mA for 5 min. The protein content of the original antigen after destruction by sonication was about 200 mg/dl. Macrophages alone were sealed in the micropipet tube as previously described, and the antigen was concentrated at 1/2, 1/4, 1/8, 1/20, 1/40, 1/80 of the original concentration. Incubation was

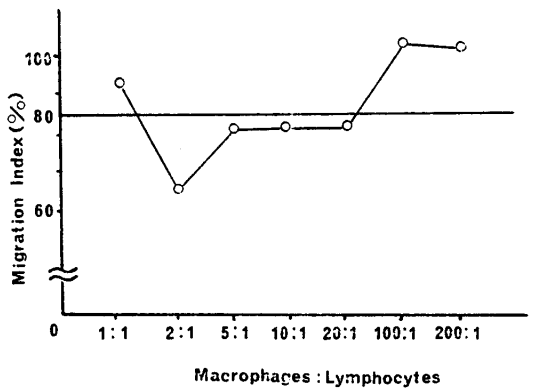

Fig. 2. Macrophage migration inhibition activity and ratio of macrophages-to-sensitized lymphocytes.

conducted with each sample, and the migration index was calculated against the migration area of the respective non-antigen control. It was found that nonspecific migration was inhibited in antigen of 1/2 concentration by 23%, at 1/4 concentration by 32%, at 1/8 concentration by 43%, at 1/20 cocentration by 97%, 1/40 concentration by 120%, at 1/80 concentration by 113%, and at concentrations on 1/8, a strong nonspecific migration inhibition was observed. Therefore, the highest concentration of antigen at which no nonspecific migration inhibition occurred was 1/20 concentration of original antigen (antigen concentration of 100 μg/ml protein) (Fig. 3). The PPD concentration used were

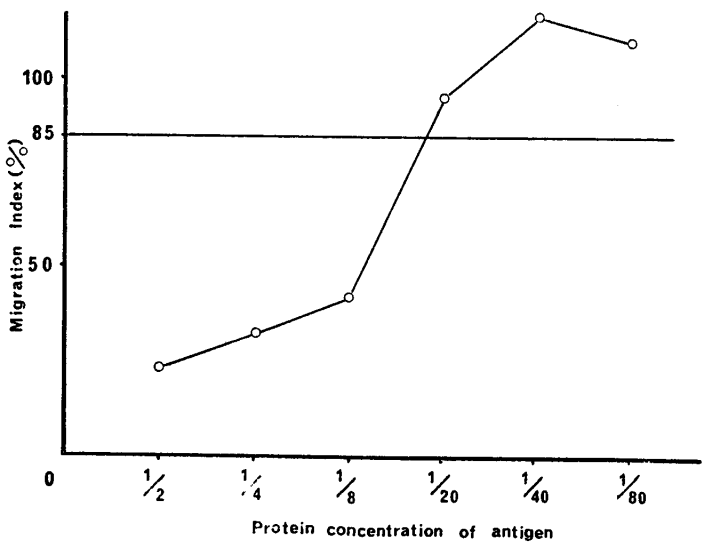

Fig. 3. Macrophage migration inhibition activity and protein concentration of antigen.

7.5 μg, 15 μg, or 30 μg/ml, and these concentration showed no significant differences, as reported by George and Vaughan (2) on MI tests with guinea pigs sensitized by BCG.

Final MI activity measurement. The MI activity measurement was identical with the method used in the preliminary experiment; namely, lymph node cells and macrophages were mixed in a ratio of 1 : 5; the mixture was sealed in a capillary glass tube; and in the case of tumor antigen, the medium was adjusted to a concentration of about 100 μg/ml, and in the case of PPD the medium concentration was adjusted to about 25 μg/ml. Both the antigen-added group and non-antigen control group were incubated for about 24 hours before measuring the cell migration area. The long diameter multified by the short diameter of macrophage migration was taken as the migration area. The ratio of the area of the antigen-added group to that of the control group was taken as the migration index. Migration indices below 85% were interpreted as positive since the migration indices in the mixture of non sensitized normal lymphocytes and macrophages ranged between 90%-110%. The formula for calculation was,

$$\text{Migration index (MI)} = \frac{\text{Average migration area of antigen-added group}}{\text{Average migration area of non-antigen control group}} \times 100\ (\%)$$

For this calculation, the migration areas of four capillary tubes were taken from each group, and the average was used in the formula.

Experimental Group

Experiment 1. C3H mice were injected subcutaneously in a dorsal site with 4 mg BCG. The axillary lymph node cells were collected weekly, and the MI activity of the lymph node cells was calculated.

Experiment 2. C3H mice were transplanted subcutaneously in a dorsal site with 2×10^4 MH-134 cells. The growth of tumor and the survival time were monitored, and the MI activity of the axillary lymph-node cells was measured daily.

Experiment 3. A mixture of 2×10^4 MH-134 cells and 4 mg BCG was injected subcutaneously into a dorsal site of C3H mice. Tumor growth was monitored, and the MI activity of the axillary lymph-node cells was measured daily.

Experiment 4. MI activity was measured in DDS mice after a dorsal subcutaneous injection of mitomycin-C-treated Ehrlich Cancer cells at 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8. Ehrlich cancer cells were treated with 25 μg/ml of mitomycin for 30 minutes at room temperature.

RESULTS

Experiment 1. The MI activity of C3H mouse axillary lymph node cells was positive at 74% one week after sensitization, and increased gradually thereafter reaching a maximum by the fifth week. The MI index then gradually decreased, maintaining a positive value to the 15th week. At around the 17th week the index was negative and by the 22nd week in disappeared (Table 1, Fig. 4).

TABLE 1. MACROPHAGE MIGRATION TEST AFTER BCG SENSITIZATION

Week	PPD	Macrophage migration area				Mean±SE	Migration Index (%)
1	+	1350,	1000,	1200,	1200	1188±228.3	74.3
	−	1500,	1750,	1650,	1500	1600±194.7	
2	+	4400,	3850,	4225,	4200	4170±366.3	68.7
	−	6000,	6300,	6000,	5955	6162±273.6	
3	+	5610,	4875,	4125,	4875	4869±957.7	68.9
	−	7125,	7655,	7125,	6375	7169±837.2	
4	+	4825,	5110,	3750,	4520	4537±921.2	70.8
	−	6325,	5424,	6340,	7524	6406±1296.8	
5	+	320,	360,	340,	480	367±125.4	61.8
	−	620,	682,	640,	496	594±121.2	
10	+	420,	504,	510,		478±92.4	65.0
	−	748,	828,	594,	770	735±214.7	
12	+	2310,	2275.	2384,	2295	2310±78.8	74.5
	−	3375,	2800.	3220,	3024	3199±391.8	
15	+	1340,	1120,	1050,	1300	1202±221.9	80.4
	−	1480,	1650,	1580,	1350	1495±195.4	
22	+	1275,	1430,	1050		1251±474.6	104.3
	−	1220,	1270,	1130		1200±173.8	

PPD (Purified protein derivative); macrophage migration area=long diameter (μm) × short diameter (μm).

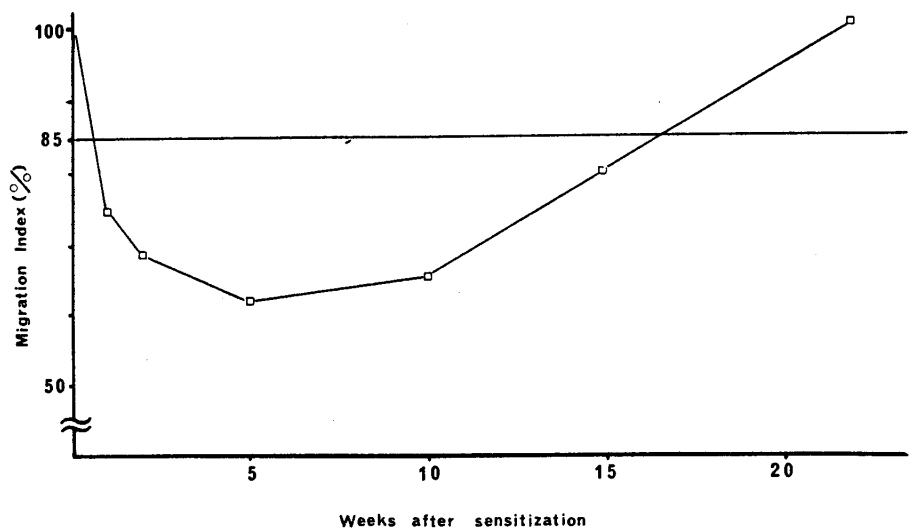

Fig. 4. Macrophage migration inhibition activity after BCG sensitization.

Experiment 2. By transplantation of 2×10^4 MH-134 cells to C3H mice, solid tumors became palpable at about one week and grew rapidly around the second week. Mice began to die of the tumor effects around the 17th or 18th day, and by the 42nd day all animals were dead. The average survival time was 29 days and the 50% survival median was at 31 days. The MI activity was 76% on the fourth day, reached a maximum of 66% on the sixth day. The activity decreased gradually thereafter: 75% on the eighth day, 79% on the tenth day, 85% on the 14th day and 100% by the 20th day, to complete disappearance (Fig. 5).

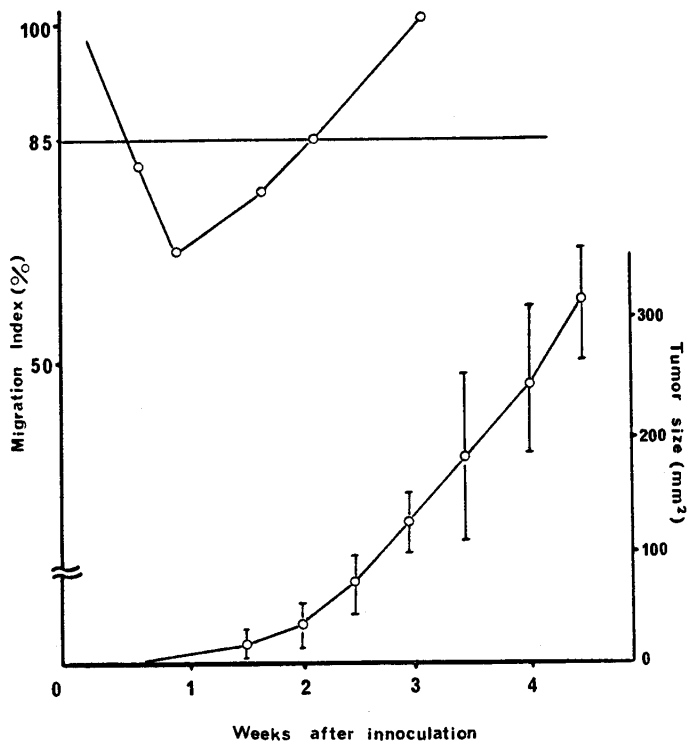

Fig. 5. Macrophages migration inhibition activity and tumor growth after MH-134 implantation.

Experiment 3. The combined injection of 2×10^4 MH-134 cells and 4 mg BCG produced palpable tumors but proliferation was usually absent, or even if some proliferation was initially present, shrinkage followed. MI activity became positive (75%) by the seventh post-injection day, reached a maximum of 60% by the 17th day and became negative by the 21st day (Fig. 6).

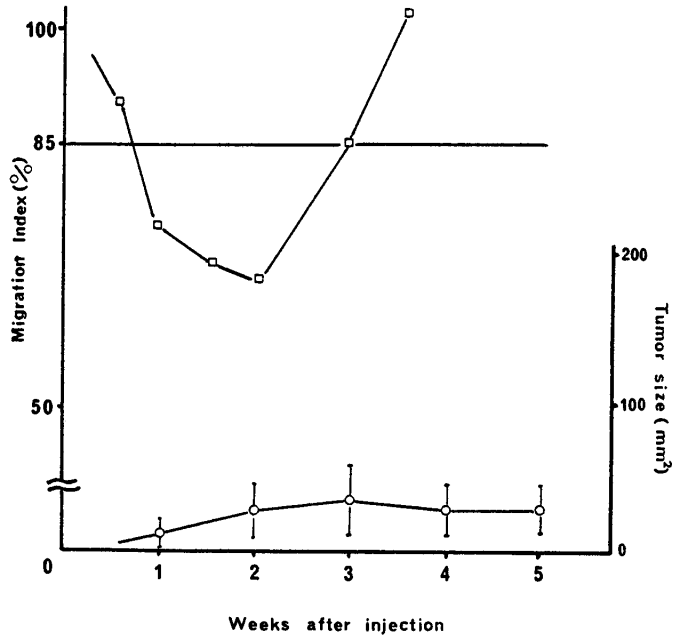

Fig. 6. Macrophage migration inhibition activity and tumor growth after intracutaneous BCG-MH-134 injection.

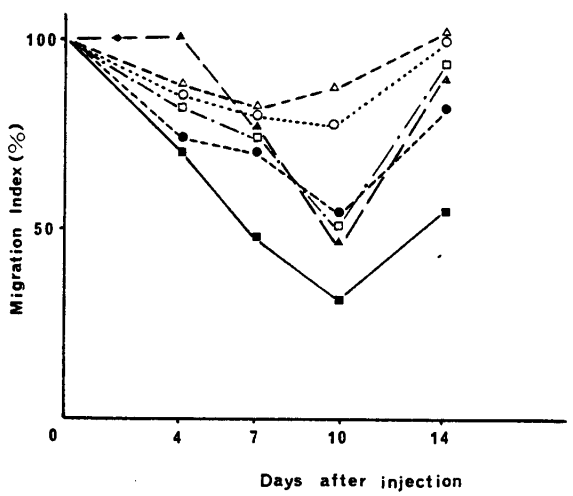

Fig. 7. Macrophage migration inhibition activity after a single injection of MMC treated Ehrlich ascites tumor cells. Concentrations injected: ·····○·····, 10^3; ·····●·····, 10^4; ——■——, 10^5; - - -▲- - -, 10^6; —·—□—·—, 10^7; - - -△- - -, 10^8.

Experiment 4. The MI activity was lowest in the group with 10^5 MH-134 cells, followed in sequence by the 10^6 group, 10^7 group and 10^4 group. The 10^3 cell group and the 10^8 cell group showed very weak activity (Fig. 7). When the MI activity on the tenth day is represented graphically, the 10^5 cell group was strongest, followed by the 10^6 group, 10^7 group, 10^4 group, 10^3 group and 10^8 group (Fig. 8).

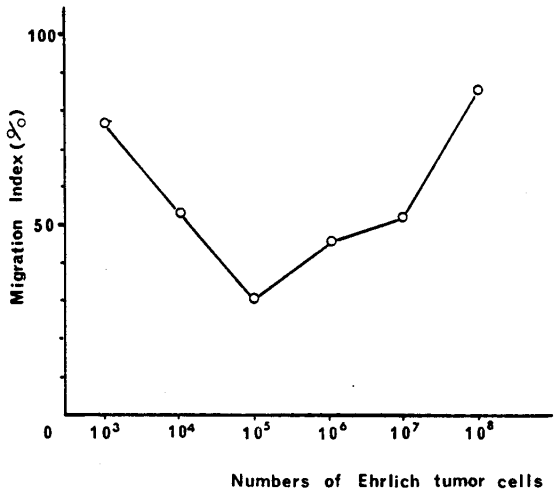

Fig. 8. Macrophage migration inhibition activity on day 10 after a single injection of different concentrations of MMC treated Ehrlich ascites tumor cells.

DISCUSSION

Aside from MIF (macrophage migration inhibitory factor) the known soluble mediators released by contact of sensitized lymphocytes with specific antigen in culture supernatant are lymphotoxin (5), chemotactic factor (6), skin reactive factor (7) and interferon (8). MIF has been used in many investigations as an *in vitro* model of delayed allergy, because of its reproducibility and simplicity. Nonetheless, the antigen dose and the ratio of macrophages-to-sensitized lymphocytes have not yet been sufficiently clarified. For this reason the author studied the optimal quantity of antigen with MH-134 and the most appropriate ratio of macrophages-to-lymphocytes before embarking on the final experiment. The determination of antigen concentration is especially important, and the protein content differs according to different kinds of antigens. It is necessary to determine the optimal concentration that would not elicit nonspecific migration inhibition with each antigen used.

It is difficult to isolate MIF from the culture supernatant after its reaction with antigen. Dumonde, Howson and Wolstencroft (9) reported that this may

be due to the adsorption of MIF to macrophage. In their morphological studies of direct action by MIF, Salvin, Nishio and Gribik (10) observed that macrophages acted upon by MIF became spherical, with slower movements, resulting in the inhibition of wavy movements of pseudopodial membrane.

In the past, the cellular immune reaction differing from humoral immune reaction was considered to be a specific *in vivo* reaction against factors, such as tubercle bacillus, but it has recently been recognized (11) that the cellular immune reaction is basically similar to delayed allergic reaction, rejection in organ transplantation, immune reaction against tumor and autoimmune lesions. Thus, it may be possible to estimate (13) the strength of the cellular immunity *in vivo* by using the MI test as a barometer. The same can be said with cellular transplantation immunity and tumor immunity.

In Experiment 1 the degree of reaction, i.e., the delayed allergic reaction to PPD, was measured with lapse of time. The skin reaction in mice is difficult to measure, but according to Ferraresi, Dedrick and Raffel (12) in guinea pigs sensitized by diphtheria toxioid, the delayed skin reaction appeared earlier and more rapidly than MI activity, and it paralleled the latter about 2 weeks later and persisted in this parallel state for about 8 weeks. At around the 15th week both MI activity and skin reaction ceased.

In Experiment 2 the MI activity of regional lymphocytes was measured with lapse of time in C3H mice homotransplanted with MH 134. In the delayed allergic reaction the strength of MI activity could be pursued. However, the transplantation immunity to the delayed allergic reaction caused by vaccine, such as BCG, disappeared within a very short period of time. At about the time when the MI activity decreased, the tumor enlarged rapidly. Conversely, it can be assumed that if MI activity persists strongly for a long time, tumor proliferation would be inhibited.

In Experiment 3 the combination of BCG (live bovine tuberculous bacillus) and MH-134 cells was injected. This experiment was conducted for the purpose of determining the inhibitory action of BCG from two view points, i.e., its power to inhibit the survival and proliferation of tumor cells at a given locus and its action of enhancing the antigenicity of regional lymphocytes. Compared with the group receiving tumor cells alone (Exp. 2), the results revealed that the MI activity reached a peak about 10 days later and the duration of positive action was prolonged to 21 days which was 7 days longer than the 14 days of Experiment 2. During this period tumor cells hardly proliferated and even if proliferation occurred once, regression by shrinkage followed.

In Experiment 4 Ehrlich ascites tumor cells were treated with mitomycin C to inhibit DNA synthesis. Those cells were injected subcutaneously to a dorsal site of DDS mice in dosages of 10^3 cells, 10^4 cells, 10^5 cells, 10^6 cells,

10^7 cells or 10^8 cells. Subsequently, the MI activity of lymphocytes from the regional axillary lymph nodes were measured. The results revealed that on the tenth postinjection day MI activity was highest in the group injected with 10^5 cells. and the MI activity weakened where the cell number was just above or less than 10^5 cells. When the quantity of sensitized antigen was too low, regional lymphocytes did not acquire antitumor activity, but the decrease in antitumorigenicity of regional lymphocytes along with the progress of cancer seemes to be due to the inflow of excess tumor antigen, and under excess antigen, sensitized T-cell reached an anergy state resulting in decreased function or inactivation.

These findings indicate that there is a close relationship between tumor proliferation and MI activity and that the tumorous state of the tumor-bearing body can be monitored fairly closely by MI activity. In other words, when tumor develops *in vivo* to a certain size, antitumorigenicity starting at the regional lymph nodes, and as the tumor grows larger, the antitumor activity also becomes marked. However, when the tumor becomes progressively cancerous and a terminal cancer stage is reached, antitumorigenicity is lost and the tumor proliferates *ad infinitum*. The restoration of antitumor activity before such a tumorous state and whether the antitumor activity can be maintained and strengthened are major problems (15). One helpful procedure is to administer the cancer bearing individuals an optimal dose of tumor antigen using adjuvants, such as BCG, as soon as possible after removing the tumor tissue.

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