Studies on Tumor Immunity


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Introduction

The essential nature of neoplastic disease is the autonomous proliferation of abnormal cells which continues until the host dies. The presence of cancerous cells should be distinguished from cancerous disease (neoplastic disease) since tumor cells do not always induce neoplastic disease; when Yoshida sarcoma cells are transplanted into rats, they continuously proliferate until the hosts die, but when the same tumor cells are transplanted into mice, they spontaneously regress after transient proliferation. It is well known that the resistance against transplanted Yoshida sarcoma cells in mice is due to immune response, and it is now well understood that cell-host relationship is an important factor determining whether or not a tumor cell will cause a neoplastic disease.

Recently, many experimental results have been reported which suggest the importance of immune response in the process of carcinogenesis and subsequent tumor growth. While, it has been demonstrated that a carcinogen has two characters, i.e. a cytotoxic effect and a depressive effect on immunologic response. Based on these facts, the process of chemical carcinogenesis can be assumed as follows: 1) A carcinogen acts toxically on a definite organ or tissue, affected tissue falls into degeneration and necrosis, followed by regenerative proliferation, and 4) if the immune mechanism (immunological surveillance) of the host is disturbed, this variant cell may progressively proliferate and result in a neoplastic disease. It is believed that disturbance of this immune mechanism is attributable to the nature of the carcinogen itself.

Reported here are the results of experiments designed to examine the role of the immune mechanism in carcinogenesis as follows: 1) Tumor incidence was observed in rats administered a carcinogen combined with various immunosuppressant treatments. 2) To obtain an index of the immune mechanism in the hosts, the carbon phagocytic activity of intra-
Materials and Methods

1. Animals: Donryu rats, 6 to 8 weeks old at the beginning of the experiments, were employed. They had been fed on the basal semisynthetic diet (MF diet) of the Oriental Co.

2. Carcinogen: 3'-methyl-Dimethylaminoazobenzen (DAB) was used; this was mixed at 0.06% with the basal diet (DAB-diet).

3. Immunosuppressants: Azathioprine (AP) and β-methasone (BM) were used. These two agents are well known as strong depressants of the reticulo-endothelial system.18) 17) 18) 19) 20) 21) 22) 23) 24) 25)

4. Experimental groups (summarized in Fig. 1): The animals were divided into following groups to receive different immuno-suppressive treatments:

   Group I : DAB-diet alone was given for 3 or 4.5 months.

   Group II : BM was combined with the DAB-diet. This group was further divided into following three subgroups:

   Subgroup a: One month after the beginning of the DAB-diet, the subcutaneous injection of BM (18 mcg 3 times a week) was started and continued for 2 or 3.5 months.

   Subgroup b: 2.5 months after the beginning of the DAB-diet, the BM treatment (same schedule as subgroup a) was started, and continued for 2 months.

   Subgroup c: 2.5 months after the beginning of the DAB-diet, the BM (36 mcg) treatment (same schedule as subgroup a) was started and continued for 2 months.

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**Fig 1 Method of Treatment**

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Group II: AP, instead of BM, was combined with the DAB-diet. After one month of DAB-diet, the AP (3 mg) treatment was started and continued at the same intervals as the Group II treatments for 2 or 3.5 months.

Control groups: In the controls, BM injection (9 mcg x 3/w, 18 mcg x 3/w and 36 mcg x 3/w) or AP injection (3 mg x 3/w and 6 mg x 3/w) were given to rats fed on the basal diet (without DAB) for 4.5 or 5 months. Another control group was fed on the basal diet alone for 5 months.

The animals were killed by ether 3, 4.5, or 5 months after the beginning of the experiments and their livers were examined and classified histologically with hematoxylin-eosin stain as follows: normal liver, liver fibrosis, liver cirrhosis, hepatoma, cholangioma and mixed type (Tab. 1, Fig. 7).

Table 1  Histological Classification

<table>
<thead>
<tr>
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<th>Tumor</th>
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<tr>
<td>Hepatoma</td>
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<td>Cholangioma</td>
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<td>Mixed type</td>
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<td>Liver cirrhosis</td>
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<td>Liver fibrosis</td>
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<td>Normal</td>
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5. Carbon phagocytic activity: Although there are many methods for evaluating RES (reticulo-endothelial system) function, 26) 27) in this experiment, examination of the carbon phagocytic activity of intraperitoneal phagocytes was used for this purpose, because this examination can be easily performed whenever it is needed and it is of slight burden to the rats. The phagocytic activity was measured at one month intervals by the following procedure (summarized in Fig. 2): 28) An ink stick of superior quality was rubbed down one hundred times at average pressure on the ink slab, using 5 ml physiological saline. The obtained fluid was filtrated twice through the same filter paper. The filtrate was

![Diagram](image-url)

**Fig 2** Measurement and Classification of Phagocytic Activity
smeared on the warmed object glasses in the same manner as the smearing of blood for hemogram study. One or two drops of the ascites obtained from rats by glass capillary was dropped on the center of the smear, and covered with a cover glass whose edges were enclosed with vaseline. After the preparation was incubated at 37°C for 1 to 2 hours, the phagocytic activity was microscopically examined. A criterion of 5 grades representing the degrees of phagocytic activity was established (Fig. 2): Cells containing no carbon particles were expressed by 0, cells containing a few carbon particles by 1, cells obviously containing carbon particles or containing granular carbon particles by 2, cells in which the carbon particles occupy 1/4 to 1/2 of the total cell area of the cell by 3, and cells in which the carbon particles occupy more than 1/2 of the cell area by 4. Thus each phagocytic activity was measured in each of 50 cells, and the phagocytic activity of the animal was expressed by the average of the 50 cells.

6. Experiments with methylcholanthren (MC): Some animals received a dorsal subcutaneous injection of 10mg MC in liquid paraffin and their subsequent phagocytic activity was observed at intervals of 1 to 6 months after MC-injection (Fig. 5). The MC-injection site of each animal was examined and the average diameters \( \frac{\text{short} + \text{long diameter}}{2} \) of the indurations were recorded every two weeks from 3 months after the MC injection. An average diameter of over 20mm or a growth more than two fold within two weeks was regarded as evidence of tumor occurrence, as mentioned in Part 1.

**Results**

1) Average intake of DAB (Table 2)

Between the groups, there were no remarkable differences in DAB intake.

2) Tumor incidence (Tables 3 and 4)

(a) Tumor incidence after 3 months' DAB-diet

No liver carcinomas were observed in Groups I (DAB alone) and II a (DAB combined with 18mcg of BM). In Group III (DAB combined with AP), one of the 11 rats

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<tr>
<td>3 months after administration of DAB</td>
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<td>4.5 months after administration of DAB</td>
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developed a liver carcinoma (9%).

(b) Tumor incidence after 4.5 months' DAB-diet

Liver carcinomas were observed 3 of the 11 rats of Group I (27%), in 9 of the 11 rats of Group I a (82%), in 5 of the 8 rats of Group I b (63%) and in 5 of the 8 rats of Group I c (63%). These findings show that tumor incidence increases when BM combined with the DAB-diet. In Group II, however, the tumor incidence was 25% (3/12). There is no significant difference between the tumor incidence in Group II and that in Group I. In the control groups (BM injection alone, AP injection alone, and basal diet alone), no liver carcinoma developed.

3) Carbon phagocytic activity (Figs 3, 4, and 5)

At the beginning of the experiments, carbon phagocytic activity was 1.82 (before DAB-diet administration). At the end of 2 months' DAB-diet, it was reduced to 1.05 in Group I a and to 1.18 in Group I a and to 1.18 in Group I. Group I a always

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<tr>
<th>Tumor Incidence</th>
<th>(3 Months after The Beginning of 3'-Me-DAB Administration)</th>
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<tr>
<td>(Histological Classification)</td>
<td>Cirrhosis Fibrosis Normal</td>
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<tr>
<td>Hepatoma Cholangioma Mixed Type</td>
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<tr>
<td>Group I</td>
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<td>Group II, a</td>
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<th>Tumor Incidence</th>
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<td>(Histological Classification)</td>
<td>Cirrhosis Fibrosis Normal</td>
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<td>Group III</td>
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<td>BM</td>
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<td>AP</td>
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<td>6 mg</td>
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showed a lower activity than Group I. At the end of the 3rd month, the lowest activity was seen in Group II, but the subsequent recovery rate was to some extent contrary to that of Group II. The BM-only group generally showed an activity which was higher than that of Group I but lower than that of the AP-injection group. The basal diet group, too, showed a reduction in activity as the rats became older; however, the degree of this reduction was much lower than those of all the other groups. This result agrees with the report that old animals produced less antibody than young adults.80) *The phagocytic activity of MC-injected rats (Fig.5):

The activity which was 1.68 before the
MC injection gradually diminished. The degree of this reduction was higher than that of the basal diet group. At the end of the 3rd month after the MC injection, 3 out of the 6 rats developed tumors. These tumor bearing rats showed a lower activity than non-tumor bearing rats. In retrospection, the rats which were to develop tumors at a following observation showed lower activity levels than those which were to remain tumor-free.

Discussion

As seen in Fig. 3 and Table 4, the group which showed the most marked reduction in carbon phagocytic activity showed the highest tumor incidence. That is, the 3.5 months' DAB administration yielded higher tumor incidence in Group I (combined with BM) than in Group I (DAB-diet alone), and the former showed a greater reduction in phagocytic activity than latter. These observations may suggest that the host's immune mechanism may play an important role in carcinogenesis. Carcinogenesis is generally held to consist of two phases, i.e. initiation and promotion (or proliferation). From these findings obtained, it can not be determined in which phase the host's immune response participates. However, in the MC-injected rats, the phagocytic activity tended to diminish antecedently to tumor development, a phenomenon which suggests that the host's immune response participates in the first phase of carcinogenesis (initiation). On the other hand, there are considerable evidences that immunosuppressive pretreatment such as cortisone and X-radiation increases tumor transplantability to homologous or heterologous animals. This fact suggests that the host's immune mechanism may also participate in the second phase (promotion). Thus it may be concluded that host's immune mechanism may play an important role in both phases of carcinogenesis.

No significant difference was found between tumor incidence in the DAB alone group and in the AP combined group (Group II), although the phagocytic activity of the latter was higher than that of the BM combined group (Group I), which showed the highest tumor incidence. From these findings, the following three possibilities should be considered: 1) AP may be so toxic that the cell might lose its proliferation potency; 2) AP may act against tumor development as an anti-tumor agent; and 3) AP may have no immunosuppressive action in rats (unsusceptive). The first possibility seems to be inadequate since the degree of histological change of the liver in the AP-only group was almost same as that of basal-diet group. The second possibility is also unlikely as AP is generally well known to be more potent as an immunosuppressant than as an anti-tumor agent. Therefore, the third possibility seems to be most adequate. Assuming that rats
were unsusceptive to AP, it is possible to give an interpretation as to why the AP-
combined rats showed a higher phagocytic activity and a lower tumor incidence. Actually,
Sugiyama had\(^3\)\(^6\) failed to transplant a skin graft in a rat by using AP. There is some
reports that AP has an immunosuppressive action in dogs, mice, rabbits, and hamsters,
but not in rats.\(^3\)\(^7\)\(^8\)

**Immunological consideration on carcinogenesis** (Fig. 6 : (1) ……(6))

It seems to be well established that chemical carcinogens have an immunosuppressive
action as well as a toxic effect on cells, \(^1\)\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\) that chemically induced
tumors possess some specific antigen not present in normal cells, \(^3\)\(^9\)\(^4\)\(^0\) and that there is
a loss of organ specific antigen in tumor cells. \(^4\)\(^1\)\(^4\)\(^2\)\(^4\)\(^3\)\(^4\)

From these facts, the following hypothesis on carcinogenesis can be drawn (summarized
in Fig. 6). When a carcinogenic agent is given to an animal, neoplasma is mainly in-
duced in a definite organ or tissue. This means that the given carcinogenic agent has a
certain affinity for a definite organ, and it may be considered that the carcinogen acts
cytotoxically by binding itself to that organ. In this hypothesis, it is assumed that such
a carcinogen binds itself with an organ specific protein (antigen) (1), not with an isoan-

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**Fig 6** Process of Carcinogenesis

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tigen, which is commonly contained in all of the cells constituting the organism. If bound with the isoantigen, the cytotoxic effect could be seen in all organs to a similar degree. DAB, as reported by Salzberg, binds itself specifically with liver protein in rats, and Miller and Miller demonstrated that the carcinogenic potency of DAB and its various related compounds in rats is closely associated with the amount of those dyes bound to the liver protein. Successive or repeated administrations of carcinogen may cause degeneration and necrosis, followed by regenerative proliferation in an organ. During this period, it seems that there is an opportunity for the appearance of variants containing less organ specific antigen. These variants may be resistant against carcinogen according to the above described assumption. Thus, gradual loss of organ specific antigen may be induced by successive or repeated administrations of carcinogen as a result of adaptation to its cytotoxicity, and it may be assumed that, when the amount of organ specific antigen (protein) comes down below a threshold level, progressive growth may begin (cancer). The threshold level may be determined by the immune responsibility of the host, i.e. when the immunological mechanism in the host is disturbed, progressive growth may occur at the locus where the organ specific antigen is deleted. The period of gradual antigen loss corresponds to the precancerous stage. This process may well explain the reason why tumor cells possess the isoantigen of the originating host, but do not possess its organ specific antigen. Prehn assumed, in his clonal selection theory of carcinogenesis, that carcinogens combine with a cell control factor (CCF). This is, the toxicity of a carcinogen is due to its combination with cellular entities involved in the regulation of growth, and a deficiency in these entities below a threshold level results in progressive growth (→cancer). However, a cell control factor need not be assumed if the organ specific antigen (protein) and the cell control factor be of the same nature, for it is considered that the quantity of organ specific antigen shows the degree of maturity of the cells, while, on the other hand, immaturity usually parallels the proliferative potency. Although the variant cell containing organ specific antigen of the amount below the threshold can be induced even by single administration of a carcinogen (conversion of a normal cell into a tumor cell: initiation), subsequent proliferation may not occur, if the immune mechanism in the host is normally functioning. This hypothesis is supported by much evidence that chemical carcinogens exert a depressive effect on the immune mechanism, as well as by the fact that tumor transplantability increases in animals pretreated with such an immunosuppressive factor as X-radiation or cortison administration. Malmgren et al. reported lowered antibody (sheep cell hemolysin) production in mice treated with several chemical carcinogens. Stjernswärd
2) definitely demonstrated the immunosuppressive effect of three carcinogenic hydrocarbons, i.e. methylcholanthrene, benz-a-pyrene, and dimethyl-benzanthracene, by counting plaque-forming cells after immunization with sheep red cells. Stern searched for the possible effects on the immune responses in mice to such carcinogenic stimuli as X-irradiation or dorsal painting with methylcholanthrene, or the feeding of rats with dimethylaminoazobenzene; he could demonstrate that, in some of his experiments, a decline in antibody formation preceded or was accompanied by the development of neoplastic disease. Odashima reported he development of liver carcinoma in rats by dermal painting with 20-methylcholanthrene following initial feeding with 4-dimethylaminoazobenzene (DAB) for certain period; DAB was regarded as the initiating factor and MC as the promoting factor, when adapted to Berenblum's two stage theory of carcinogenesis. However, as MC has a depressive effect on the immune mechanism, it can be considered that this depressive effect might permit subsequent proliferative growth. On the other hand, it should be kept in mind that X-ray is a mutagen (i.e. it acts directly in causing cytochemical alterations) as well as an immunosuppressant.

As above mentioned, it seems reasonable to conclude that the immune response of the host may participate in the first phase (change from normal cell to tumor cell) as determinant factor of the threshold level of the organ specific antigen. But it remains a problem as to whether this first change is induced by the direct effect of a carcinogen causing cytochemical alterations, which lead to changes in the hereditary informational content of the affected cells, or by an indirect effect. After all, the immune response may play a more important role in subsequent autonomous proliferation (clinical cancer). The important concept of neoplastic disease is that, at first, one or more cells convert from normal cells to neoplastic ones and then a subsequent progressive proliferation takes place. According to the above described hypothesis, the possibility of carcinogenesis from auto-immune diseases or phenomena may be drawn. Auto-immune phenomena are caused by the production of a tissue specific antibody which acts cytotoxically to the tissue itself. Therefore, the attack of this auto-antibody on the tissue may introduce variant cells involving less organ specific antigen, followed by its gradual loss to induce the appearance of neoplastic cells.

The author is indebted to Dr. Y. Aosaki for his invaluable suggestions and criticism during the course of this investigation.

(The results were presented at the 30th Annual Meeting of the Japanese Cancer Association, Tokyo, October, 1971.)
References


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1. The liver fibrosis (Group I) after the administration of DAB for 3 months.
2. The liver cirrhosis (Group I) after the administration of DAB for 4.5 months.
3. The hepatoma (Group II a) after the administration of DAB for 4.5 months and BM for 3.5 months.
4. The cholangioma (Group II a) after the administration of DAB for 4.5 months and BM for 3.5 months.
5. The mixed type (Group II a) after the administration of DAB for 4.5 months and BM for 3.5 months.

Fig 7 Histological Pictures