Lymphocyte Blast Formation in Rats with DAB-Induced Hepatic Carcinoma

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Summary: Lymphocytes derived from rats bearing DAB-induced hepatic carcinoma at progressive stages (micro- and macroscopically) were stimulated by three mitogens; PHA, Con A and PWM. A remarkable increase in $^{3}$H-thymidine uptake was found over the period from the stage preceding the appearance of carcinoma to the early stage of carcinogenesis, while a remarkable decrease of $^{3}$H-thymidine uptake was revealed in lymphocytes of advanced tumor-bearing rats. This result is very remarkable, for it questions the generally believed assumption that immune ability decreases from the early stage of carcinogenesis.

Key words: DAB hepatoma—PHA—Con A—PWM—blast formation—cancer stage—immunity at stages

Introduction

Each living organism possesses a defense mechanism by which it can discriminate between self and nonself. It recognizes determinants on pathogenic antigens and initiates a complex immune response to exclude them. What response, then, does a carcinoma-bearing organism exhibit toward carcinomatous cells borne in itself? Burnet (1970) proposed the concept of immunological surveillance, a mechanism for eliminating or inactivating dangerous mutant cells. Once cells have escaped from this mechanism, they may still be immunological targets of the tumor-bearing host. The major host defense against carcinoma is effected by cellular immunity, similar to the reaction of a recipient to a tissue allograft (Bell, 1972). Activated T lymphocytes, natural killer cells and antibody-dependent K cells all contribute to the maintenance of a cellular immune effector mechanism (Herberman et al. 1978; Zinkernagel et al. 1974).

It has been well documented that a significant decrease in immune response occurs in patients with certain malignances (Fairly 1969; Sharman et al. 1966; Trubowits et al. 1966; Brooks et al. 1972). Such a subnormal response in number and function of lymphocytes seems to correlate with suppressive cells and serological factors in tumor-bearing host (Brooks et al. 1972; Berendt et al. 1980; Sample et al. 1971; McMaster et al. 1977; Krant et al. 1968) In the case of human carcinoma the more advanced the stage of disease, the lower the level of cellular immunity. It is of interest to understand the relationship between the immune ability of carcinoma-bearing host and the tumor. We may begin this process by studying the chronological changes in the immune ability of
the tumor-bearing host, including proliferation, infiltration and metastasis of carcinoma. Further developments would not only lead to early diagnosis and improved prognosis but also new treatments, including immunological therapy of carcinoma (Savel 1963). Great differences in the mode of and velocity of development, the original sites of carcinogenesis, and the histological types have made it difficult to assess properly the host's immune ability even though they are classified by genetic difference, sex, age and stage of illness. We overcame this problem by employing an inbred strain of rat and orally administrating p-dimethylaminoazobenzene (DAB) mixed with food to induce a hepatic carcinoma at a high frequency. Lymphocytes proliferate and synthesize RNA and DNA when stimulated by a variety of mitogens as well as by antigens. This phenomenon, called lymphocyte transformation has been used to assess immunologic competence (Stobo, 1972; Veit et al. 1976). Several mitogens selectively act on different lymphocyte subpopulations in a polyclonal fashion. Con A stimulates both immature and mature T cells, whereas PHA only stimulates mature T cells. PWM is a T cell dependent-B cell activator. Peripheral T lymphocytes are also activated by these mitogens. Lymphocyte transformations by mitogens were examined with the lapse of time as an indicator of immunologic reactivity. As reported by several authors in the response of lymphocytes of cancer patients to phytohemagglutinin (PHA) (Garrioch et al. 1970; Ducos et al. 1970; Orita et al. 1973), the blast formation of lymphocytes in rats during advanced stage of DAB hepatic carcinoma was suppressed. However, a marked increase in mitogen response was observed during the early stage of carcinomatosis. Such a high reactivity of lymphocytes at this stage seems to suggest that the host's immune is stimulated by the presence of carcinomatous cells to inhibit their proliferation.

**Materials and Methods**

1. Experimental animals and DAB hepatic carcinoma

Forty male rats of JAC Wistar strain, 5 weeks of age weighing about 110 g, were used. Thirty rats were used for the production of DAB hepatic carcinoma (experimental group) and fed a compressed CLEA-CE2 diet containing 0.06% DAB up to 275 days from the beginning of the experiment. Food intake was about 12 g - 13 g per day per an animal. Ten rats were fed a normal CLEA-CE2 diet as a control group.

Surviving rats (Table 1) from the experimental group were examined for the degree of proliferation and development of hepatic tumors. The following criteria were used to discriminate the stage or grade of tumor development:

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>tumors of 0.2 - 0.3 cm in diameter scattered.</td>
</tr>
<tr>
<td>I</td>
<td>tumors of 0.4 - 0.5 cm in diameter scattered</td>
</tr>
<tr>
<td>II</td>
<td>tumors of 0.6 - 1.0 cm in diameter</td>
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<tr>
<td>III</td>
<td>tumors of 1.1 - 3.0 cm in diameter</td>
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<tr>
<td>IV</td>
<td>tumors 3.1 - 5.0 cm in diameter</td>
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<tr>
<td>V</td>
<td>tumors greater than 5 cm in diameter</td>
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</tbody>
</table>

2. Mitogen simulation

Blood specimens were collected with heparin from the abdominal vena cava and diluted with two volumes of Eagle's minimum essential medium (EMEM, GIBCO, NY). This mixture was layered over 2.5 ml of Ficoll-Hypaque (Pharmacia), centrifuged at 1000 G for 30 min at room temperature and the mononuclear cell layer collected. The cells were washed three times in EMEM by centrifugation at 1000 G for 7 min and suspended in RPMI-
TABLE 1
Monthly incidence of deaths among DAB administered rats

<table>
<thead>
<tr>
<th>stage</th>
<th>Group of DAB administered rats</th>
<th>0</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>total</th>
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<tr>
<td>months</td>
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<td></td>
<td>1</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>deaths</td>
<td></td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td>10</td>
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<tr>
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<td>20</td>
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<td>9</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>

1640 medium (Nissui Seiyaku, Tokyo) supplemented with 15% fetal calf serum (FCS, GIBCO, NY), 100 U/ml penicillin and 100 g/ml streptomycin. Triplicate cultures containing $1 \times 10^5$ viable lymphocytes in 0.1 ml and optimal dose of mitogens (0.1 ml) were incubated at 37°C in 96 well microtiter plate (Linbro Scientific Inc., Hamden, Conn.) for 72 hours in 5% CO$_2$-95% humidified air. Previously determined optimal concentration of mitogens were 1.25 mg/ml for phytohemagglutinin (PHA), 5.0 mg/ml for pokeweed mitogen (PWM) and 10.0 mg/ml for concanabalin A (Con A). Each culture received 0.1 μCi of $^3$H-thymidine (methyl-$^3$H-thymidine, specific activity, 2.0 mCi/mmole, New England Nuclear, Boston, Mass.) 3 hours before the end of incubation period. Cells were collected on a glass-fiber filter by an automatic cell harvester WT-MH (Wakayaku Co. Kyoto). The filter was washed with phosphate-buffered saline (PBS), 5% trichloroacetic acid (TCA) and 95% ethanol, successively. The dried filters were transferred into glass vials, to which were added 0.2 ml of Soluen 350 (Packard) and 5.0 ml of scintillation solution, and the radioactivity was measured in a Packard liquid scintillation counter.

Mitogen stimulation was assessed by a stimulation index (S.I.), the ratio of the mean cpm of experimental cultures to the mean cpm of control cultures.

The degree of cellular proliferation (transformation) is conveniently determined by measuring the uptake of $^3$H-thymidine into TCA-insoluble DNA of blastoid cells. This method was used to evaluate the competence of T cells of a tumor-carrying host. DNA synthesis stimulated by PWM is known to be a measure of helper T cells response (Waxdal, 1975; Morisawa et al. 1979). A preliminary experiment to determine the optimal mitogen concentrations indicated that lymphocytes from normal controls and DAB-treated rats in several stages of carcinogenesis showed a similar dose requirement for the blast transformation by PHA, Con A or PWM.

Results

**PHA response**: Rats were sacrificed after 275 days and the grade of carcinoma was determined. Concomitantly, the mitogen response was assayed. Fig. 1 shows that rats with advanced tumor (grade III or IV) gave a much lower index than normal animals. On the other hand, it should be noted that some rats with an early stage of carcinogenesis (grade I or II of severity) gave a several times higher S.I. than control animals. The S.I. for each grade of carcinoma is given in Table 2. The mean S.I. value of normal rats was $5.4 \pm 2.9$.

**Con A response**: The results obtained by con A stimulation were comparable to those from PHA response (Fig. 2). The values were smaller than those of controls.
Markedly small response was observed in rats with carcinoma of Grade IV. Again, one can see relatively large S.I. values in the animals bearing grade I or II carcinoma, especially in the case of grade II (Fig. 2). The mean S.I. ± SD for grade of tumor is given in Table 2. Control rats gave 35.8 ± 15.9.

*PWM response:* Very similar results to PHA and Con A response were obtained in the stimulation of rat lymphocytes by PWM. A greatly enhanced reactivity of lymphocytes from animals with grade I or II tumor is evident in Fig. 3. Values for each stage of carcinoma are given in Table 2. Control rats gave a value of 6.4 ± 4.5.
TABLE 2

<table>
<thead>
<tr>
<th>S.I. ± S.D. for 3 experimental mitogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>grade</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>I</td>
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<tr>
<td>II</td>
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<tr>
<td>III</td>
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<td>IV</td>
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</table>

**Discussion**

Rapid progress has been made in immunologic studies on tumor. Nevertheless, little is known about the presence of specific immunity to human carcinoma. There is ample evidence supporting a decrease in immune ability of patients with the progression in severity of cancer. Carcinoma cells possess tumor-specific transplantation antigens (TSTA) or tumor-associated antigens (TAA), to which animals can respond often resulting in death of the malignant cells in vitro. It is believed that the body's rejection of mutant or neoplastic cells is performed by cellular immunity mechanisms for which the action of several types of lymphocytes is responsible.

Immune response is a well-organized reaction involving T cells, B cells, macrophages and other cells. In particular, some defined subpopulation of T cells, such as the effector cells in delayed type hypersensitivity and killer T cells, exert various functions on tumor immunity (Zinkernagel et al. 1974). Involvement of non-T, non-B lymphocytes, termed natural killer (NK) cells and K cells, in the rejection of tumors has also been proposed (Herberman et al. 1978). In addition, activated lymphocytes in response to carcinomatous antigens produce many mediators which are destructive to tumor cells. They include mitogenic factor, macrophage inhibitory factor (MIF), lymphotoxin, and inflammatory and transfer factors.

Despite the development of these immune responses, tumors carrying TSTA are not suppressed in vivo but allowed to grow in some cases. This has been explained by the production of immunosuppressive factors (Hellström et al. 1977; Baldwin et al. 1976; Sjögren et al. 1971), immunological tolerance, and immunological enhancement in cancer patients (Prehn et al. 1971). Other investigators have reported suppression of immunocompetent cells in tumor-bearing hosts that were mediated by suppressor cells (Berendt et al. 1980; Fujimoto et al. 1976; Han et al. 1979; Gautam et al. 1979; Hubbard et al. 1981). Immunological enhancement seems to be best explained by this immunosuppressor cell mechanism (Hellström et al. 1978).

There is no doubt that TSTA is present on carcinomatous cells. The authors have already reported the presence of TSTA or TAA from the biochemical and immunohistological studies (Kawasaki et al. 1971, 1977). In many cases TSTA plays an important role in tumor immunity for the suppression of carcinomatous cells (Foley, 1953; Hellström et al. 1967, 1970; Parmiani et al. 1975).

Comparison of immature abilities will not give any reliable results in human carcinoma for the reasons mentioned above. In the present model, DAB induced hepatic carcinoma developed in inbred rat strains. The whole course of disease, including the stage before the appearance of carcinoma and the subsequent stages of onset, proliferation, infiltration, and metastasis, was divided into six stages (0 through V) macroscopically and histologically. Lymphocytes were prepared from these rats as well as from normal control rats and incubated with PHA, Con A and PWM. The rise and fall of blastoid cell transformation as an indicator of immune ability demonstrates a remarkable increase in the uptake of \(^3\)H-thymidine in the early stages of carcinogenesis. However, lym-
phocytes from advanced tumor-bearing rats (grade III and IV) showed a remarkable decrease in transformation by all mitogens. In these stages many animals died of tumor.

As mentioned, the blastoid cell transformation was unusually prevalent in stages I and II in the present experiment. This result is noticeable, since it is quite different from the results by many previous investigators who had observed constant decreases of immune ability at similar periods in the development of carcinomas. Mekori et al. demonstrated no difference in rats of lymphocyte transformation between patients and normal individual when gastric carcinoma was localized (Mekori et al. 1974).

It is evident that an immune response is clearly inhibited against tumor in the late stage of tumor development. This might be explained by the decreased function of host effector T cells and the increase of suppressor cells in this stage.

In tumor-bearing hosts, a stage exhibiting a strong immune ability is presented, whereby animals may respond to and attempt to exclude the carcinoma or any other tumors which occur, similar to the defense reaction against the usual antigens in the early stage of infection. Diagnostic value of this phenomenon is uncertain at present and further demonstration of this kind of enhancement in other tumors or, if possible, in human is needed.

References


