Induction of Experimental Allergic Encephalomyelitis and Tumor Occurrence in Rats Treated with 4-Dimethylaminoazobenzene and 3,4-Benzpyrene

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Summary

Antagonisms between 2 simultaneously developing processes were studied by inducing experimental allergic encephalomyelitis in rats bearing liver tumors produced by feeding 4-dimethylaminoazobenzene (4-DAB) or in nontumorous rats which either received the 4-DAB tumorigenic diet or were treated with a tumorigenic dose of 3,4-benzpyrene.

It was shown that tumorous and pretumorous rats were less susceptible to sensitization with the encephalitogenic vaccine. The occurrence of 4-DAB liver tumors was lower if the encephalitogenic vaccine was injected within about 1 month after the end of the azo dye feeding. The results are discussed in view of the assumption that processes similar to the competition of antigens may be the cause of described antagonisms.

Introduction

Immunologic enhancing effect is mainly considered as related to homologous transplanted tumors as reviewed by Snell (20, 21) and Kaliss (14).

After original observations of Gross (10, 11) and Foley (9), many investigators have demonstrated specific antigenicity in several types of experimental tumors (see recent reviews, Refs. 12, 16). It may therefore be expected that the immunologic enhancing effect also acts in promoting the growth of autotrophicous tumors.

Attempts to induce immunologic enhancing effect in the mouse strain of tumor origin have been scarce. Casey et al. (3-7) reported on the enhanced growth of E0771 carcinoma originating in C57BL/6 mice. Recipients of the same line of C57BL/6 strain were previously injected with lyophilized or long-frozen tumor tissue. Shear et al. (18, 19) have demonstrated the same in C3H mice with the Z8352 mammary tumor. Feldman and Globerson (8) obtained enhancement of the autologous tumor after having injected C57BL mice with the anti-C57BL serum, and then challenged this effect with a s.c. isograft of SBL1 sarcoma which was strain-specific and originated from C57BL mice injected with benzo(a)pyrene. Enhancement of the tumor isograft was used to support the concept of direct action of antibodies in stimulating the augmented growth of tumor cells. According to their own results, Casey and Gunn (5) suggested "the possibility that spontaneous tumor could be enhanced in the host of origin by XYZ factors if such were present in them."

Hirsch and Iversen (13) were able to produce accelerated development of spontaneous mammary tumors in C3H mice pretreated with isologous spontaneous mammary tumor tissue admixed with Freund's adjuvant. The same result was obtained in rats fed with 4-dimethylaminoazobenzene (4-DAB) and injected i.p. with the homogenized rat liver tumor (produced by the same azo dye) mixed with Freund's adjuvant (1). Both results offer certain evidence that Casey's suggestion may be correct.

To date several attempts have been made in our laboratories to suppress the development of experimental allergic encephalomyelitis (EAE) (2). A marked suppression of EAE was observed in rats injected i.p. with the adjuvant alone within a period of 10 days after sensitization with the encephalitogenic vaccine. The main and most impressive result of the i.p. injection of the adjuvant alone in normal rats was an extensive proliferative peritonitis. It seems interesting to note that, in the animals in which EAE has developed, abdominal proliferations were minimal or completely absent; and vice versa, the animals with suppressed EAE showed a marked inflammatory proliferation in the abdominal cavity. According to these observations, the working hypothesis about processes similar to the competition of antigens was used in order to explain the dissociation of 2 particular processes.

Two presented hypotheses were the starting point in these experiments. It appeared therefore appropriate to examine the "competition of antigens" hypothesis to show the deviation of presumed immune reaction from the liver tumor tissue toward the neural tissue and vice versa. The present study is concerned with the testing of this concept by analyzing (a) the incidence of EAE in tumorous and pretumorous 4-DAB-fed and 3,4-benzpyrene-treated rats and (b) the development of tumor in 4-DAB-fed and 3,4-benzpyrene-treated rats injected with the encephalitogenic vaccine.
Materials and Methods

Animals

Rats used in the present study were random bred of the Y stock. The rats of both sexes were 3-5 months old at the beginning of the treatment, weighing about 180-270 gm.

Induction of Tumors

Tumors were induced in rats either by supplying food with 4-DAB (Merck or British Drug Houses) or by s.c. injections of 3,4-benzyperylene (Fluka).

4-DAB was dissolved in methanol and admixed with the standard laboratory food pellets which consisted of maize, white corn, casein, dry brewers' yeast, salts, and 0.6 gm of the dye/kg of the food. The animals were fed ad libitum. Several times during the feeding period lasting 203 days the food consumed in 7 days by 6 animals housed in 1 cage was determined, and the amount of the ingested azo dye/day/animal was calculated. The food consumption increased with the age and growth of the animals. The mean azo dye consumption was about 3.5 mg (3-4 mg)/day/rat; thus the total amount of the ingested 4-DAB was about 700 mg.

3,4-Benzpyrene was dissolved in olive oil (1% solution) and injected i.e. into each rat in 10 separate doses each amounting to 1 mg of the drug. Ten injections were given in the same place, at the flank of the body, every 2nd day.

Preparation of the Encephalitogenic Vaccine and Injection Procedure

The rat brain tissue was homogenized in 0.25% phenol in water and mixed with Freund's adjuvant consisting of the lyophilized bovine type Mycobacterium tuberculosis (kindly supplied by the Serum zavod Kalinovica) in paraffin oil to which adeps lanae (Aquaphor) was added. The vaccine was heated in a water bath for 45 min at 60°C. The rats simultaneously received 8 intracutaneous injections and 2 into the hindfoot pads (0.1 ml/injection).

Methods of Scoring the Incidence of Experimental Allergic Encephalomyelitis

The clinical symptoms of EAE were used as signs for diagnosis. The symptoms were: (a) ataxic gait, (b) incontinence of urine, and (c) flaccid paralysis of hindlegs and, often, forelegs.

Methods of Scoring Tumors

4-DAB hepatoma and 3,4-benzyperylene tumors were detected clinically by inspection and palpation. After the end of the observation period, the tumors were scored by autopsies and histologic examinations.

Histology

Brain, spinal cord, liver and tumor tissues were fixed in formol and embedded in paraffin; 4-μ sections were stained with hematoxylin and eosin.

Results

Incidence of the Liver Tumor in 4-DAB-fed Rats

In order to determine the incidence of liver tumor and the time of tumor appearance (detected clinically), 2 sets of 100 male rats were fed with the azo dye diet. The feeding of the 1st set started in January, and of the 2nd in October of the same year. The animals were surveyed for 1 year after having completed the 4-DAB diet. The results are presented in Table 1.

In the 1st set 68%, and in the 2nd, 86% of the animals developed liver tumor. The evaluation of these data determined by the x² test revealed a significant difference (P < 0.005) in the total tumor incidence between the 2 groups. This difference should not be ascribed to seasonal influence, since similar differences have been observed between groups of rats submitted to the 4-DAB diet in the same season. As seen from Table 1, about 54-56% of rats fed with the described diet developed liver tumor and 50% of the tumorous animals were morbid at Days 115-130 after the azo dye was discontinued. From the 210th day following the end of the carcinogenic diet, until the end of the observation period, no more tumors appeared.

<table>
<thead>
<tr>
<th>INTERVALS FOLLOWING END OF CARCINOGENIC DIET (DAYS)</th>
<th>1ST SET—JANUARY (100 RATS)</th>
<th>2ND SET—OCTOBER (100 RATS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-60</td>
<td>0</td>
<td>0</td>
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<td>61-70</td>
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<td>101-110</td>
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<td>66</td>
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<td>181-190</td>
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<td>67</td>
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<td>191-200</td>
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<td>67</td>
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<td>201-210</td>
<td>1</td>
<td>68</td>
</tr>
<tr>
<td>211-360</td>
<td>0</td>
<td>68</td>
</tr>
</tbody>
</table>

TABLE 2

Incidence of Experimental Allergic Encephalomyelitis (EAE) in Rats with Liver Tumors

<table>
<thead>
<tr>
<th>Group of rats</th>
<th>No. of rats</th>
<th>No. of rats developing EAE</th>
<th>% of rats developing EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20</td>
<td>17</td>
<td>85</td>
</tr>
<tr>
<td>Tumorous</td>
<td>50</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>
Incidence of EAE in Rats with Liver Tumor

Fifty male rats with palpable, well-developed liver tumors were injected with the encephalitogenic vaccine according to the described procedure. Twenty normal rats of similar age were also injected. The appearance of EAE was checked until the 40th day after sensitization. The results are presented in Table 2.

As seen, rats bearing liver tumor are less susceptible to the injected vaccine than the normal ones. The difference in susceptibility is highly significant. This susceptibility was determined on the basis of the development of specific lesions in the nervous tissue and non-specific lesions associated with “adjuvant disease” (2). It should be mentioned that not one of the tumorous rats injected with the encephalitogenic vaccine developed any characteristic sign of arthritis.

Rats used in this experiment were in good condition, with body weights similar to weights of normal rats of the same age, and without any sign of cachexia. Thus the lowered ability of tumorous organisms to react toward the injected vaccine cannot be ascribed to general body weakness. Since Malmgren et al. (15) showed the immunosuppressive effect of several carcinogens, 4-DAB included, it could be assumed that accumulation of the drug in the body may be responsible for lower immune reaction.

What also should be taken into account is that tumorous organisms may, in general, be less reactive toward foreign antigens. As seen, rats bearing liver tumor are less susceptible to the injected vaccine than the normal ones. The difference in susceptibility is highly significant. This susceptibility was determined on the basis of the development of specific lesions in the nervous tissue and non-specific lesions associated with “adjuvant disease” (2). It should be mentioned that not one of the tumorous rats injected with the encephalitogenic vaccine developed any characteristic sign of arthritis.

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Incidence of EAE in 4-DAB-fed Rats without Clinically Manifested Tumors

As shown in Table 1, nearly 50% of all rats sensitized on the 122nd day following the completion of the 4-DAB diet showed a similar incidence of tumor in both groups (63 and 66%). It should be noted, however, that rats in both groups were selected on the basis of the clinical diagnosis of the absence of tumor. As shown in Table 1, nearly 50% of all tumorous animals developed a palpable liver tumor at the 122nd day following the end of the azo dye diet, and at the end of the observation period 68-86% of all 4-DAB-fed animals were recovered from EAE. Therefore 2 more control groups, one of nonfed sensitized animals and another of fed nonsensitized animals, were used. The results are presented in Table 4.

Incidence of EAE in 4-DAB-fed Rats Injected with the Encephalitogenic Vaccine at Various Times after Discontinuation of the Carcinogenic Diet

The 4-DAB-fed rats were divided into 3 groups. They were injected with the encephalitogenic vaccine at Days 1, 35, and 122, respectively, after discontinuation of the carcinogenic diet. The animals were observed for a year. This also made it possible to establish the incidence of the liver tumor in sensitized rats.

As shown in Table 4, the incidence of EAE decreased with increase in the time interval elapsing between the end of the 4-DAB diet and the injection of encephalitogenic vaccine. In order to check the development of liver tumors, the rats that were resistant to the encephalitogenic vaccine and those that recovered from EAE were inspected during a 1-year period after completion of the diet. It was observed that the sooner the sensitization took place, the lower was the incidence of the liver tumor. There was, however, no statistically significant difference in tumor development between EAE-susceptible and EAE-resistant rats.

Rats sensitized on the 122nd day following the completion of the 4-DAB diet showed a similar incidence of tumor in both groups (63 and 66%). It should be noted, however, that rats in both groups were selected on the basis of the clinical diagnosis of the absence of tumor. As shown in Table 1, nearly 50% of all tumorous animals developed a palpable liver tumor at the 122nd day following the end of the azo dye diet, and at the end of the observation period 68-86% of all 4-DAB-fed animals were recovered from EAE.

Incidence of EAE and Liver Tumors in 4-DAB-Fed Rats Sensitized with the Encephalitogenic Vaccine at Various Times After the End of the Azo Dye Feeding

The numbers given in parentheses are the percentages.

A few rats died during the course of EAE.

<table>
<thead>
<tr>
<th>Sensitization with the encephalitogenic vaccine (days after end of azo dye feeding)</th>
<th>No. of rats</th>
<th>No. of rats developing EAE</th>
<th>Incidence of tumor in rats recovered from EAE</th>
<th>No. of rats resistant to sensitization</th>
<th>Incidence of tumor in EAE-resistant rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfed controls</td>
<td>60</td>
<td>51 (85)</td>
<td>18 (30)</td>
<td>4/18 (22)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>42 (70)</td>
<td>4/39* (10)</td>
<td>13/28 (46)</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>60</td>
<td>32 (53)</td>
<td>5/26* (19)</td>
<td>28/47 (60)</td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>60</td>
<td>18 (30)</td>
<td>10/16* (63)</td>
<td>28/42 (66)</td>
<td></td>
</tr>
<tr>
<td>Nonsensitized controls</td>
<td>60</td>
<td>18 (30)</td>
<td>10/16* (63)</td>
<td>48/60 (80)</td>
<td></td>
</tr>
</tbody>
</table>

* EAE, Experimental allergic encephalomyelitis; 4-DAB, 4-dimethylaminoazobenzene.

The numbers given in parentheses are the percentages.

A few rats died during the course of EAE.
Incidence of EAE in 3,4-Benzpyrene-treated Rats

Sensitization with the encephalitogenic vaccine (days after end of carcinogenic drug inoculation) | No. of treated rats | No. of rats with EAE<sup>a</sup> | No. of rats with tumor<sup>b</sup>
--- | --- | --- | ---
Nontreated controls | 25 | 18 (72) | 23/25 (92)
2 | 25 | 0 | 18/25 (72)
20 | 25 | 1 (4) | 20/25 (80)
Nonsensitized controls | 25 | 0 | 22/25 (88)

<sup>a</sup> EAE, Experimental allergic encephalomyelitis.
<sup>b</sup> Numbers in parentheses are percentages.

Incidence of EAE in 3,4-Benzpyrene-treated Rats

To verify the described results, another tumor with a similar latent period between the end of the treatment and the tumor appearance was observed. 3,4-Benzpyrene treatment at the site of injection produces a cutaneous tumor in 80-90% of animals. The tumors begin to appear by the 3rd month after the end of the treatment in about 10% of all treated rats. This incidence increases during the 4th and 5th months, reaching the final incidence of 80-90% at the end of that period. The size of the tumor is often about 1% of the whole rat’s body.

Three groups of the treated rats comprising 25 animals each were injected with the encephalitogenic vaccine at Days 2, 20, or 40 following the end of the drug inoculation. The results are presented in Table 5.

It is evident from Table 5 that 3,4-benzpyrene-treated rats showed greater resistance to the encephalitogenic vaccine sensitization than did the 4-DAB-fed rats. During the latent period all drug-treated and sensitized animals were in good condition, showing a permanent progress in body weight. With the appearance and the growth of the tumor, the rats were gradually wasting. As the time elapsed, the incidence of tumors in these animals was about the same as that of the nonsensitized controls. The conclusion can be drawn that sensitization of 3,4-benzpyrene-treated rats with encephalitogenic vaccine caused neither a paralytogenic effect nor any influence upon the appearance and growth of tumors.

Discussion

The experiments presented suggest the existence of 2 simultaneous “competitive” processes, i.e., the development of experimental allergic encephalomyelitis and tumor disease. The idea of an antagonism between tumor disease and tuberculosis was emphasized by Rokitansky (17) in the 19th century. According to this theory, tubercle bacilli antagonize the development of tumor by their pathogenicity and antigenicity, and vice versa.

As is well known, the main constituent of Freund’s adjuvant is killed tubercle bacilli which have lost their pathogenicity but retained most of their antigenicity. This quality, among others, is manifested by a strong stimulation of reticuloendothelial activity. Stimulated activity of the reticuloendothelial system seems to be general, thus providing the organism with an increased resistance and heightened fighting potency against different noxious agents. However, if an additional particular antigen is added to the tubercle bacilli suspension, as is in encephalitogenic vaccine, a more specific immune response can be elicited, but this immune response can also be deleterious if the organism’s own antigens are admired. One of such effects is visible if the neural tissue admixed with Freund’s adjuvant is injected. Because of its strikingly visible symptoms the induction of EAE was used as a process presumably “competitive” with the development of tumor.

In a previous paper (2) the experiments described supported the idea of a “competition” of 2 processes, one of them being due to the inoculation of Freund’s adjuvant alone and the other to the inoculation of the neural tissue added to the same mixture.

Regardless of the mechanisms by which the enhancement of the tumor growth was produced, it seems evident that the immune response directed toward tumor antigens can promote the tumor growth. It seemed therefore reasonable to test the assumed “competition of antigens” in the model of autologous (autochthonous) tumor growth. It was of particular interest to investigate this possibility, because autologous tumors were reported to be enhanced by injection of the tumor tissue added to Freund’s adjuvant mixture (1, 13).

The results presented seem to corroborate the above assumption by making visible the antagonism between 2 simultaneous processes, i.e., EAE and tumor development. This antagonism was demonstrated in rats in which the liver hepatoma was produced by 4-DAB feeding only if the inoculation of the encephalitogenic vaccine was given early during the latent period after the completion of the azo dye diet. If a longer period of time elapsed after the completion of the 4-DAB diet, the percentage of EAE-susceptible rats decreased, although the drug concentration was obviously lower than in the rats injected with the encephalitogenic vaccine immediately after the carcinogen diet was stopped. For this reason the lower incidence of EAE in 4-DAB-fed rats with or without clinically manifested tumors cannot be ascribed to the immunosuppressive effect of the azo dye.

It seems that the deviation of the immune reaction occurs only during the initial period of tumorigenesis, or during the tumor growth. It may therefore be concluded that the process of tumor growth is likely to produce a gradual incapacity of the organism to react toward the injected antigens and that this, among others, is due to the deviation of the immune response toward the neoplastic tissue.

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