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Inhibition of Chemically Induced Neoplasia by Immunization with an Antigenic Carcinogen-Protein Conjugate[1]

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SUMMARY

Over 50% tumor inhibition was achieved by immunizing Sprague-Dawley female rats with small amounts of a carcinogen-protein conjugate prior to a single intragastric feeding of 2-anthrylamine, on the basis of numbers of animals developing persistent, palpable tumors. In addition, immunized rats developed fewer tumors, and induction was delayed.

INTRODUCTION

In attacking the human cancer problem, perhaps immunization will be a practical means to prevent cancer. In 1939, Franks and Creech (5) reported some inhibition of tumor appearance subsequent to local immunization of mice with carcinogen-protein conjugate followed by s.c.-administered carcinogen. The results of Homburger and Tregier in 1960 (8), with small amounts of i.p.-injected carcinogen prior to a larger s.c. dose of the same carcinogen, in mice, indicated a delay in tumor appearance.

An exquisitely sensitive experimental model for testing the possibility of immunization against cancer-causing chemicals is presented by the well-documented susceptibility of the young Sprague-Dawley female rat to mammary tumorigenesis caused by a single small dose of any of a number of carcinogens (7, 9, 10). Previous experience on the preparation (2, 13) and antigenicity (1) of carcinogen-protein conjugates has been utilized to preimmunize rats against the carcinogen 2-anthrylamine by means of a conjugate synthesized from HSA[2] and 2-anthrylisocyanate (4).

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats with nursing mothers were obtained from A. R. Schmidt Company, Madison, Wis. They were ear punched for individual identification and weaned during treatment. Rats were housed in stainless steel cages 20 x 18 x 11 inches (no more than 13 rats/cage) and were fed Wayne Lab-Blox and water *ad libitum*.

Chemicals. Antigen was 2-anthrylcarbamido-HSA-41 (β-HSA-41). To a stirred, cooled solution of 1 g of HSA (Fraction V; Pentex Biochemicals, Kankakee, Ill.) in 29 ml of water, 5 ml each of 1 N $NaHCO_3$ and 1 N Na_2CO_3 and 10 ml of purified, sodium-distilled dioxane, was added, over a 1.5-hr period, a solution of 0.25 g of 2-anthrylisocyanate, m.p. 209–211° (4) in 17.5 ml of dioxane. Internal temperature was maintained during addition and for another 10 min at −5° to −10°, and then the solution was dialyzed exhaustively against ice water (4 days) and centrifuged to give 180 ml of clear solution containing 4.7 mg/ml of protein (Kjeldahl method). Ammonium sulfate was gradually added to 0.5 M, and the resultant turbid solution was left standing overnight at 5° and centrifuged. The centrifugate was dialyzed first against ice water and then against distilled water, giving on centrifugation 49 ml of a clear solution containing 12.0 mg/ml. UV analysis of a 1:50 dilution gave E_{max} = 0.565 at 358 mμ. Calculation by principles previously set forth (2) gives a value of 41 2-anthryl groups per molecule of protein.

Immunization. Our schedule for immunization of the rats was suggested by Dr. Eberhardt Weiler, a former associate at the Institute for Cancer Research.[3] The courses were as follows:

Group I (Chart 1) received no immunization.

Group II (Chart 2) HSA controls, received 0.75 mg of unaltered HSA in 1:1 0.9% NaCl solution:complete Freund's adjuvant at 14 days of age, 1.0 mg HSA (complete Freund's) at 42 days of age, and 1.0 mg HSA (no Freund's) at 49 days. All injections were i.p.

Group III (Chart 3) received β-HSA-41, 0.75 mg in 1:1 0.9% NaCl solution:complete Freund's at 14 days of age, 1.0 mg β-HSA-41 (complete Freund's) at 42 days of age, and 1.0 mg β-HSA-41 (no Freund's) at 49 days.

Administration of Carcinogen. At 56 days of age, all animals (3 groups) were fed 18 mg of 2-anthrylamine (twice recrystallized commercial product, m.p. 243–244°, with decomposition) as a fine suspension in 1 ml of sesame oil. Feeding was via gastric intubation with a No. 8 French Neleton rubber catheter. The animals were subsequently weighed weekly and examined for tumors.

After the 2nd and to a lesser extent after the 3rd immunizing dose, most rats developed an appreciable ascitic secretion which, when tapped, presented a convenient means of assaying the current antibody titer of the animals by use of

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[2] The abbreviation used is: HSA, horse serum albumin.

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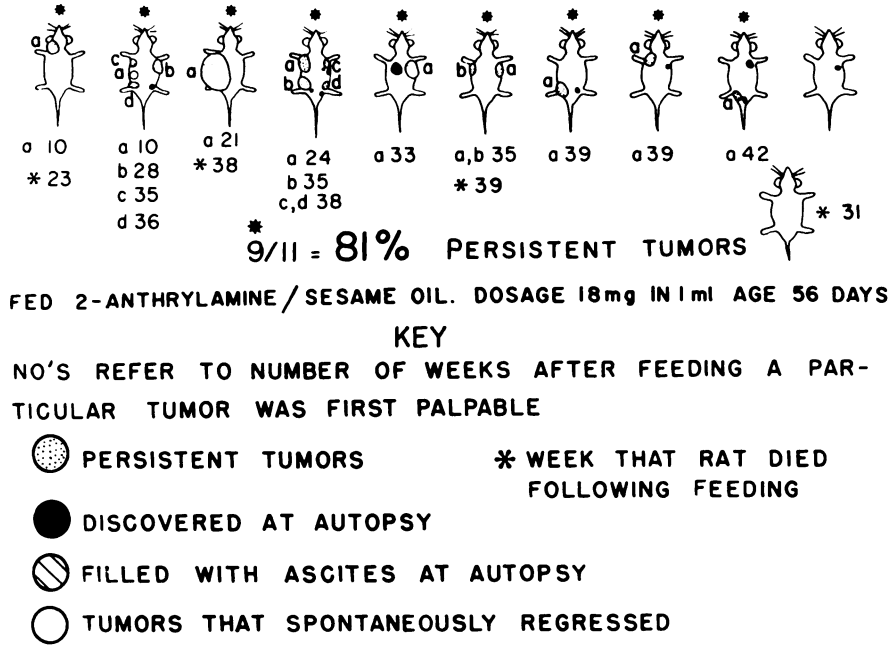

Chart 1. Effect of 2-anthrylamine on untreated rats, Group I. Key applies also to Charts 2 and 3.

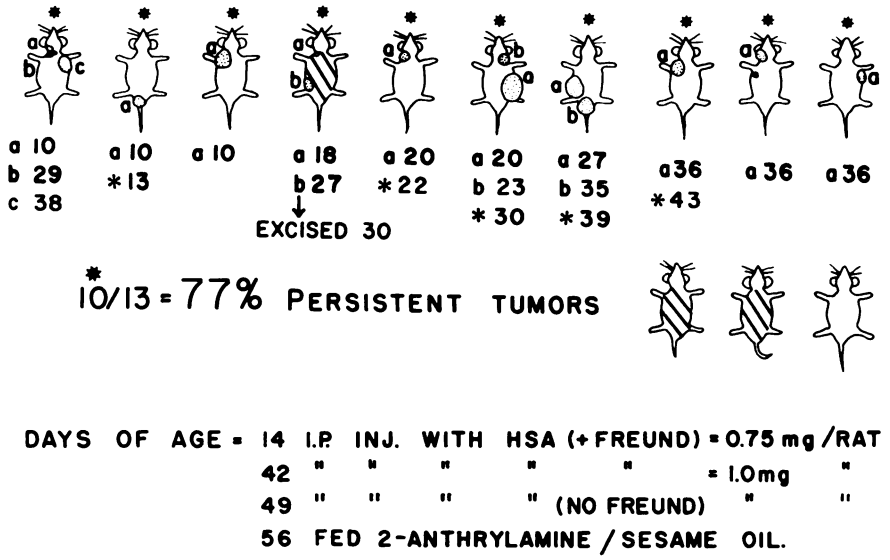

Chart 2. Effect of 2-anthrylamine on rats, Group II, immunized with native HSA. *Inj,* injected.

the micro agar immunodiffusion test. In order not to alter the immune potentials of the rats, samples taken were ~0.2 ml.

Weight gain was comparable for animals in all 3 groups although Groups II and III were temporarily heavier prior to feeding due to the accumulation of ascitic fluid which resulted from the immunization regimen with Freund's adjuvant.

Approximately 48 weeks after feeding carcinogen, all remaining animals were autopsied.

RESULTS

Immunity. In samples of ascitic fluid taken from 13/22 animals of Group III on Day 48, every sample showed strong determinant group activity by testing *versus* a similarly prepared carcinogen-bovine serum albumin conjugate; much stronger bands were produced than when tested against native HSA (the homologous protein component). On the day prior to feeding, 4 animals were tapped for retesting; strengthened immunity as evidenced by much heavier bands farther from the antibody origin was indicated in all cases. At this time, most of the gross ascitic seretions had been resorbed.

Tumors. Charts 1 through 3 graphically represent the status of animals at time of autopsy or prior death, as well as time of appearance of palpable tumors and whether or not spontaneous regression occurred. Ultimate relative tumor size is also graphically depicted. One animal from Group I and 3

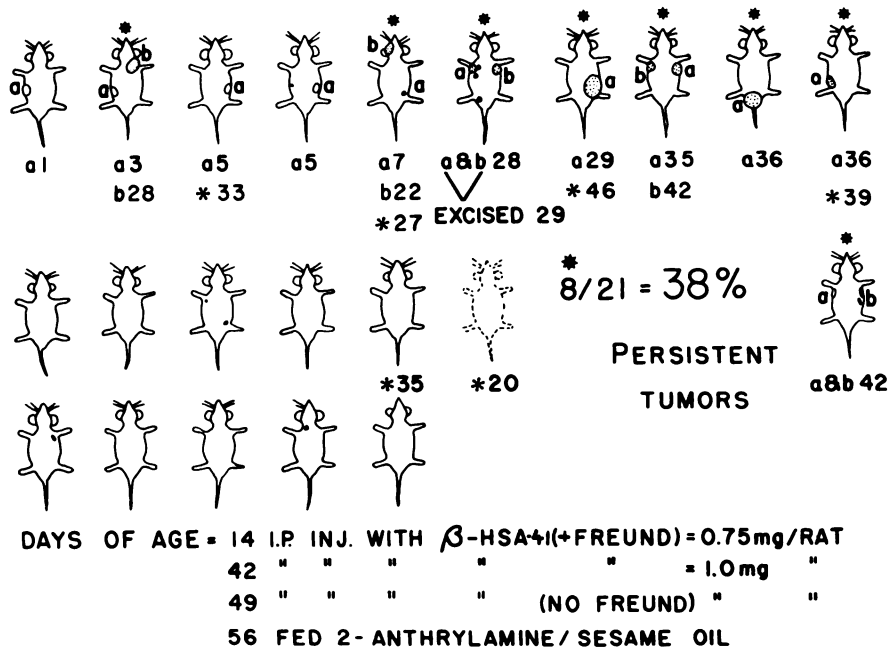

Chart 3. Effect of 2-anthrylamine on rats, Group III, immunized with 2-anthrylcarbamido-HSA conjugate. *Inj*, injected.

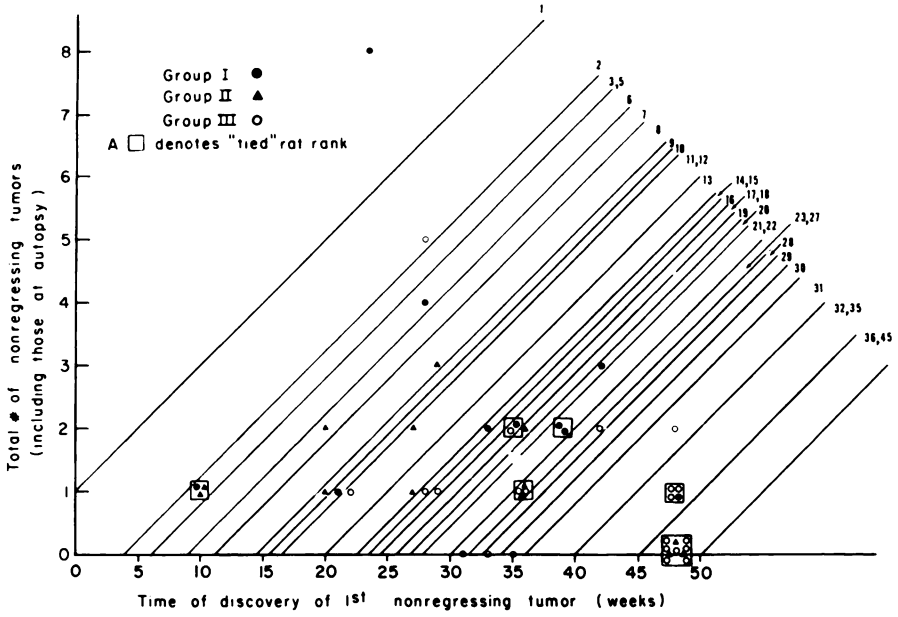

Chart 4. Mann-Whitney nonparametric analysis.

animals from Group III which died during the immunization regimen are not included. Shown in Group III but not tabulated is 1 animal which died too early to claim as tumor-free. No autopsy was possible.

Statistics. Tests of hypothesis for difference in proportions were applied first to the numbers of nonregressing palpable tumors in Group I *versus* those in Group III, 9/11 *versus* 8/21, giving a p value equal to 0.0093 or a validity at higher than the 99% level.

An identical calculation applied to Group II *versus* Group III gives $p = 0.0137$, slightly less than the 99% level, and a calculation comparing the combined control Groups I and II with Group III gives $p = 0.0026$, a much more powerful validity.

These figures make the negative assumption of accepting as tumor-free 1 rat in Group I which died early and 2 animals in Group II bearing ascites fluid. After the further negative assumption is made that nonpalpable tumors found at autopsy are the equal of the larger tumors, identical analysis for Group III *versus* Groups I and II taken as combination control gives a p value of 0.027 or a validity above the 97% level.

In an analysis with parameters neglected in the simple

Table 1
Results of Mann-Whitney Analysis

	Smaller group	Larger group	Mann-Whitney statistic U	Normalized U statistic Z	*p* value
I – III	11	21	43.5	−2.857	0.00214
II – III	13	21	78.0	−2.07	0.0192
(I + II) – III	21	24	121.5	−2.97	0.0015

percentages of tumor-bearing *versus* nontumor-bearing rats, Chart 4 is a plot where each point represents 1 rat. The x axis displacement is the time of discovery of the first nonregressing tumor (in weeks). The coordinate is the total number of nonregressed tumors found at autopsy or present at time of death, including nonpalpable tumors. Points at the upper left of the chart represent more tumors sooner discovered than points at the lower right. In order to rank the tumor status of the rats, a series of 45° lines were drawn so as to separate each of the points (unless several fall at the same point or along the same 45° line, in which case they are declared "tied"). The lowest rank, 1, was assigned to the point in the most upper left position, and succeeding points were ranked 2, 3, 4, etc., as they progressed to the lower right. The set of ranks produced this way is equivalent to those that would be produced by assigning the number $N - (t/5)$ to each rat, where N is the number of nonregressing tumors at autopsy (or prior death) and t is the time in weeks of discovery of the first such tumor. The equation $N - (t/5) =$ constant will produce a 45° line on the graph. Tied points such as ($t = 22$, $N = 1$) and ($t = 27$, $N = 2$) are both assigned the average rank of the 2 ranks that would be assigned to them if they were slightly different. The Mann-Whitney nonparametric rank test (16) was applied to these ranked data. The results show that Groups I and III differ significantly, as do Groups II and III. Tests of hypothesis that Groups I and III (or II and III) come from the same population are rejected at very satisfactory levels of significance. These results are shown in Table 1.

DISCUSSION

In the nonimmunized control group (Chart 1), 9/11 animals developed persistent palpable tumors during the course of the experiment. One additional animal (and several others in Chart 3) were found at autopsy to have tumors too small to palpate. Autopsy disclosed small tumors in some tumor-bearing rats in all groups.

In the group immunized with HSA (Chart 2), 10/13 rats developed persistent palpable tumors. No additional tumor bearers were found at autopsy. However, other tumors were discovered and 2 of the 3 scored as nontumor bearers were filled with ascitic fluid, as was a 3rd tumor bearer the tumor of which had been excised.

In the carcinogen-immunized group (Chart 3), 8/21 retained palpable tumors at autopsy; 3 additional animals had experienced complete regression of palpable tumors; 2 of these were confirmed at autopsy to be free of any tumor.

As a frame of reference, data of Huggins and Yang (10) and of Davis *et al.* (3) indicate that under similar conditions untreated Sprague-Dawley rats develop spontaneous mammary tumors to the extent of 5 to 10%.

The data show that we have produced animals resistant to the action of 2-anthrylamine in producing neoplasia. We believe that this is due to an immune status produced by the prosthetic groups of the antigen. Predication of a nonspecific or nonimmunological mechanism as responsible for the reduced number of tumors in the immunized group requires assigning unique properties to the carcinogen-protein conjugate on the basis that it is an altered protein and that a protein so altered *per se* changes the susceptibility of the animals to carcinogenesis. Alternatively, if the immunization produced abortive tumors due to the carcinogen moiety involved, the possibility exists that an antigenic response to these produced protection in the animal against tumors subsequently formed after the p.o. dose. This would require the hypothesis of a common antigenic response to tumors in separate animals, as claimed by Reiner and Southam (14, 15). However, they obtained evidence for common antigenic properties only by combining tissues from up to 4 separate tumors, and other workers (11) have not found cross-resistance.

Other data of interest but possibly of moot value from the immunoprotection thesis follow.

The 5 earliest tumors to appear were actually in Group III, but all these underwent complete and spontaneous regression. At present, we do not care to commit ourselves to speculative explanations for this phenomenon.

Data indicate but do not demonstrate that the immunization regimen undergone by Group III was more rigorous than that of Group II on the basis of 3 deaths during this period *versus* none in Group II.

Alteration of the Sprague-Dawley female from a carcinogen-susceptible animal to a (relatively) immune animal may have a relation to recent work on contact sensitivity. According to a widely held theory, the guinea pig, an animal relatively resistant to chemical carcinogenesis, is able to form antigenic conjugates with carcinogenic chemicals, thereby acquiring an immune status. This is supported by data on contact sensitivity developed by the guinea pig (and not by the mouse) on immunization with the aromatic hydrocarbons methylcholanthrene, benzo(a)pyrene, and 7,12-dimethylbenz(a)anthracene, in work by Old *et al.* (12). Appreciable cross-reaction between these related hydrocarbons was noted. This finding was extended to the aminoazo dyes by Gordon (6). Those dyes which are carcinogenic in the rat generally induced contact sensitivity in the guinea pig, in contrast to the behavior of noncarcinogenic analogs. Again, appreciable cross-sensitivity was noted.

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