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Influence of Age and Mammary Development upon Mammary Carcinogenesis Following Injection of "Milk Agent."*

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It has been observed that when mice of a genetically high tumor strain but lacking the milk agent are infected with this virus after they are a few months of age they develop a much lower incidence of mammary carcinoma than if infection occurs when they are under a month of age.[1,2] Because of this observation and that of Van Gulik and Korteweg[3] that the degree of lateral bud formation in the mammary gland is determined by the presence or absence of the milk agent, it was suggested[4] that in order to be effective in the genesis of mammary cancer the milk agent virus has to be present at the time the mammary gland develops. More recently Dmochowski[5] has been able to overcome this relative "non-susceptibility" of older mice by increasing the amount of virus injected and by injecting it weekly over a 3 month period. This, together with our inability to confirm the above mentioned observations of Van Gulik and Korteweg,[6] prompted the present investigation. Since the mammae of male mice remain essentially rudimentary throughout life, the demonstration that castrate male mice bearing ovarian grafts may develop a high incidence of mammary cancer[7] suggested a suitable method for testing the relationship of the state of mammary gland development at the time of the introduction of the milk agent to mammary carcinogenesis.

Materials and methods. Male mice of the Zb stock (fostered C_3H), being genetically susceptible to the development of breast cancer but lacking the milk agent, were used for this experiment since their mammae normally show almost no development throughout life (less than those of A x Z hybrid males). Three groups of mice were employed. In the first, castration and ovarian transplantation were performed when the mice were approximately one month of age, and on the following day they were injected intraperitoneally with an extract of a spontaneous mammary tumor containing the milk agent. In the second, castration and ovarian transplantation were carried out when the animals were one month of age, but the injection of milk agent virus was postponed until the animals were 4 months of age in order to allow mammary gland development prior to the introduction of the virus. In the third group, castration and ovarian transplantation were performed when the mice were 4 months of age, and on the following day the animals were injected with a tumor extract. In the second and third groups, the animals received the milk agent when approximately the same age, but in Group 2 the mammae had developed prior to the introduction of the virus while in Group 3 mammary gland development occurred in the presence of the milk agent.

The methods of castration and ovarian transplantation were the same as described in

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3 van Gulik, P. J., and Korteweg, R., *Proc. Nederl. Akad. v. Wetenschappen*, 1940, **43**, 891.

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the previous paper, and ovaries of one month old Zb females were used in all instances. To obtain active virus preparations, fresh mammary tumors arising spontaneously in Z strain females were employed. The tumors were ground in a mortar with sand, extracted with distilled water, and centrifuged at relatively low speeds to remove viable cells, as well as larger cellular and connective tissue fragments. Each animal received 1 cc of a 10% extract intraperitoneally. The animals were maintained on Purina Fox Chow, and inspected weekly for the development of subcutaneous tumors. All animals developing such tumors were autopsied.

Results. As a preliminary study to determine the extent of glandular development at the time of the introduction of the milk agent in the second group of experimental animals, glands of Zb mice castrated and transplanted with ovaries at a month of age were studied histologically when the mice were 4 months of age. From the study of whole mount preparations it was evident that by this time several of the glands of each animal had developed extensively (all 10 glands seldom develop in male mice in response to estrogenic stimulation). The ducts of the developed glands had extended throughout the fat pads to a degree similar to that seen in virgin female mice of the same age. In addition to ductular and lateral bud development, however, there were many areas of alveolar development, a situation not encountered in virgin female mice lacking the milk agent. These areas occurred mainly near the peripheri of the glands and resembled normal alveolar lobules as seen in pregnant mice approaching parturition. Morphologically they differed from the typical precancerous lesions seen in glands of high tumor strains of mice in that the epithelium showed considerable secretory activity, and there was not the slight increase of connective tissue usually seen in precancerous hyperplastic nodules. Because of the presence of these areas of alveolar development, however, a fourth group of Zb males were castrated and transplanted with ovaries when one month of age, but they were not injected with the milk agent

TABLE I.

Summary of Tumor Data Obtained in the Various Groups of Male Mice That Were Castrated, Transplanted with Ovaries, and Injected with Milk Agent Virus at Different Ages.

Group	Age when transplanted with ovaries, mo.	Age when injected with milk agent, mo.	No. of animals/ No. tumorous	% tumorous		Avg age of tumor development		Avg age of non-tumorous death, mo.
				uncorr.	corr.	from birth, mo.	from inject. of milk agent, mo.	
I	1	1	34/25	73.5	78.1	11.5	10.5	14.8
II	1	4	30/8	26.7	32.2	14.9	10.7	17.1
III	4	4	34/8	23.5	29.1	15.1	10.7	16.8
IV	1	None injected	16/0	0	0	—	—	20.1

virus in order to make certain that mammary tumors would not develop in such lobules in the absence of the virus.

The mammae of male mice castrated and transplanted with ovaries when four months of age were also studied three months after transplantation to determine if their glands had developed to the same extent as those of the younger mice. As far as could be ascertained from the inspection of whole mount preparations, this was the case.

The tumor data are summarized in Table I. In the group receiving both ovaries and milk agent at one month of age, mammary tumors developed at an earlier average age than in virgin females of the Z strain maintained in this laboratory[8] confirming the observations previously reported employing hybrid males.[7] In the 2 groups that received the milk agent at 4 months of age the incidence of mammary tumors was only about one third that seen in the group that received virus when one month of age, but there would appear to be no difference in the age at which tumors developed or in the frequency of tumor development in these latter 2 groups of animals. It is interesting that the average latent period of tumor development after the injection of the milk agent was the same in all 3 groups of animals.

Since the age distribution of tumor development after the injection of milk agent was the same for all 3 groups of mice, it would appear that the correction factor developed by Bryan and Shimkin[9] would be applicable to these data and would give some indication whether or not the lower incidence of tumors observed in the 2 groups of mice injected at 4 months of age was entirely due to the fact that these animals had a somewhat shorter time in which to develop cancer. The per cent of tumors developing during each 30 day period following the injection of the milk agent was calculated, and the mice dying non-tumorous weighted accordingly. As can be seen from the "corrected" data given in the sixth column of Table I, only a small portion of these lower incidences of tumors can be attributed to differences in the life span following the injection of the virus.

Discussion. The results of this experiment emphasize again that as they grow older genetically susceptible mice that lack the milk agent become less "susceptible" to the milk agent virus, at least as far as the subsequent development of mammary tumors is concerned. These data would also seem to indicate that this relative "non-susceptibility" is not due to the greater mammary gland development in female mice after one month of age since glands of 4 month old male mice that developed after the injection of milk agent had no more tumors than did glands that developed prior to the introduction of the virus.

It has been shown that young mice are more susceptible to chemical and physical carcinogens than are older mice.[10–13] As yet we have been unable to demonstrate in older mice lacking the milk agent a humoral agent capable of neutralizing active milk agent virus either *in vitro* or *in vivo*. It has been observed, furthermore, that although mice which obtain the milk agent late in life may die non-cancerous, such mice may pass the agent to their offspring which then may show a high incidence of tumors. This relative "non-susceptibility" to the development of mammary tumors following the late introduction of the milk agent may not, then, be a specific resistance to the virus but may reflect a more general increased resistance of older mice to the influence of "carcinogens." Studies are now under way in an attempt to shed more light on this possibility.

Summary. Employing male mice, transplanting ovaries and injecting active milk agent virus at different ages, an experiment

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was carried out to specifically test the relationship of the state of mammary gland development at the time of the introduction of the milk agent to mammary carcinogenesis. The results would indicate that the relative non-susceptibility of older mice is not due to greater mammary gland development at the time of virus infection.

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Effects of Excess Dietary *dl*-Methionine and/or *l*-Arginine on Rats.*

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The addition of low concentrations of *dl*-methionine to a casein diet increases the nitrogen balance index in the rat while the addition of higher concentrations of the amino acid decreases the index and causes a marked loss in body weight.[1,2] Loss in body weight and inhibition in growth in animals receiving excess methionine has been reported from a number of laboratories.[3-5] Excess methionine could alter the need for other amino acids, for example, by increasing the demand for them in methylating reactions,[5,6] and by promoting the formation of one type of tissue protein at the expense of others. The question is raised as to whether or not the addition of other amino acids to the diet would reduce the drain on tissue sources which excess methionine could exert. The following experiments on rats were designed to answer this question in part.

Methods. Nitrogen balance indexes were determined using the diet and technics previously described for the dog.[7] The diet contained 12% casein (dry weight basis). Rats were pair-fed with those fed excess methionine (4.8%) where food intake was markedly restricted. The experiments were continued for a period of 20 days with urine and fecal collections throughout the period. At the end of the period the animals were autopsied and the livers and kidneys were dried at 95° C to constant weight. The dry livers and kidneys were analyzed for nitrogen by the micro-Kjeldahl procedure and were also analyzed for total fat. The urine was analyzed for creatinine and creatine by the Folin method.[8]

Results. The effects of relatively large addition of *l*-arginine and/or *dl*-methionine, to casein, on the nitrogen balance index and on the excretion of creatinine in the rat are recorded in Table I.

The average nitrogen balance index for casein was 0.81. The creatinine excretion on the casein diet was 28.3 mg/day/kg body weight. The addition of *l*-arginine to casein decreased the nitrogen balance index to 0.73 so that this amount of *l*-arginine did not improve the pattern of amino acids in the diet. Addition of excess *dl*-methionine to casein decreased the nitrogen balance index markedly so that the animal was in negative nitrogen balance and increased the excretion of creatinine significantly (34.3 mg), changes which

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