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THE EFFECTS OF PRETREATMENT WITH NITROGEN MUSTARD N-OXIDE AND STRAIN-SPECIFIC TUMOR TISSUE ON THE DEVELOPMENT OF SPONTANEOUS MAMMARY TUMOR

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ABSTRACT

In order to investigate host-tumor relationship, the effects of pretreatment with nitrogen mustrad N-oxide and strain-specific tumor tissue on spontaneous mammary tumor development were studied.

In neither group was the pretreatment effective in preventing tumor development of virgin female C3H/He mice. There were no reduction in spontaneous tumor incidence, no retardation in age at which tumor appeared, nor prolongation in survival time following onset of tumor and in total survival time.

On the contrary, the development of the consecutive tumor of tumorous mice increased significantly in the group immunized with isologous tumor homogenate.

Further, tumor-unspecific antibody was estimated by hemagglutination test.

The data are discussed on the point of host-tumor relationship.

INTRODUCTION

The administration of carcinostatic agents has sometimes been followed by an increase in tumor growth or by the production of metastases. This phenomenon was observed by Kondo and Tsukui[1] on Yoshida sarcoma after pretreatment with nitrogen mustard N-oxide. In addition, several groups of investigators have reported that the use of carcinostatic agents[2)3)4)5)], X-rays[1)6)7)8)9)], or cortisone[1)10)11)12)] may result in increased number of metastases and tumor growth. These investigators assume that this enhancement is mainely due to a reduction in host resistance.

On the other hand, immunization of inbred mice against a strain-specific tumor has been reported in a few literatures, although no unequivocal evidence of the presence of tumor-specific has so far been fully established.

The purpose of this investigation is to study the effects of pretreatment with carcinostatic agent and of immunization with isologous tumor homogenate of very recent spontaneous origin on the inicdence of spontaneous tumor.

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MATERIALS AND METHODS

Virgin female C3H/He mice, F3 and F4 generations, derived from one pregnant mouse from the Mishima Genetic Institute, were used as experimental animals. They were bred by mothers for one month and then isolated.

Nitrogen mustard N-oxide (Nitromin) was injected intraperitoneally, in a daily dosage of 0.5 mg for 10 consecutive days, 5, 6, and 7 months after birth, respectively.

Tumor homogenate was obtained from spontaneous mammary carcinoma of F1 or F2 mice after homogenization and lyophilization. Then the homogenate was adjusted to contain 100 mg wet weight of tumor tissue per ml in the Ringer's solution and stored in a refrigerator at $-20°C$ before use. For immunization, 0.1 ml of the homogenate was injected subcutaneously for 3 consecutive days into mice of 5, 6, and 7 months of age respectively, before the tumor appeared.

Development of tumors was observed weekly by palpation. When palpable tumors developed, their locations and sizes were recorded at each inspection by measuring the two main diameters of each tumor by means of caliper until the mice were dead. When they died, the subcutaneous regions were examined for the presence of smaller tumors.

Hemagglutination test was performed on a few treated and non-treated groups of mice. They were immunized with 6 injections of 1 mg of bovine albumin (Fraction V) intermuscularly and intraperitoneally, alternately. All the animals were bled on the 14th day after the last injection. The bloods were diluted with the same volumes of saline and then centrifuged. After inactivation in a water bath at 56°C, the sera to be tested were absorbed with sheep erythrocytes and titration was started with a serum dilution of 1 in 4. The method used for titration was similar to the technique described by Stavitsky[15], but the use of formalinized sheep erythrocytes. Intensity of agglutination from negative to 3 positives was graded by his criteria[15].

RESULTS

In assessing the effect of pretreatment with mammary tumor tissue and nitrogen mustard N-oxide on subsequent spontaneous mammary tumor development, the following variables were considered in the experimental and control groups: (1) tumor incidence; (2) age at which tumor appeared; (3) survival time of the tumorous mice; (4) survival time of the tumorous mice following onset of the tumor. In addition, tumorous mice were inspected for further development of other tumor.

A summary of the results obtained is shown in Table 1. No significant differences were found in tumor incidence, mean age of tumor appearance, mean survival time, or mean survival time after onset of tumor, between the control

TABLE 1. Effect of Pretreatment with Strain-Specific Mammary Tumor Tissue and Nitrogen Mustard N-Oxide on Spontaneous Mammary Tumor Development

	Homogenate* injected	Nitromin** injected	Control
Total no. of mice observed	30	30	31
No. of mice with tumors	22	20	19
Tumor incidence	73%	67%	61%
No. of mice with more than 2 tumors	15	5	6
Mean age of tumor appearance***	302.7±38.7	332.2±44.7	322.5±36.0
Mean survival time***	358.6±55.9	396.7±40.5	386.2±29.4
Mean survival time after tumor onset***	55.9±19.5	64.5±19.6	63.7±16.7

* Homogenate was made from removed spontaneous tumor after homogenization and lyophilization.

** Nitromin is a commercial name of nitrogen mustard N-oxide.

*** Days ± standard deviation

TABLE 2. Age at which Tumor Appeared

(Days)

Homogenate injected	Nitromin injected	Control
221	235	340
271	378	320
272	342	347
343	371	290
273	302	388
243	389	293
321	332	387
295	310	325
386	262	270
252	348	310
325	390	312
351	356	331
332	280	292
301	362	312
298	292	394
312	345	280
295	355	295
342	388	321
321	331	310
280	376	
316		
310		
$\bar{X}$=302.7	332.2	322.5
N=22	20	19
S.D.=38.7	44.7	36.0

TABLE 3. Survival Time of Tumorous Mice

(Days)

Homogenate injected	Nitromin injected	Control
268	302	387
362	438	400
337	396	407
364	448	376
315	362	415
338	415	340
384	398	445
348	382	375
431	345	333
301	390	385
410	428	375
408	421	413
382	352	372
363	432	385
352	368	432
363	407	338
332	399	383
413	442	394
352	402	382
343	406	
371		
352		
$\bar{X}$=358.6	396.7	386 2
N=22	20	19
S.D.=55.9	40.5	29.4

TABLE 4. Survival Time After Onset of Tiumor

(Days)

Homogenate injected	Nitromin injected	Control
47	67	47
91	60	80
65	54	60
21	77	86
42	60	27
95	26	47
63	66	58
89	72	50
45	82	63
49	42	75
85	38	65
57	65	82
50	72	80
62	70	73
54	76	38
51	62	58
37	44	88
71	54	73
31	71	72
63	30	
54		
52		
$\bar{X}$=55.9	64.5	63.7
N=22	20	19
S.D.=19.5	19.6	16.7

$\bar{X}$=average; N=number of animal; S.D.=standard deviation.

TABLE 5. Site of Tumor Development

Homogenate injected		Nitromin injected		Control	
primary site of tumor development	consecutive site of tumor development	primary site of tumor development	consecutive site of tumor development	primary site of tumor development	consecutive site of tumor development
l-inguinal	*r*-inguinal	*m*-back		*r*-axillary	*l*-axillary
l-back	*r*-axillary *r*-back *r*-inguinal	*r*-flank		*r*-axillary	*r*-flank
r-inguinal		*r*-back		*m*-back	
l-flank	*l*-inguinal *r*-back	*l*-axillary		*r*-inguinal	*l*-flank
l-axillary		*l*-axillary		*l*-axillary	
r-axillary	*r*-inguinal	*l*-back		*r*-axillary	
l-flank		*l*-axillary	*r*-axillary	*l*-flank	
r-axillary	*l*-axillary *m*-back	*r*-back	*r*-flank	*r*-inguinal	
r-inguinal		*l*-inguinal	*r*-inguinal	*l*-inguinal	
r-axillary	*r*-flank	*m*-back		*l*-flank	*m*-back
r-inguinal					
l-axillary	*r*-inguinal	*l*-back		*r*-axillary	
l-axillary	*l*-flank	*r*-flank	*r*-axillary	*l*-axillary	*l*-flank
r-neck	*l*-flank	*r*-axillary		*r*-flank	
l-flank		*l*-neck		*r*-inguinal	*l*-inguinal *l*-flank
r-axillary	*l*-axillary	*r*-inguinal	*l*-inguinal	*m*-back	
r-axillary	*l*-axillary	*r*-flank		*l*-axillary	
r-flank	*r*-axillary	*l*-flank		*l*-axillary	
l-back		*r*-axillary		*r*-axillary	
m-back	*l*-back	*l*-axillary			
r-inguinal	*l*-inguinal *r*-axillary				
l-inguinal	*r*-inguinal				
22	15	20	5	19	6
22/30 (73%)	15/22 (68%)	20/30 (67%)	5/20 (25%)	19/31 (61%)	6/19 (32%)
$X^2=1.0$ $p>0.05$ (v.s. Control)	$X^2=6.9$ $p<0.01$ (v.s. Control)	$X^2=0.2$ $p>0.5$ (v.s. Control)	$X^2=0.7$ $p>0.25$ (v.s. Control)		

and the two experimental groups. The results for individual animals on the ages at which tumor appeared are shown in Table 2, on the survival times of tumorous mice in Table 3, and on the survival times after onset of tumor in Table 4. The tumor incidences and sites of tumor development are shown in Table 5. A X^2 test for a comparison between the proportion of tumors in the control group and the experimental groups was made. The proportion of

animals developing tumors in the experimental groups did not differ significantly from the proportion of animals developing tumors in the control group, where X^2 was 1.0 ($P>0.05$) in the homogenate treated group and X^2 0.2 ($P>0.5$) in the nitrogen mustard N-oxide treated group. However, there was a highly significant difference in the proportion of consecutive tumor development of tumorous animals pretreated with isologous tumor homogenate. By analysis, X^2 of the homogenate treated group against the control group was 6.9 ($P<0.01$). The difference between the proportion of consecutive tumor development of tumorous animals pretreated with nitrogen mustard N-oxide and that of the control was not so clear-cut as the corresponding difference in the homogenate treated group, where X^2 was 0.7 ($P>0.25$).

Circulating antibody was demonstrated in 2 of the tested 15 mice immunized with albumin, as shown in Table 6. These two sera were all from non-treated mice bearing tumor; one from a mouse with left axillary tumor, 26 × 24 mm in size, and the other from a mouse with left inguinal tumor, 25 × 27 mm in size. From this result, no difference in immune response could be drawn either between tumor-bearing animals and animals without tumor, or between the pretreated group and the non-treated controls.

TABLE 6. Hemagglutination Titers of Tumorous and Non-Tumorous Animals 14 Days after the Injections of Bovine Albumin

				Hemagglutination in antisera diluted**		
	No.	Size of tumor* (mm)	Treatment	1:4	1:8	1:16
tumor bearings	1	15×15	Nitromin	—***	—	—
	2	20×18	Nitromin	—	—	—
	3	21×16	none	—	—	—
	4	25×32, 5×5	none	—	—	—
	5	26×24	none	##	#	+
	6	30×30, 12×24	none	—	—	—
	7	25×25, 20×20	none	—	—	—
	8	25×27	none	#	#	±
no tumors	9	none	Nitromin	—	—	—
	10	none	Nitromin	—	—	—
	11	none	Nitromin	—	—	—
	12	none	none	—	—	—
	13	none	none	—	—	—
	14	none	none	±	—	—
	15	none	none	—	—	—

* on day sacrificed

** each blood was diluted with the same volume of saline, then the serum was obtained after centrifugation.

*** —, ±, #, ## represent the intensity of hemagglutination after the Stavitsky's criteria[15].

DISCUSSION

The inbred spontaneous tumor bearing mouse, with its rate of growth, its homogeneity of tumor tissue, and its resemblance to human cancer, has been considered a potentially more accurate guideline to human cancer than transplantable tumors.

Enhancing effect on tumor growth after the administration of carcinostatic agents has been obtained with genetically unspecific transplantable tumors.[1)2)3)4)5)] In the specific host-tumor system, however, there were no differences observed on the subsequent occurrence of spontaneous tumor between the group pretreated with nitrogen mustard N-oxide and the controls. Schmid *et al.*[16)] also reported that pretreatment with cyclophosphamide failed to enhance pulmonary formation in C3H mice receiving intravenous injections of isologous tumor cells from spontaneous mammary adenocarcinomas, in contrast to the effect on Ehrlich ascites tumor. They assumed that a different attitude existed in host-tumor system between the animal bearing spontaneous tumor and the animal with transplantable tumor. On the other hand, it has been well established that the poor response of spontaneous tumors of mice to agents that are effective against transplants of these tumors may be a general phenomenon[17)18)19)]. From these points of view, it is considered that ineffective agent against tumor might not so remarkably influence its host as has been observed in the mice bearing tumor treated with effective agents. With regard to this, Kondo *et al.*[20)] have reported in their experiment on tumor sensitivity test and clinical observation that effective drugs for tumor have less side effects on the host. In the present experiment on host resistance, no conclusive answer was drawn from the formation of antibody, whether the immune response of mice treated with nitrogen mustard N-oxide might be decreased.

Concerning the immunization of inbred mice with their strain specific tumors against the occurrence of spontaneous tumors, so far only a few experiments have been reported, because of the considerable duration of such experiments. Hackmann[21)] attempted to induce active immunity against spontaneous tumor development in an inbred mouse strain. No difference in spontaneous tumor incidence in the control and experimental group was noted. Klein *et al.*[22)] and Prehn[23)] showed that immunization with carcinogen-induced tumor contained tumor-specific antigens. No resistance seemed apparent, although Isojima *et al.*[24)] reported that active immunization of young virgin females with isologous mammary tumor homogenates plus Freund's adjuvant appeared effective in prolongation of the survival time. However, they failed in preventing tumor growth. In contrast, Hirsch and Iversen[25)] obtained earlier tumor appearance and a reduction in survival time of the mice pretreated with strain-specific or strain-related tumor tissue. Later, Nutini *et al.*[26)] reported that the injections of alcohol-soluble, protein-free fractions from a transplantable and a spontaneous

tumor lowered the incidence of spontaneous tumor in virgin female C3H, but did not cause significant differences in mean age at which tumors appeared, mean survival time after tumor onset, or mean total survival time.

In our observation, the results accorded with these authors' for the most part, though not in part. Pretreatment with isologous mammary tumor tissue did not lead to a significant reduction in spontaneous mammary tumor incidence, did not delay age at which tumor appeared, nor prolong survival time of the tumorous mice and survival time of mice following onset of the tumor, but led to a significant production of the consecutive tumor development. The explanation of this promoting effect may be quite complicated. One concept implies that, if an antigenic substance exists as much as in excess, it creates an "immunologic paralysis" with no effective circulating antibodies. This, indeed, has been demonstrated by tumor transplantation in somewhat incompatible hosts. Some investigators, on the other hand, postulate that the fundamental defect in cancer is a defect in the host's antibody-forming capacity. Martinez *et al.*[27] described circumstances in which mice proved immune to retransplantation of a tumor, even while lung metastases from the original tumor were growing. Hirsch *et al.*[23] explained this phenomenon on the basis of the possibility that the metastases became established at a time when no immunity was present. Therefore, the other possibility is considered that this enhancement is not due to some sort of autoimmunization against tumor development, but to additional mammary tumor agent introduced with the injections, such as milk factors and the like.

Whether different circumstances of the host exist between solitary tumor and multiple tumors is quite obscure. It would be of great interest to study this matter, comparing a host bearing a large tumor and a host bearing smaller multiple tumors.

One of the aims of the present study was to observe the host-tumor relationship in spontaneous tumor. Negative results obtained here in the pretreatment of a carcinostatic agent do not necessarily indicate that the inbred of mice are not decreased their host resistances by the administration of such drugs. It is possible that the use of another agent or another method of administration might have produced different results. Concerning the tumor preparations for immunization, the use of preparations consisting entirely of cells (X-ray or otherwise inactivated), fractions of cells, or extracts might have given dissimilar results.

The host defence mechanisms involved in these aspects remain to be determined.

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