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Reduced Antibody Titers in Mice Treated with Carcinogenic and Cancer Chemotherapeutic Agents. (19419)

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(Introduced by H. B. Andervont.)
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It has long been known that x-radiation, in addition to inhibiting(1) and initiating(2) neoplastic growth, depresses antibody levels in experimental animals(3). More recently this triad of effects has been demonstrated for nitrogen mustard(4-6). Further it has been reported that the Rous sarcoma virus in addition to initiating tumors will also depress antibody levels(7). Chemotherapeutic substances which have recently been shown to inhibit both tumor growth and antibody production are A-methopterin(8), benzene(9), cortisone (10) and 8-azaguanine(11).

In view of the foregoing observations the following work was undertaken to determine whether or not antibody inhibition is a property common to other substances involved in tumor production and inhibition.

Materials and methods. The data are based chiefly on results obtained in homozygous strain C (B alb C) mice 2 to 6 months old. However, the tests were frequently repeated in homozygous strain C3H mice also 2 to 6 months old, and the same results were obtained. The antibody forming mechanism was evaluated by determining sheep cell hemolysin titers of mouse serum harvested 5 days after the intraperitoneal injection of 0.5 cc of a 4% suspension of washed sheep cells in saline. The hemolysin titers were determined by noting the degree of hemolysis resulting from 30 minute incubation at 37°C of 0.5 cc of serial 2-fold dilutions of test sera with 1.0 cc of 1:20 guinea pig serum and 0.5 cc of 1% sheep cells. Carcinogenic compounds and their non-carcinogenic analogues from three major groups of carcinogens were studied. These included 1) methylcholanthrene, 1,2,5,6-dibenzanthracene, 1,2-benzanthracene‡ and phenanthrene,§ 2) butter yellow (*p*-dimethylaminoazobenzene) and *p*-diethylaminoazobenzene,§ 3) urethan (ethyl carbamate), isopropyl carbamate,‡ *β*-chloroethylcarbamate,§ and methyl carbamate.§‖ Test sub-

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† National Institutes of Health, Federal Security Agency.

‡ An analogue with weak carcinogenic activity.

§ Non-carcinogenic analogue.

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TABLE I. Effect of Carcinogens and Their Non-Carcinogenic Analogues Given Over a Period of 12 Days on Hemolysin Titer of Strain C and C3H Mice.

Test substance	No. of inj.	Dose, mg/kg/inj.	Hemolysis* in anti sera diluted 1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Corn oil (control)	3		0	0	0	0	0	0	4+	4+
Methylcholanthrene†	3	25	0	0	0	0	4+	4+	4+	4+
		50	0	0	4+	4+	4+	4+	4+	4+
		100	0	4+	4+	4+	4+	4+	4+	4+
1,2,5,6-dibenzanthracene†	3	50	0	0	0	0	2+	3+	4+	4+
		100	0	0	0	0	3+	4+	4+	4+
		400	0	0	2+	4+	4+	4+	4+	4+
1,2-benzanthrene†	3	100	0	0	0	0	0	3+	4+	4+
		400	0	0	4+	4+	4+	4+	4+	4+
Phenanthrene†	3	800	0	0	0	0	0	0	4+	4+
		1600	0	0	0	0	0	0	4+	4+
Butter yellow (ρ-dimethylaminoazobenzene)†	3	250	0	0	0	0	0	0	4+	4+
		500	0	0	0	2+	3+	4+	4+	4+
		1000	4+	4+	4+	4+	4+	4+	4+	4+
ρ-diethylaminoazobenzene†	3	1000	0	0	0	0	0	2+	4+	4+
		2000	0	0	0	0	0	3+	4+	4+
		4000	0	0	0	0	0	3+	4+	4+
Saline (control)	12		0	0	0	0	0	0	4+	4+
Urethan	12	100	0	0	0	0	0	4+	4+	4+
		200	0	0	0	0	4+	4+	4+	4+
		400	4+	4+	4+	4+	4+	4+	4+	4+
β-chloroethylcarbamate	12	200	0	0	0	0	0	3+	4+	4+
		400	0	0	3+	4+	4+	4+	4+	4+
Isopropyl carbamate	12	800	0	0	0	0	2+	3+	4+	4+
		1000	0	0	0	2+	3+	4+	4+	4+
Methyl carbamate	12	400	0	0	0	0	0	2+	3+	4+
		800	0	0	0	0	0	0	3+	4+

* Zero indicates no turbidity or complete hemolysis and 4+ indicates complete turbidity or no hemolysis; 1+, 2+, 3+ represent intermediate grades of turbidity.
† In corn oil.

stances from the chemotherapeutic group were 1) alpha peltatin, 2) A-methopterin, 3) benzene, 4) colchicine, 5) 2,6-diaminopurine, 6) furacin, 7) podophyllotoxin, 8) sodium arsenite, 9) triethylene melamine, and 10) urethan.¶‖

To show whether or not the effect of the compounds was related to the amount injected, each test substance was administered at several dose levels using 3 to 4 mice for each group. Particularly in the carcinogenic group the maximum tolerated dose was determined for each compound in an attempt to provide a standard by which to compare the toxicities of the carcinogens with their non-carcinogenic analogues. In all experiments control mice were treated with the appropriate vehicle. Test substances were given subcutaneously in a volume of 0.01 cc/g body weight once a day for 5 days starting the day the antigen was administered. Because of the probable slower absorption of the oil soluble carcinogens, these compounds were given in 3 injections spread evenly in a 12-day period which included the 5 days following administration of the antigen. Since urethan is classed as both a carcinogen and a chemotherapeutic agent it was tested on the 5- as well as the 12-day schedule. Because of its rapid rate of breakdown(12) urethan was given daily in all tests. To determine whether all or part of the decreased antibody titers were due to direct effects of the test substances on the antisera, the maximum daily dose of each compound was combined *in vitro* with the antisera before an hemolysin titer was performed. The possibility that the lowered hemolysin titer was due to increased

the American Cyanamid Co., Pearl River, N. Y. Furacin was kindly provided by Eaton Laboratories, Norwich, N. Y., and 2,6-diaminopurine by the Wellcome Research Laboratories, Tuckahoe, N. Y.

¶ Also carcinogenic.

TABLE II. Effect of Chemotherapeutic Agents Given Daily Over a Period of 5 Days on Hemolysin Titers in Strains C and C3H Mice.

Test substance	Dose, mg/kg/day	Hemolysis* in antisera diluted 1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Control†		0	0	0	0	0	0	3+	4+	4+
α-peltatin	1.25	0	0	0	0	2+	4+	4+	4+	4+
	2.5	0	4+	4+	4+	4+	4+	4+	4+	4+
	5	4+	4+	4+	4+	4+	4+	4+	4+	4+
A-methopterin	.18	0	0	0	0	3+	4+	4+	4+	4+
	.36	0	0	3+	4+	4+	4+	4+	4+	4+
	.75	0	3+	4+	4+	4+	4+	4+	4+	4+
	1.5	4+	4+	4+	4+	4+	4+	4+	4+	4+
Benzene	3200	0	0	0	0	0	3+	4+	4+	4+
	4800	0	0	0	0	3+	4+	4+	4+	4+
Colchicine	.3	0	0	0	0	0	0	0	3+	4+
	.6	0	0	0	0	2+	4+	4+	4+	4+
	1.2	4+	4+	4+	4+	4+	4+	4+	4+	4+
2,6-diamino-purine	25	0	0	0	0	0	2+	4+	4+	4+
	50	0	0	0	0	2+	4+	4+	4+	4+
	100	0	2+	4+	4+	4+	4+	4+	4+	4+
Furacin	500	0	0	0	0	0	0	0	3+	4+
	1000	0	0	0	0	0	0	0	3+	4+
Podophyllo-toxin	1.25	0	0	0	0	4+	4+	4+	4+	4+
	2.5	4+	4+	4+	4+	4+	4+	4+	4+	4+
Sodium arsenite	2.5	0	0	0	0	0	3+	4+	4+	4+
	5	0	0	0	0	3+	4+	4+	4+	4+
	10	0	0	0	3+	4+	4+	4+	4+	4+
Triethylene melamine	.25	0	0	0	2+	3+	4+	4+	4+	4+
	.5	0	3+	4+	4+	4+	4+	4+	4+	4+
Urethan	250	0	0	0	0	0	0	0	0	3+
	500	0	0	0	0	0	3+	4+	4+	4+
	1000	0	0	0	0	3+	4+	4+	4+	4+

* Zero indicates no turbidity or complete hemolysis and 4+ indicates complete turbidity or no hemolysis; 1+, 2+, 3+ represent intermediate grades of turbidity.

† Results obtained with the separate vehicles are indicated by one control group because the titers obtained were the same for all.

destruction of the antibody by the compound *in vivo* was evaluated by administering the test material once a day for 5 days to animals which were injected intraperitoneally on the third day of treatment with 0.5 cc of a mouse anti-sheep red cell serum previously prepared in the same strain of mice. Forty-eight hours later the sera were harvested by decapitation and hemolysin titers performed.

Results and discussion. As shown in Tables I and II, treatment with both the carcinogens and the chemotherapeutic agents, with the exception of furacin, resulted in a significant decrease in hemolysin titers. The degree of inhibition varied from one compound to another but a direct relationship between the amount of any one compound administered and the inhibition it produced was apparent. These results suggest that antibody inhibition may be a property common to substances associated with the initiation and the inhibition of neoplasms. The fact that the non-carcinogenic analogues had little or no effect on the hemolysin titers when administered in quantities several times greater than the corresponding carcinogens brings out a parallelism between the chemical specificity of antibody inhibition and carcinogenic effect.

Although urethan reacted as did all the other substances in the carcinogenic group, its dual role as a carcinogen and a chemotherapeutic agent and the varied biological activity of its analogues makes a more detailed observation interesting. When contrasted with methyl carbamate, which is neither carcinogenic nor chemotherapeutic, the antibody inhibiting ability of urethan and the lack of effect of methyl carbamate follows the pattern

TABLE III. Example* of *in vivo* and *in vitro* Effect of Carcinogenic and Chemotherapeutic Agents on Hemolysin Titers.

Type of test	Sera	Hemolysis† by dilutions of antisera							
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
In vivo	Urethan treated	0	0	2+	4+	4+	4+	4+	4+
	Control	0	0	2+	4+	4+	4+	4+	4+
In vitro	Urethan treated	0	0	0	0	0	0	4+	4+
	Control	0	0	0	0	0	0	4+	4+

* Since none of the compounds tested effected the hemolysin titers *in vivo* or *in vitro* the results obtained with urethan are presented as typical.

† Zero indicates no turbidity or complete hemolysis and 4+ indicates complete turbidity or no hemolysis; 1+, 2+, 3+ representing intermediate grades of turbidity.

of results obtained with other compounds. However, isopropyl carbamate, which has been shown to have some carcinogenic activity(13), and β-chloroethyl carbamate, which interferes with mitosis in a manner and degree similar to urethan(14), both reduced the hemolysin titers although to a lesser degree than the corresponding dose of urethan.

No data are available to explain the inability of furacin to inhibit the hemolysin titer. It might be conjectured that its mechanism of action is different from that of the other compounds or, although it seems unlikely, the ability of all the other agents to reduce hemolysin titers is an unusual coincidence.

The failure of the test substances to inhibit passive antibodies *in vivo* (Table III) suggests that all these compounds act at the site of antibody production. This does not, however, imply that they all achieve their inhibition in the same manner. Table III also indicates that antibodies were not affected *in vitro* by any of the test substances. The observed depression of the antibody titer was, therefore, not a result of interference with the hemolytic test system.

No attempt was made in this work to clarify the way in which these compounds act. However, the question of toxicity was explored partly to establish a standard by which to measure test substances and also to determine whether or not toxicity *per se* was responsible for antibody depression. With this in mind, sodium cyanide, which is not associated with malignancy but which is highly toxic, was given in the maximum tolerated dose of 4 mg/kg body wt/day. At this level sodium cyanide did not lower the hemolysin titer.

A further comparison was made with methylcholanthrene, which was found to have 4 times the lethal effect of its analogue phenanthrene. Four hundred mg/kg body weight given 3 times in a 12-day period was found to be the maximum tolerated dose for methylcholanthrene and 1600 mg/kg body weight given 3 times in a 12-day period the maximum tolerated dose for phenanthrene. As shown in Table I, however, 3 injections of 100 mg/kg body weight methylcholanthrene which is ¼ the toxic dose produced marked antibody inhibition while 3 injections of 1600 mg/kg body weight phenanthrene which equals the maximum tolerated dose of this substance produced no inhibition. On the basis of these findings it would not appear that general systemic toxicity is responsible for the decrease in hemolysin titer.

The marked lowering of the antibody levels by the carcinogens may or may not be related to their carcinogenic activity. However, this reduction of hemolysin titers does suggest that in addition to a very gradual, localized change, thought to be associated with their tumor inducing ability, these compounds also produce a rather sudden systemic alteration which is reflected by the antibody producing system.

Summary. A number of cancer chemotherapeutic agents, carcinogens and their non-carcinogenic analogues were tested for their effect on sheep cell hemolysin production in mice. All of the chemotherapeutic agents, with the exception of furacin, depressed the antibody titers. All of the carcinogens also produced a marked depression of the antibody levels but their non-carcinogenic analogues were without effect. None of the substances

tested inhibited preformed antibodies *in vivo* or *in vitro*.

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Pancreatic Disease in Mothers of Suckling Mice Infected with Connecticut No. 5 Strain of Coxsackie Virus.* (19420)

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It has been the practice in this laboratory, when infecting suckling mice with one or another strain of Coxsackie virus, to discard the mother at the completion of the experiment. But when it was shown that adult mice are susceptible to infection with the Connecticut No. 5 strain(1), it was decided to keep some of the mothers under observation in order to determine whether or not they might become spontaneously infected.

Thus, the mothers of 19 litters were examined microscopically at various intervals after infection of their young with Conn.-5 virus. Seven of these showed histologic evidence of pancreatic disease, which appears to be the principal pathologic effect of infection of adult mice with the Conn.-5 strain. Microscopic examination of the pancreas and liver of mothers naturally infected through their offspring reveals the same pathologic lesions which are characteristic of pancreatic disease produced experimentally in adult mice(1). Fig. 1 is a photomicrograph of a section of pancreas of a mother mouse which was found dead 9 days after inoculation of her young and 6 days after she had ingested 2 of them. There is a massive early necrosis of the entire pancreas. There was also severe hepatitis, with hyaline necrosis of many liver cells, and fatty infiltration of others, and many phospholipid containing cells in the sinusoids. In general, the character of the pancreatic lesions coincided fairly well with the period elapsing between the presumed ingestion of the infected young and the day of death or sacrifice. Thus there was massive necrosis in those mice surviving only 6 days, whereas those which lived 10 to 12 days presented subacute lesions (Fig. 2). At this time the necrotic acinar cells have been removed and the remaining pancreas consists almost entirely of islands and ducts embedded in loose areolar or fatty tissue thickly infiltrated with histiocytes. Hepatitis of varying degrees of severity was found in some animals. Fig. 3 shows extreme fatty infiltration of the liver, with necrosis of individual liver cells.

The more obvious methods by which the mothers might acquire this viral infection from their offspring are: 1) by ingestion of the excreta of the sucklings, 2) by eating the sick or dead mice in their litters, and 3) by inhalation of infectious materials. In addition to the infected baby mouse carcass, both urine

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