Acceptance of Tumor Homografts by Mice Injected with Antiserum. I. Activity of Serum Fractions.*† (22185)

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With certain combinations of transplantable tumors and inbred strains of mice, a normally resistant host can be induced to accept a tumor homograft if it is pretreated with preparations of mouse tissues prior to tumor grafting(1-3). Tumor homografts will also survive in mice injected with *antiserums* to mouse tissues, produced in either rabbits or mice(4-6).

Since the effect of these antiserums is opposite to that expected from a passive transfer of antibody, namely, *inhibition* of tumor growth, the study to be reported in the present paper was conducted to determine whether the serums had the chemical characteristics usually associated with antibodies, or whether they resembled the active substances in the tissues used to induce homograft acceptance. To this end, the serums were subjected to salt fractionation or zone electrophoresis, and the various fractions were tested for their ability to effect the survival of tumor homografts. The effects of heat and of sodium periodate on the activity of the serums were also determined, since it had been shown(2,3,7) that such treatments will destroy the ability of tissue preparations to abrogate host resistance.

Materials and methods. Antiserums were produced in rabbits or in C57BL/6Ks (C57 Black) mice. The rabbits were immunized with saline homogenates of freshly secured spleens from strain A mice. The first injection, which was made subcutaneously, consisted of 250 mg of spleen in 1 ml of Freund's adjuvant, containing 3.3 mg of heat-killed BCG. One week later, a series of intraperitoneal injections with saline homogenates of spleen (100 mg/ml) was started. Injections were given every third day, for a total of 5 injections. The amounts per injection were, respectively: 1.0, 1.5, 2.0, 2.0, and 2.5 ml. Bleeding for serum was done from the ear artery, by a method described by Cohen(8). The serums were stored in the deep-freeze, until needed. These serums, and those produced in mice (described below), exhibited hemagglutinating activity when tested with red blood cells of strain A mice.

Mice were immunized with an extract of a transplantable tumor (Sarcoma I), indigenous to strain A mice. The extract was prepared by homogenizing previously frozen tumor with an approximately equal weight of sterile 0.85% NaCl in a high speed blendor. The homogenate was centrifuged at 8500 x g for one-half hour, at room temperature. The supernatant was then diluted 1:10 with sterile 0.85% NaCl, and the diluted material was used for immunization. The diluted supernatant was stored at −26°C. The mice received a series of 5 intraperitoneal injections, 0.5 ml per injection, over a period of 2½ weeks. Twelve days after the last injection, the mice were bled by incision of the lateral tail vein and the serum was separated from the blood clot. In addition to these methods, the antiserum used for zone electrophoresis was produced in mice (C57BL/6Ks strain) inoculated subcutaneously with a tumor homograft (Sarcoma I). The mice were bled from the lateral tail vein 10 days after inoculation. The serum was separated from the clotted blood, freeze-dried in ampoules, and sealed *in vacuo*. Zone electrophoresis was carried out for us by Dr. Peter Bernfeld,‡ by a method described by Bernfeld and Nisselbaum(9). Five globulin fractions, thus prepared and freeze-dried, were reconstituted by

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TABLE I. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Antiserum Fractions Precipitated by Ammonium Sulphate.

Fraction injected		Amt inj. per mouse	No. of mice dying with tumors*	
$(NH_4)_2SO_4$ conc., %	Material ppt.			
Antiserums produced in rabbits				
50–75	Albumin	25 mg	3/5	
"	"	15	0/5	
"	"	5	0/5	
33–50	α, β-globulin	5	5/5	
0–33	γ -globulin	5	5/5	
"	"	3	4/5	
"	"	1	5/5	
—	Unfractionated serum	.5 ml	6/6	
Antiserums produced in C57BL/6Ks mice			Exp. 1	Exp. 2
50–75	Albumin	10 mg	0/5	1/ 5
33–50	α, β-globulin	5	4/5	5/ 5
"	"	2	—	5/ 5
"	"	1	1/5	—
"	"	.5	—	4/ 5
0–33	γ-globulin	5	5/5	—
"	"	2	4/5	10/10
"	"	.5	1/5	3/ 5
—	Unfractionated serum	1 ml	15/15	

* Numerators give the No. of mice dying with tumors; denominators, the total No. of mice in each group.

us with sterile 0.85% NaCl and dialyzed against 0.85% NaCl in autoclaved Visking cellulose sacks for 18 hours, at 4°C, to remove excess salt (since the fractions had been freeze-dried from a large volume of solution). The contents of the sacks were removed and diluted with sterile 0.85% NaCl to the concentration desired for injection into the mice. Salt fractionation of the serum proteins was carried out with a saturated solution of ammonium sulfate which was added dropwise to 20 to 30 ml of serum, maintained at a low temperature in an ice bath. The proteins precipitating in the salt concentrations of 0-33, 33-50, and 50-75% saturation respectively, were collected. Each fraction was then dissolved in distilled water, reprecipitated at least twice more, dialyzed against distilled water in a refrigerator, and freeze-dried. For injection, the freeze-dried material was reconstituted with sterile 0.85% NaCl to the desired concentrations. Treatment with sodium periodate was carried out on the globulin fractions of an antiserum that had been produced in mice. The globulins had been prepared by salt fractionation, as described above. The globulins, in a final concentration of 3.9 mg/ml, were mixed with $NaIO_4$ dissolved in 0.85% NaCl, and buffered with phosphate to pH 7.0. The final concentration of $NaIO_4$ was either 0.01 M, or 0.001 M, and the final concentration of buffer was 0.03 M. The mixtures were kept for one hour at a temperature of approximateely 21°C. An equal volume of glucose solution (10 mg/ml) was then added to inactivate the remaining $NaIO_4$. The measure of biological activity of treated serums and serum fractions was their ability to effect survival of tumor homografts. The various preparations were injected intraperitoneally into the prospective hosts several hours before grafting of the tumor. The transplantable mouse tumor used as the test homograft was the strain A tumor Sarcoma I (SaI). This tumor grows in 100% of A/Ks mice, killing the animals within 3 to 5 weeks after inoculation. Hosts were male mice (except for one experiment in which females were used to test heated serums) of the C57BL/6Ks strain, which is genetically unrelated to the A strain. SaI rarely grows in an untreated C57BL/6Ks mouse. All mice were 2 to 4 months old at the start of the experiments. The grafts were placed subcutaneously, by trocar, in the suprascapular region. Growth of the grafts was followed by means of per-

TABLE II. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Antiserum Fractions Prepared by Zone Electrophoresis.*

Material inj., 3 mg/mouse		No. of mice dying with tumors
α-globulin	Fraction II	0/5
Intermediate fraction	III	"
β-globulins	IV	"
Main β-globulin	V	1/5
γ-globulin	VI	5/5

* Antiserum prepared in C57BL/6Ks mice.

iodic palpation, until the mice either died with a progressively growing tumor, or remained without a sign of growth for at least 2 months, at which time they were killed and classified as "negative".

Results. The 2 serum fractions precipitated by 0-33% saturated $(NH_4)_2SO_4$ (mainly gamma-globulin) and 33-50% saturated $(NH_4)_2SO_4$ (mainly alpha and beta-globulin) appeared to be equally active (Table I). The fraction precipitated by 50-75% saturated $(NH_4)_2SO_4$ (mainly albumin) was almost completely inactive. That the activity of the globulin fractions was not due to some possible alteration in the proteins during salt fractionation, was demonstrated by the lack of activity of fractions similarly obtained from normal serum, derived from C57BL/6Ks mice.

The fractions obtained by zone electrophoresis can be assumed to have a degree of purity which cannot be achieved by salt fractionation. Fraction VI (practically pure gamma-globulin) was fully active, while a slight activity was displayed by the neighboring fraction V (the main beta-globulin) (Table II). Fractions II, III, and IV, which respectively are alpha-globulin, an intermediate fraction between alpha and beta-globulin, and lastly mostly beta-globulin (actually 2 beta-globulins), did not effect homograft survival. One point of interest is the observation that the homografts in the mice that had received fraction VI showed a marked initial growth (as compared with uninjected controls or with the animals receiving the other fractions). This was followed by a marked regression and then a resumption of growth, with eventual death of the host. The possible significance of this is considered below in the discussion.

The results obtained with the heated serums (Table III) show that the antiserums produced in both rabbits and mice are fully stable when heated to 56°C for one hour. Whole rabbit antiserum heated to 70°C for one hour retained its activity, though there was a slight impairment, as shown by a slower initial growth rate of the tumors. In the case of the antiserums produced in mice, heating to 70°C inactivated the whole serum to a large extent, though not completely, but did not impair the activity of the globulin fractions (produced by salt fractionation).

Sodium periodate did not markedly alter the effectiveness of the antiserum (Table III). There was a slight indication of impairment at the higher concentration (0.01 M $NaIO_4$), in terms of slower growth of the homografts in the mice receiving the serum treated at this level of sodium periodate. This, however, is in contrast to the complete destruction of the activity of tissue treated with $NaIO_4$(7). Though there was a lower number of "takes" in the mice receiving the dextrose-serum mixture, this is not considered significant, since only 4 of the 5 mice which received untreated

TABLE III. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Heated Antiserum, or Antiserum Treated with Sodium Periodate.

Material inj. and treatment		Amt inj. per mouse	No. fatal /No. inj.†
Antiserum produced in rabbits			
Whole serum,	100°C/20 min.	1 ml	0/ 5‡
	70 / 1 hr	1	5/ 5‡
	70 / 1	.5	4/ 5
	56 / 1	1	14/14§
Antiserum produced in C57BL/6Ks mice			
α, β-globulins,*	70°C/1 hr	2 mg	3/ 5
γ -globulin,*	70 /1	2	5/ 5
Whole serum,	100 /1	1 ml	0/ 5
	70 /1	1	2/ 5
	56 /1	1	5/ 5
	.01 M $NaIO_4$	3.9 mg	4/ 5
	.001M $NaIO_4$	3.9	5/ 5
	Dextrose	3.9	2/ 5
	Untreated	1.0 ml	4/ 5
	Dextrose	1.0	15/15

* Prepared by fractionation with ammonium sulphate.
† Males used throughout, except as indicated.
‡ Females.
§ 9 females, 5 males.

serum, in this experiment, grew the homografts. Furthermore, in another experiment, in which dextrose was added to the serum in even larger quantity, the effectiveness of the serum was not diminished.

Discussion. These data, together with others to be published later, indicate that the substance in the antiserums which is responsible for the survival of the tumor homografts was not introduced by the injection of the tissue extracts used for immunization, but is an antibody component (or components). This conclusion is supported by the observation that the antiserums, unlike tissue preparations, were not inactivated by sodium periodate, and that tissue preparations are labile at temperatures above 60°C(2,3), while the serums are comparatively stable at 70°C. The partial loss of activity of the whole serums heated to 70°C, as compared with the full retention of activity of the globulin fractions similarly treated, is in accord with previously reported observations(10) that isolated protein fractions are more stable to heat than the unisolated proteins in whole serum.

Although the results obtained with the fractions produced by zone electrophoresis would indicate that the activity is most likely associated with the gamma-globulin portion, it is possible that at higher dosage levels than those we used, activity might have been demonstrated by some of the fractions other than fraction VI (gamma-globulin). The limitation on the dosages used was determined by the amount of material on hand, and the inaccessibility of the equipment used for fractionating the serums. The possible importance of dosage is emphasized by the comparatively slow rate of growth of the homografts in the mice pretreated with fraction VI. The lower activity of this material, as compared with the gamma-globulins produced by salt fractionation, may be attributed to a lower potency of the antiserum used for fractionation. This antiserum had been taken from mice bled 10 days after inoculation with the immunizing tumor, and it is known (data to be published) that the peak potency of the antiserums is first reached at between 14 to 21 days.

Summary. Antiserums to mouse tissues, produced in rabbits or mice, induce acceptance of an otherwise incompatible tumor homograft in mice, when injected into the host prior to grafting of the tumor. Activity of antiserums was associated with the globulin fractions, and was most concentrated in the gamma-globulin fraction. The activity was not impaired by treatment with sodium periodate, or by heating to 56°C. It was slightly impaired by heating to 70°C, and was destroyed at 100°C.

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