Tumor Neutralization by Anti Ehrlich Tumor and Anti Mouse Tissue Sera.* (27909)

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The production of tumor neutralizing antibodies in rabbits following injection with tumor cell preparations has been noted by numerous investigators(1,2,3,4,5). Whether the neutralizing ability results from stimulation of the antibody forming cells by a specific tumor antigen or by normal tissue antigens is still unclear. Results following absorption of antisera with normal tissues have been equivocal(6,7), possibly due to a tumor antibody which differs to such a small degree from normal tissue antibody that it is absorbed along with normal tissue antibody by normal tissue antigens. Therefore, it was decided to compare the Ehrlich tumor neutralizing power of sera from rabbits injected with normal mouse tissues with that of sera from animals injected with Ehrlich ascites cells. If there is a specific tumor antigen, then antisera from rabbits exposed to this antigen should neutralize tumor cells while antisera from rabbits injected with normal tissue antigens should have very little, if any, tumor neutralizing ability.

Materials and methods. Mouse tissue antigen. Lung, spleen, liver and kidney were removed from Swiss mice and with no attempt to remove the blood from the organs. Each tissue was weighed and an amount pressed through electromesh screen† to give a suspension equivalent to 2.5% cells by volume. Alsever's solution was used to wash the cells through the screen. The cell suspensions from each tissue were combined to make a final suspension of 10% by volume. Microscopic examination of the screened suspensions showed many intact cells. Antigen was used immediately after preparation. *Ehrlich tumor cell antigen.* Antigen was prepared on the day to be used by removing ascites tumor which had been growing for 7 days in Swiss mice and repeatedly washing the cells with 0.9% sodium chloride solution containing 0.1% gelatin. A large number of the erythrocytes in the ascitic fluid were removed by this procedure and the packed Ehrlich cells were resuspended in saline to a concentration of 10% by volume. Heparin (8) was used as an adjuvant and added in a final concentration of 5 mg/ml. Both mouse tissue and tumor antigens were filtered through nylon mesh immediately before use.

Immunization schedule. Three strains of rabbits were used for immunization; 40 New Zealand, 20 Ginger Sandy Giants and 20 Dutch Pee Wee rabbits were injected with Ehrlich ascites antigen while 20 New Zealand and 20 Dutch rabbits were inoculated with normal mouse tissue antigen. Both sexes were represented in each strain and all animals were between 11 and 12 weeks old. Pre-immunization blood was obtained from each rabbit by cardiac puncture, and after centrifugation the sera were stored at –38°C. Within 1 to 2 hours after bleeding, the rabbits were injected intravenously with 1.0 ml of 10% antigen. Dutch rabbits were given 2.5% cell suspensions since their average weight was ¼ that of the other strains. Heparin was not mixed with the normal mouse antigen but was given to each animal immediately before the antigen injection. Seven days after the "normal bleeding" and the first injection of antigen, 30 ml of blood were removed from each of the large strains and 10 ml from the small strain of rabbits. The serum from each rabbit was labeled with the animal's number and "A" to indicate the first stage of immunization. "A" sera as well as succeeding sera were stored at –38°C. Within 2 hours after bleeding 0.5 ml of antigen was injected subcutaneously, followed 4 hours later by 0.5 ml intravenously. Thus the total amount of antigen inoculated re-

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† Lectromesh .015 hole size C. O. Jelliff Mfg. Corp., Southport, Conn.

TABLE I. Immunization Results as Shown by Neutralization Tests (Serum Dilutions Ranging from 1:160 Through 1:640).

Serum	No. of mice with tumor/ No. of mice inoculated			% protection		
	New Zealand	Ginger	Dutch	New Zealand	Ginger	Dutch
Anti Ehrlich						
N	190/192	87/87	82/83	1	0	2
A	541/584	184/249	227/249	8	27	9
B	392/573	234/262	194/223	32	11	13
C	293/533	112/231	41/223	46	52	82
D	362/434	136/153	127/141	17	6	15
Anti normal tissue						
N	99/99		92/92	0		0
A	244/283		251/282	14		11
B	259/293		232/278	12		17
C	237/282		204/246	16		18
D	253/266		200/208	5		4
Tumor cell controls		146/155 or 6 % takes				

mained the same but the danger of deaths due to anaphylaxis was lessened. This procedure of bleeding and injecting was continued at weekly intervals through 2 more bleedings, "B" and "C", after which injections at 7-day intervals were continued until the animals had received a total of 9.0 ml of antigen. One week after the ninth injection the animals were bled and the sera labeled "D". *Neutralization test.* All sera were inactivated at 56°C for 30 minutes before diluting and testing. Ehrlich ascites cells were removed from mice which had been inoculated intraperitoneally 7 days earlier and were washed with saline to remove erythrocytes. The packed cells were resuspended in normal guinea pig serum(9) to a concentration of 2% by volume. Equal amounts of pre-immunization serum and 2% cells were mixed and incubated at room temperature for 30 minutes before injecting each of 5 (12-14 g) mice subcutaneously with 0.2 ml. The post-injection sera which had been stored at −38°C for 7 days were diluted 1:80, 1:160, and 1:320 before mixing with the cell suspension and incubating as before. Five mice were injected with 0.2 ml of each dilution, thus making a total of 15 mice for each serum of each bleeding. Tumor cell controls were run for every protection test by injecting 5 mice with 0.2 ml of 1% cells suspended in guinea pig serum. Furthermore, the viability of the cells was determined by injecting the control mice with the same cell suspension, after the injection of the test mice. Cell counts of each cell control suspension were made in a hemocytometer and the number of tumor cells varied between 7.3 $\times 10^5$ and 1.3 $\times 10^6$. Results were recorded as $\frac{\text{number tumor takes}}{\text{number animals injected}}$. Neutralization was evaluated on the basis of total number of tumors appearing in a 14-day period after injection.

Results. In Table I are recorded the ratios between total number of mice developing tumors and total number of mice injected with tumor cells treated with 3 different dilutions of serum from each bleeding. This accumulation of the results obtained with the 3 dilutions of sera gives a broad estimate of the antitumor properties of the sera collected from all of the rabbits of a given strain and injected with either tumor antigen or mouse tissue antigen, at a particular period in the immunization procedure. The data show 3 significant trends: 1) The peak of tumor neutralizing antibody is reached after 3 injections of antigen (C serum) followed by a decided drop in the activity of sera after 9 injections (D serum). 2) Dutch Pee Wee rabbits produce a much greater amount of tumor neutralizing antibody than either New Zealand or Ginger Giant rabbits. 3) Both strains of rabbits injected with normal mouse tissue produce a small amount of tumor neu-

TABLE II. Per Cent Rabbits Whose Sera Showed 80-100 % Protection* at Dilutions of 1:160, 1:320, or 1:640.

		% rabbit sera showing 80% protection									
		New Zealand				Ginger				Pee Wee Dutch	
		Serum dilution				Serum dilution				Serum dilution	
Antigen	Serum	160	320	640		160	320	640		160	320	640
Tumor cells	N (40)†	0	0	0	(18)†	0	0	0	(18)†	0	0	0
	A (40)	0	0	0	(18)	11	5	0	(18)	0	0	0
	B (39)	28	0	0	(18)	11	0	0	(16)	0	0	0
	C (35)	37	20	5	(16)	81	12	5	(15)	100	86	40
	D (35)	—	2	0	(16)	—	0	0	(15)	—	0	0
Normal tissue cells	N (20)	0	0	0					(19)	0	0	0
	A (20)	0	0	0					(19)	0	0	0
	B (20)	5	0	0					(19)	0	0	0
	C (19)	0	5	0					(16)	0	0	0
	D (18)	0	0	0					(14)	0	0	0

* 0-1 mice with tumors/5 mice inoculated.
† No. of rabbits from which sera were obtained shown in parenthesis.

tralizing antibody after 1 injection of antigen and the amount does not increase significantly after 2 additional injections. However, in no case does the amount of antibody approximate the quantity which is produced by the animals injected with tumor cells. When sera from individual rabbits are examined for strong tumor neutralizing ability *i.e.*, 80% or greater at any given dilution of serum (Table II) the differences in neutralizing ability of sera from rabbits injected with tumor cells and those from animals injected with normal mouse tissue become more apparent. It is especially evident in the case of the Dutch rabbits where 100% of the animals produced sera which showed this high tumor neutralizing ability at a dilution of 1:160 and 40% of the animals at a dilution of 1:640. However, none of the animals injected with normal mouse tissue produced serum which showed this degree of protection at any of the 3 dilutions.

Summary. Sera produced by rabbits injected with Ehrlich ascites cells had 3 to 4 times the tumor neutralizing power of sera from rabbits injected with normal mouse tissues. The peak of tumor neutralizing titers was reached after 3 injections of antigen and dropped significantly following 9 injections with tumor cells. Dutch Pee Wee rabbits produced higher titer antibody than did either New Zealand or Ginger Giant rabbits.

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1. Ishikura, H., Itakura, S., Usubuchi, I., Suzuki, K., Aizawa, M., Imamura, T., Mori, S., *Gann,* 1950, v41, 177.
2. Nungester, W. J., Fisher, Helen, *Cancer Res.,* 1954, v14, 284.
3. Wissler, R. W., Barker, P. A., Flax, M. H., LaVia, M. F., Talmage, D. W., *ibid.,* 1956, v16, 761.
4. Horn, Edward C., *ibid.,* 1956, v16, 595.
5. Easty, G. C., Ambrose, E. J., *Brit. J. Cancer,* 1957, v11, 287.
6. Gorer, P. A., *J. Path. Bact.,* 1942, v54, 51.
7. Fuson, R. B., Rambo, O. N., *Transplant. Bull.,* 1958, v5, 388.
8. Knisely, M. H., Bloch, E. H., Brooks, F., Warner, L., *Am. J. Med. Sci.,* 1950, v219, 249.
9. Kalfayan, B., Kidd, J. G., *J. Exp. Med.,* 1953, v97, 145.

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