Immunological Homograft Enhancement. Interactions of Antiserum and Skin and Tumor Homografts. (29805)

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The phenomenon of immunological homograft enhancement consists of a prolongation of the survival of a homograft as a result of pre-treatment of the host with donor tissue or with an antiserum against donor tissue, and has been the subject of much recent investigation(1-6). It can be demonstrated most readily when a tumor is used as the homograft but can also be elicited in the case of homografts of normal tissue, such as skin (7,8).

Hypotheses concerning the mechanism of enhancement have postulated either an alteration of graft cells by the antibodies or an interference with the host immune response. The latter might occur at the "afferent" level, at which antigen released from the graft might be prevented from adequately sensitizing the host by virtue of its prior union with antibodies; at the "central" level, at which antibodies or antigen-antibody complexes(9,10) might depress the host's specific immune response; or at the "efferent" level, where antibodies might protect the graft from cellular or other destructive aspects of the immune response.

It seemed possible that important clues as to the nature of the phenomenon might be obtained if enhancement were attempted in an animal bearing both a skin and a tumor homograft from the same donor strain. The present report describes results of such experiments and of attempts to analyze the phenomena observed.

Materials and methods. The C3H mouse sarcoma B.P.8, which can be either enhanced or inhibited by antiserum(11), was kindly supplied in the ascites form by Dr. J. R. Batchelor. All mice used were obtained from Jackson Memorial Laboratories, Bar Harbor, Maine. C3H/HeJ (H-2k) male mice were used as skin donors and for weekly tumor passage. Recipients in all experiments were adult male B6D2F_1J mice, the F_1 hybrid of a C57/B1/6J × DBA/2J cross (H-2b × H-2d).

Pools of antiserum were produced by the inoculation into the foot pads of B6D2F_1J males 0.05 ml of a 1:1 emulsion in Freund's adjuvant of a 20% suspension of C3H/HeJ liver and spleen cells (10:1 by weight, dissociated by repeated passage through a syringe and needle) in Mixture 199†, and by subcutaneous inoculation of B.P.8 ascites tumor cells in each flank. Further foot pad injections of liver and spleen suspensions, and subcutaneous and intraperitoneal inoculations of liver, spleen and B.P.8 cells were given as boosters at 2, 5, 7, and 11 weeks after the initial antigenic stimuli. The mice were bled from the retro-orbital venous plexus from 9 to 20 days after each of the last 3 booster injections. After clot retraction, serum was pipetted off and pooled, cells were removed by centrifugation, and the sera were frozen and stored at —20°C until use.

Skin grafts of 2 cm × 1.5 cm were used in the inactive phase of the hair cycle, with subcutaneous fat, fascia, and vascular tissue removed. Grafts were sutured over a bed in the dorsal midline without bandaging and were observed daily, rejection being recorded when the entire graft appeared hard and dry or crusted. Test tumors for enhancement studies were produced by injection of 1.0 × 10^6 B.P.8 ascites tumor cells diluted in heparinized saline (3 units/ml) given intradermally in a volume of 0.05 ml in the flank of B6D2F_1 males. Antiserum was injected intraperitoneally.

Results. Simultaneous administration of antiserum and homografts. To explore the effects of giving antiserum, tumor, and skin grafts on the same day, 15 B6D2F_1J males received 1.0 ml of antiserum. Five of these at once received intradermal injections of B.P.8 and were grafted with C3H/HeJ skin,

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TABLE I. Tumor Growth in Mice Receiving Tumor Grafts, Skin Grafts, and Antiserum on Same Day.

Animal No.	Antiserum given	Skin graft applied	Tumor size* (days after tumor inoculation) 6	9	13	17	22	28	41	71	Enhancement†
1			12	32	60	24	0	0	0	0	None
2			16	34	63	140	180	276	large		Present
3	Yes	Yes	9	20	42	56	42	30	30	165	"
4			9	9	16	25	36	25	30	120	"
5			0	6	9	12	16	30	35	324	"
6			16	20	49	64	100	died			"
7			0	9	12	12	0	0	0	0	None
8	"	No	16	25	42	24	6	0	0	0	"
9			9	9	16	76	221	large			Present
10			18	24	51	80	130	"			"
11			16	16	16	4	0	0	0	0	None
12	No	Yes	4	4	0	0	0	0	0	0	"
13			12	28	15	6	0	0	0	0	"
14			16	16	7	0	0	0	0	0	"
15	"	No	9	30	4	0	0	0	0	0	"
16			9	9	0	0	0	0	0	0	"

* Tumor size recorded as the product of the greatest diameter (in mm) and that diameter at right angles to it.

† Enhancement was considered to be present in cases in which the tumor graft grew progressively throughout the period of observation.

another group of 5 received only B.P.8, and still another group of 5 received only skin grafts. Three mice received no antiserum, but were given skin and B.P.8 grafts, 3 received only B.P.8, and 3 were given only skin grafts.

Skin grafts on all of these animals were rejected between the 9th and 13th day, with no evidence of accelerated rejection or enhanced survival. Tumor growth is recorded in Table I, and it can be seen that enhanced tumor growth occurred in those groups receiving antiserum. It is clear from this experiment that enhancement of a tumor graft by antiserum injection can be achieved without demonstrable enhancement of survival of a concomitant skin graft from the same donor strain.

Effects of prior or repeated injections of antiserum. To evaluate the effect of varying the dosage and timing of antiserum administration, the following experiments were performed. Twelve B6D2F_1J males were inoculated with antiserum at various time intervals. All received either a skin graft, an inoculation of B.P.8, or both 2 days after the first serum injection. Seven control animals received no antiserum. The treatments given and the tumor growth observed are listed in Table II. All skin grafts except that on animal #15 were rejected between the 10th and 14th day after grafting. Mouse #15, which received 13 ml of antiserum over a period of 28 days, exhibited a delayed and chronic rejection of its graft. Beginning on the 17th postoperative day, there was no drying or sloughing but hair was gradually lost from the graft, which then gradually fibrosed and shrank between the 20th and 27th days after its application, until it was finally obliterated. Enhanced tumor growth occurred in those groups of mice receiving antiserum. It should be noted that those animals which did not receive antiserum showed a greater initial tumor growth than those which did.

Abrogation of enhancement by prior application of skin homograft. To investigate the effect of a skin homograft on the immunological enhancement of growth of a tumor inoculated earlier or later, the following studies were performed. Six groups of 4 B6D2F_1J males each received skin grafts on different days. All of these and 8 other mice received both an inoculation of B.P.8 and 1.0 of antiserum on the same day. Another group of 3 mice received only B.P.8 on that day. Tumor growth is recorded in Table III.

Skin grafts applied 10 or 15 days before the tumor and antiserum were given apparently effectively immunized the recepients, and usually prevented the enhancing effect of antiserum, observed in groups C-G. As in Table II, it can be seen that the initial growth of tumors was smaller in the case of

TABLE II. Effect of Dosage and Duration of Antiserum Treatment on Survival of Tumor and Skin Homografts.

Animal No.	Ml of serum per day	Day(s) of serum injection	Total Ml of serum	Type of graft	Tumor size (days after tumor inoculation) 4	7	11	15	20	26	29	Enhancement
	1.0	−2,0	2.0	Skin								
1		Two days before		and	9	28	35	96	190	large		Present
2		grafts and at		B.P.8	9	9	30	49	90	144	large	"
3		time of grafting			0	4	4	4	4	0	0	None
	1.0	−2,0	2.0	B.P.8								
4		Two days before			9	16	36	90	196	large		Present
5		grafts and at			9	31	54	117	360	large		"
6		time of grafting			9	36	49	99		large		"
	1.0	−2	1.0	B.P.8								
7		Two days			4	4	13	49	184	large		"
8		before			0	8	60	140	196	large		"
9		grafts			6	9	25	49	100	144	large	None
	none	none	none	B.P.8								
10					16	16	12	0	0	0	0	None
11					20	63	42	16	0	0	0	"
12					16	33	20	0	0	0	0	"
	1.0	−2,−1,0,4,5,7	6.0	Skin								
13, 14		days after grafting						(None)				"
	1.0	−2,−1,0,4,5,6,8,11,	13.0	"								
15		15,18,20,22,25 days after grafting						"				Present
	none	none	none	"								
16, 17								"				None
18, 19												

Tumor size recorded as on Table I. Skin grafted and tumor inoculated on day 0.

those mice that received antiserum than in those that did not, although it was the former that eventually developed larger tumors. One animal (#1), which had received a skin graft 15 days before the antiserum and tumor injections, developed a progressively growing tumor that was first observed 18 days after tumor inoculation.

Discussion. These experiments demonstrated that antiserum in levels sufficient to enhance the growth of the C3H tumor B.P.8 was insufficient demonstrably to affect C3H skin graft survival time in the same B6D2F$_1$ host, and further showed that a simultaneously applied skin graft had no demonstrable effect on enhancement of the tumor. No significant change was seen in the behavior of skin or tumor grafts when the number of antiserum inoculations was doubled, and it appeared that antiserum injected 2 days before the inoculation of tumor had the same effect in enhancing the tumor as did simultaneous inoculation of serum and tumor.

Antiserum was capable of inhibiting as well as enhancing tumor growth in the same animal, as shown in Tables II and III, where control tumors grew faster during the first 4 to 6 days than did those in mice receiving serum. In some cases, the delay of tumor growth in the serum-treated hosts was present until the seventh day, and in one case (Table II, mouse #3) the tumor never reached the size of the control tumor, although it persisted longer.

The observation by Brent and Medawar (7) that antiserum may enhance survival of skin homografts when it is administered in large quantities throughout the period after grafting was confirmed. Lesser dosages were ineffective. No accelerated skin graft rejection could be attributed to antiserum administration at any dose range. B.P.8 thus appears to be more sensitive than C3H skin both in its ability to be enhanced and to be inhibited by antibodies.

Skin homografts placed on mice 10 or 15 days before the inoculation of tumor resulted in marked suppression of growth of the latter, even when antiserum in doses capable of

producing enhancement was given at the time of tumor inoculation.

The "afferent" hypothesis, which supposes that administration of antiserum prevents adequate antigenic stimulation of the host, can hardly account for these findings. Kaliss (2) showed that a tumor could be enhanced by antiserum given as late as 7 days after the tumor graft. Although this finding was construed to be a refutation of the afferent hypothesis, it could as well have been argued that the small tumor graft initially provided only weak antigenic stimulation, and that later administration of antiserum at a time when the tumor had become established resulted in sufficient diminution of the host immune response to permit the tumor to continue to grow progressively. Such an argument is less tenable in the studies reported here, since the skin homograft was much larger than the tumor cell inoculum and presumably contained a greater quantity of antigen. Passively administered antibodies in amounts incapable of enhancing the skin graft should thus be inadequate to prevent it from stimulating the host. The antiserum

TABLE III. Abrogation of Enhancement by Prior Application of Skin Homograft.

Animal No.	Group	Day skin grafted	Serum given	Tumor size* (days) 6	9	12	15	18	22	26	33	44	72	Enhancement
	A	−15	+											
1				4	0	0	0	9	16	20	42	63	437	Present
2				0	0	0	0	0	0	0	0	0	0	None
4				0	0	0	0	0	0	0	0	0	0	"
	B	−10	+											
5				12	8	6	0	0	0	0	0	0	0	"
6				0	0	0	0	0	0	0	0	0	0	"
7				6	6	6	0	0	0	0	0	0	0	"
8				9	6	0	0	0	0	0	0	0	0	"
	C	− 5	+											
9				25	36	42	56	81	100	82	90	272	large	Present
10				9	9	16	25	29	42	58	128	276	large	"
11				9	6	6	0	0	0	0	0	0	0	None
12				24	30	54	70	77	104	112	210	528	large	Present
	D	− 2	+											
13				25	42	72	110	120	144	196	360	650	died	"
15				6	12	32	50	66	96	160	216	384	large	"
16				9	9	15	28	32	45	66	84	160	large	"
	E	0	+											
17				12	10	6	6	6	9	died		—	—	"
18				20	24	70	120	156	136	128	80	91	0	"
19				9	died		—	—	—	—	—	—	—	"
20				6	9	20	45	60	77	120	220	360	large	"
	F	+ 3	+											
21				4	12	21	50	50	50	50	38	12	0	"
23				9	12	30	56	80	120	196	204	204	large	"
24				12	20	35	63	136	169	died		—	—	"
	G	—	+											
25				12	9	12	20	30	42	96	150	270	large	"
26				8	8	0	0	0	0	0	0	0	0	None
27				8	12	19	35	40	56	80	132	288	large	Present
28				12	12	20	30	42	56	110	140	340	large	"
29				15	15	15	18	25	20	12	4	0	0	"
30				9	6	4	0	0	0	0	0	0	0	None
31				18	18	41	60	77	117	187	264	540	died	Present
32				6	6	9	20	30	30	36	42	48	357	"
	H	—	−											
33				49	49	30	24	0	0	0	0	0	0	None
34				56	36	20	0	0	0	0	0	0	0	"
35				16	28	12	0	0	0	0	0	0	0	"

* Tumor size recorded as in Table I. Tumor and serum (1.0 ml) inoculated on Day 0. Mice dying before day 6 are omitted (Nos. 3, 14, 22).

used in these experiments required administration in much greater quantities over a longer time to induce any increase in skin graft survival time.

The hypothesis that passively administered antibody results in a "central" depression of the host response likewise fails to account adequately for the present findings, since it would be difficult to explain on this basis the behavior of the tumor in mouse #1 of Table III, which arose and grew progressively 15 days after its inoculation, and the tumors in mice 3, 4, and 5 of Table I and of 9, 17, and 32 in Table III, which initially grew rapidly but then stopped for a variable period before resuming progressive growth. Tumors of such varying size could scarcely all be expected to cause the host to maintain for such a long time the appropriate level of immunity to provide the delicate balance necessary for continued survival without growth or rejection. In the 3 years of our experience with the tumor B.P.8, it has never been observed to grow progressively or in these patterns in untreated B6D2F_1J mice. Of some 200 B6D2F_1J male mice that have received an inoculation of B.P.8 with no other treatment, early tumor growth followed by subsequent rejection have been the invariable rule. If enhancement were due to selective outgrowth of cellular mutants, such outgrowth should have been observed in some of the untreated mice, many of whose tumors grew, before regressing, to a much larger size than that at which some persistent tumors were maintained; if enhancement were due to a central inhibition, the prolonged persistence of an impalpable tumor in a presensitized host would be unlikely.

There remains the "efferent" hypothesis: that immunological enhancement is due to a protection of the graft against the effector mechanism of the host immune response. These experiments offer no contradiction to this hypothesis, which is supported by the recent studies of Möller(3-5) who notes that antiserum and immune lymphocytes can be shown to act antagonistically. Antibodies which are not greatly cytotoxic in low concentrations may locally combine with graft antigens and thus disguise the graft cells to the invading immunologically active host cells.

In conclusion, the results of tests of enhancement utilizing both skin and tumor grafts on the same animal are useful tools in the investigation of the enhancement phenomenon and the homograft reaction. These experiments support an efferent mechanism of enhancement; that is, that the presence of antibodies locally somehow protects a homograft from a normally induced and progressing immune response.

Summary. C3H mouse skin and the C3H tumor B.P.8 were both placed as homografts in the same B6D2F_1 mice, which were also treated with an antiserum directed against histocompatibility antigens of the donor tissues. The amount and duration of antiserum treatment was varied, as was the relative time of the two graftings. The tumor was readily enhanced by antiserum, but it was possible only with difficulty to demonstrate any enhancement of skin graft survival by antiserum injection. Growth of the B.P.8 tumor was enhanced by doses of antiserum that had no demonstrable effect on the rejection of a simultaneously applied C3H skin homograft. When the skin grafts were applied 10 or 15 days before the tumor cell inoculum, the tumor tended to show accelerated rejection despite simultaneous injection the growth of tumor in control animals. The results of these and other findings presented here are interpreted as supporting the hypothesis that an efferent mechanism is responsible for the immunological enhancement of homografts.

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Received September 2, 1964. P.S.E.B.M., 1965, v118.

Effect of Restricted Food Intake on Growth and Composition of Preweanling Rat Brain.* (29806)

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The weight and chemical composition of the rat brain tends to be less affected by dietary factors than most other organs of the body. Lehr(1) found that restricting food or protein intake of adult rats did not affect total nitrogen, protein, or deoxyribonucleic acid content of the cerebral cortex. Allison(2) noted that the ratio of protein to DNA in rat brain was not altered by the level of protein in the diet as opposed to liver, kidney and muscle tissue. Mandel(3) found that adult rats on protein-free diets for 2 months showed no changes in brain protein or deoxyribonucleic acid levels in spite of considerable loss in body weight. It has been demonstrated(4, 5) that the brains of rats, whose body weights were held constant from birth to 16 days of age, increased in weight by well over 100%. Most other organs of the body gained much less than this and some, such as the liver, actually lost weight. No chemical analyses were done on the brains.

Methods and materials. Litters of 5-day-old Wistar rats were split and half of the newly formed litters were left with lactating dams at all times. The remaining litters were placed with lactating dams for 3 to 6 hours daily, the time being regulated so that their body weight gain was 20-25% of that of the rats that suckled *ad libitum*. All young were weighed daily to insure adequate control of weight gain. Only one preweanling rat died during the experiment. All dams were fed *ad libitum* a nutritionally complete diet containing 30% casein as the protein source. At 20 days of age, each of the young was anesthetized with ether, the skull opened, the olfactory lobes dissected from the brain and the brain removed by severing at the level of the foramen magnum. The brain was immediately rinsed with ice water, blotted and weighed. Brain lipids were extracted(6) by homogenizing in 19 volumes of chloroform-methanol (2-1). The resulting suspension was filtered through a preweighed fine porosity sintered glass filter. The residue on the filter was washed 2 times with 3 ml portions of chloroform-methanol (2-1), after which the residue was dried to constant weight and total non-lipid solids calculated by difference. Total unwashed lipid solid weight was obtained by drying a portion of the chloroform-methanol extract. The remainder of the lipid extract was washed with .2 volumes of .1 M potassium chloride, followed by 2 washings with .2 volumes of chloroform-methanol - .1 M potassium chloride (3-48-47) and a final washing with chloroform-methanol-water (3-48-47). A portion of this washed lipid solution was dried and weighed to obtain the washed lipid weight. The washed lipid extract was used to analyze for cerebrosides(7), cholesterol(8) and phospholipid phosphorus(9). The phospholipid value was obtained by multiplying the phosphorus value by 25. Brains of *ad libitum* fed 5-day-old rats were similarly analyzed to determine the magnitude of the changes in brain growth and composition during the experimental period of 5 to 20 days of age.

Results and discussion. Brain components were calculated as percent of the wet brain weight and appear in Table I. While only the percentages of brain total lipids, cerebrosides and cholesterol were significantly lower than

* Supported in part by U.S.P.H.S. Grant NB-05262.

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