DEPRESSION BY ANTIBODY OF THE IMMUNE RESPONSE TO HOMOGRAFTS AND ITS ROLE IN IMMUNOLOGICAL ENHANCEMENT*

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When mice receiving a tumor homograft are pre-injected with killed tumor tissue, or with non-living preparations of certain normal tissues from the strain to which the tumor is indigenous, the growth of the tumor is often markedly enhanced. In tumor-host combinations favorable to the phenomenon, all, or almost all, pretreated mice die with progressively growing tumor and all untreated controls survive. The effect is surprising in that the pretreatment employs a schedule that would be expected to immunize and hence to diminish rather than to promote tumor growth.

This phenomenon has been variously called the XYZ effect, the enhancing effect, and immunological enhancement. Reviews have been written by Casey (1), by Snell and coworkers (2, 3) and by Kaliss (4). Only a few salient facts are mentioned here, with discussion deferred to the end of the paper.

An important step in the elucidation of the enhancing phenomenon was taken by Kaliss and coworkers when they showed that the enhancing effect can be passively transferred with the serum of pretreated mice. The evidence proves that antibodies directed against the isoantigens of the tumor are an essential factor in the phenomenon (5, 6, 4), and Kaliss now defines enhancement as the progressive growth of homografts, produced by specific antiserum. This is probably good usage; it limits the term to a clear effect and an experimentally well defined cause. It is worth emphasizing that in suitable tumor-host systems, progressive tumor growth in mice injected intraperitoneally with a hyperimmune antiserum is even more consistent than in mice enhanced by the pre-injection of freeze-dried tumor.

Another major advance, important in understanding not only the enhancing effect but also the homograft reaction in general, was the demonstration by Kidd (7) and later by Mitchison (8) that the major agent in the rejection of most grafts is the immune lymph node cell. Mitchison showed that homograft immunity can be "adoptively transferred" by the intraperitoneal injection of

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minced lymph nodes from the area draining a tumor transplant. Evidence from various sources indicates that the lymphocyte does not act by releasing antibody into the circulation, but rather migrates to and directly attacks the graft (3). Grafts of leukemic or normal lymphoid tissues present a special case; these, with a few possible exceptions, can be destroyed by humoral antibody and complement (9). It follows as a corollary that leukemias cannot be enhanced.

One other fact is essential for understanding the enhancing effect. Injections of killed tissue produce only, or predominantly, humoral antibody, whereas injections of living tissue tend to produce both cellular and humoral immunity (3). A close parallel to this difference in effect of living and dead tissue is provided by bacteriological studies. Holland and Pickett (10) have found that whereas *Brucella*-infected guinea pigs show both a high antibody titer and delayed skin reactions, animals vaccinated with heat-killed organisms produce antibody but do not develop delayed hypersensitivity.

In the light of these facts, two hypotheses to account for the enhancing effect can now be considered.

Hypothesis 1.—Kaliss (4) interprets enhancement as "due to some 'physiological' alteration in the tumor, induced by its contact with antiserum, which insures its survival despite the hostile responses of the host." Kaliss considers, and rejects, a variant of this hypothesis; namely, that the effect of the antiserum is to modify the tumor through "immunological selection." We agree that this is not a major factor in the enhancing phenomenon.

Hypothesis 2.—A second and entirely different mechanism has been suggested by Snell (11, 3) and independently by Billingham, Brent, and Medawar (12). Briefly, it is postulated that the effect of the antiserum is in some way to block the development of the cellular type of immunity in the nodes and other lymphoid tissues draining the graft. Since the destruction of grafts, other than leukemias, is dependent on such cellular immunity, a blockage of this sort would permit the graft to grow. Billingham *et al.* have referred to the postulated effect as an " 'afferent inhibition' of transplantation immunity," and Snell as "a 'walling off' of the graft by circulating antibody, so that antigens either do not reach the regional nodes, or reach them in an inactive state."

This paper reports tests of these two hypotheses.

To avoid circumlocution, mice with circulating isoantibody as a result of freeze-dried tissue injections will be called actively enhanced mice, and mice which have received antibody by transfer will be called passively enhanced mice.

Materials and Methods

Mice.—The mice used in tests of hypothesis 1 were mostly from two strains, A/WySn and B10.D2 (abbreviated to B.D. in Fig. 2). The latter is a subline of C57BL/10 in which $H\text{-}2^d$ has been substituted for $H\text{-}2^b$ (13). For reasons not fully understood, strain A tumors are more easily enhanced in strain B10.D2 than in strain C57BL/10. A few tests were also made

with strains A.SW (13), C57BL/10ScSn, and C3H/DiSn. All mice were born in the same month, and the youngest were 7½ weeks old when enhancing injections were started. All experimental groups contained equal numbers of males and females.

In most experiments testing hypothesis 2, strains A/WySn and B10.D2 were again employed. In one instance C57BL/Ks (also an *H-2*^{d} subline) was substituted for B10.D2. A few lymph node assays were performed in the hybrid (A × B10.D2)F_1. In all parts of experiments in which an immune response was being studied, mice were at least 8 weeks old. Mice in any one experiment were matched as to sex and age.

Tumor.—The tumor used was SaI (also called Sarcoma I), native to strain A.

In tests of hypothesis 1, the subline of SaI employed was kindly provided by Dr. Kaliss. All experimental mice were implanted subcutaneously in the right axilla by trocar. Tumors were palpated weekly and a record of tumor size made in the form of a drawing.

In tests of hypothesis 2, the Snell subline of the ascites form of the tumor (called SaI-A), was used. Whereas the Kaliss subline grew progressively in about 50 per cent of untreated B10.D2 hosts, the Snell subline rarely grew progressively in such animals. To prevent clotting, heparin from a stock solution (10 mg. heparin in 10 ml. of 0.93 per cent saline) was added to the ascites fluid in the proportion 1 in 10 to 1 in 20.

Active Enhancement.—Actively enhanced B10.D2's were used in tests of hypothesis 1. The tissue used for enhancement was lyophilized A spleen. The enhancing treatment consisted of 5 intraperitoneal injections of 5 mg. each over a 2 week period, with tumor given 14 days after the last injection.

Passive Enhancement.—In tests of hypothesis 2, passive enhancement was used. The antiserum employed was hyperimmune B10.D2 anti-A. Multiple injections of living SaI, sometimes preceded by an injection of normal or SaI tissue extract, were used for imunization. Mice were sometimes bled four times, with a booster injection of SaI after the second bleeding. The antiserum was stored in a deep-freeze. Mice to be enhanced were given an intraperitoneal injection of antiserum, usually about an hour before, but in certain specified cases, a day or more after, the subcutaneous implantation of SaI or strain A normal tissue. Except as otherwise specified, the dose per mouse was 0.3 ml.

Tumor and Tissue Injections.—The blocking effect of antiserum was tested not only against tumor SaI-A, but also against normal tissues. When tumor was used, ascites fluid was drawn from the peritoneal cavity, a cell count made, and the desired dilution prepared. The normal tissues employed were embryo, thymus (from 3- to 5-week-old mice), lymph nodes and spleen. Embryos near term were prepared as a mince and injected by trocar. Other tissues were usually prepared as a cell suspension in the cytosieve (14), and diluted to the appropriate cell count. In certain instances tissues were minced with scissors and mixed with the tumor.

The medium used for preparing cell suspensions was a buffered glucose-Ringer's solution (hereafter referred to as BGR). This was made as previously described (14) except that we now use a phosphate instead of a citrate buffer.

Injections of tumor or tissue for testing the effect of enhancement usually were made subcutaneously, either on both flanks or on the right flank only. Entry near a draining node was avoided in the case of tumor.

Red Cell Agglutination Tests.—The method used was the serum-dextran technique of Gorer and Mikulska (15).

Lymph Node Assay.—To obtain a critical test of hypothesis 2, an assay for the level of cellular immunity in the various lymphoid organs is essential. While the method used by Mitchison in earlier studies in this laboratory (8) to demonstrate cellular immunity might serve, it is neither sensitive nor quantitative. An assay recently developed by Winn (9) requires only about 0.01 to 0.03 as much lymphoid tissue as the earlier method, and, because of this greater sensitivity, allows quantitative determinations.

The general procedure is shown in Fig. 1. A cell suspension from the nodes or spleens to be assayed is prepared in the cytosieve (14), a cell count made, and serial dilutions prepared. A known number of ascites tumor cells is mixed with each dilution, and the mixture injected at once into appropriate mice. A week or more later the mice are killed and the tumors weighed. Absence of growth, or a significant reduction of growth as compared with controls, is indicative of immunity. For the assay, either the original tumor-donating strain, the original host strain, or an F_1 hybrid between the two can be employed. Experience has shown that the use of the original host strain (strain B10.D2 in Fig. 1) gives the most sensitive results. This is because the nodes to be assayed and the assay animals are mutually compatible; the animals

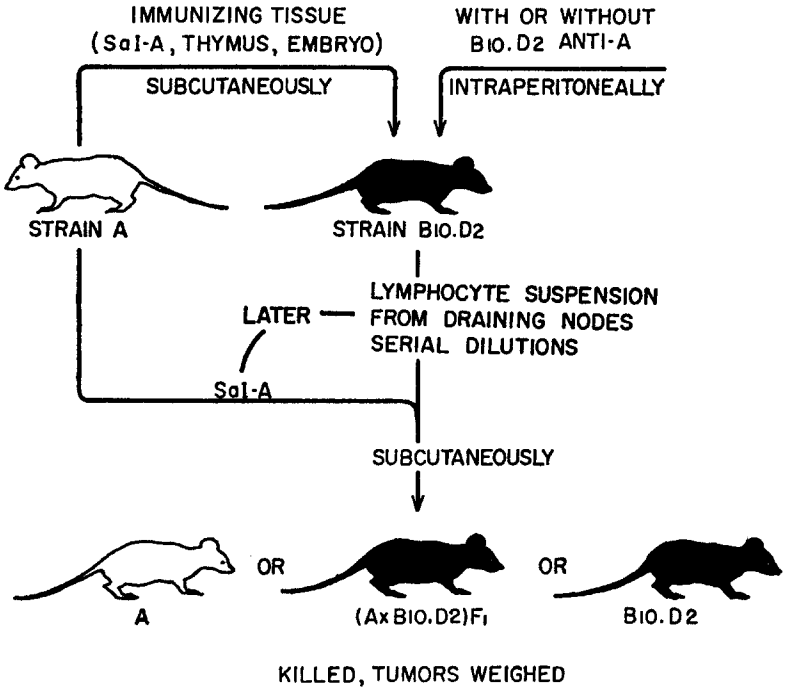

FIG. 1. Diagram showing the method of lymph node assay.

do not react against and destroy the node cells, and the cells are not diverted from the antigens of the tumor by corresponding antigens in the host. The details of the technique are as follows.

The nodes used were the axillary, brachial, and inguinal. These were taken from one or both sides according as stimulation was on one or both sides. The typical experimental group consisted of the nodes from 10 mice. These were collected in chilled BGR, placed in a cytosieve, BGR added (usually 4 ml.), and a cell suspension prepared. A cell count was then made, serial dilutions prepared (usually), SaI-A added, and each mixture injected at once in the right axilla of 10 mice.

In order that the results of different experiments might be easily compared, the dilutions were usually a standard series such that each mouse received one of the following numbers of lymph node cells: 8, 4, 2, 1, 0.5, or 0.25 million. When the cellular immunity was likely to be low, in addition to the specified dilutions, one group received undiluted node cell suspension mixed with tumor.

For assay in strain A, each mouse received lymph node cells mixed with 5,000 cells SaI-A; for assay in (A × B10.D2)F_1 or in B10.D2, with 1,000,000 cells SaI-A, usually in 0.2 cc. Tumors were removed and weighed after an appropriate interval.

In a few early experiments, the medium used was chilled BGR plus normal serum, but later chilled BGR only was used. The McCartney bottles containing the cell suspensions were kept on ice or in the refrigerator at all times.

Plan for the Test of Hypothesis 1.—The basic plan for the test of hypothesis 1 was to compare the growth in B10.D2 mice of SaI from two sources, A's and enhanced B10.D2's. If the effect of the enhancing treatment is to modify the tumor so that it can resist the hostile responses of the host, this should show up in faster or better growth of the tumor from the second of the two sources.

Four A's (with the tumor from two of them pooled) and 5 enhanced B10.D2's were used as donors, the tumor from each donor being passed to 10 or more untreated B10.D2's. Multiple donors were used to average out any subline variation of the tumor that might occur in a single donor simply as the result of chance. Tumor from 2 enhanced B10.D2's, ♀40 and ♀49, was also passed into A's and thence into untreated B10.D2'S. This was done becaues one possible source of altered growth in B10.D2's of an A tumor taken from B10.D2's is the absence of A lymphocytes and other A white blood cells. Such cells are usually abundantly present in an A tumor taken from A's, and since normal lymphoid cells are an excellent agent for inducing homograft immunity (3), an A tumor homografted from A's might on this account alone be rejected more rapidly than an A tumor homografted from B10.D2's.

Tumors were also passed from an enhanced B10.D2, ♂45, and from an A, ♀64, to groups of A.SW, C3H and C57BL/10.

RESULTS

Results of the Test of Hypothesis 1.—The results are shown in Fig. 2. A control group of 25 enhanced B10.D2's all succumbed to the tumor. Of 50 untreated B10.D2's receiving SaI directly from A's, 24, or 48 per cent, succumbed. The corresponding figure for untreated B10.D2's receiving tumor *via* enhanced B10.D2's was 22 out of 50, or 44 per cent, and for untreated B10.D2's receiving tumor which had passed in succession through enhanced B10.D2's and A's, 12 out of 28, or 43 per cent. It is evident that there was no demonstrable difference in the fraction dying as a result of the different types of passage. Residence in enhanced B10.D2's did not modify the tumor so that it grew better in other, unenhanced B10.D2's. Neither was there any improved growth of such tumors when passed to other foreign strains. Tumor from an A mouse and tumor from an enhanced B10.D2, respectively, grew in 8 out of 10 and 7 out of 9 A.SW, in 3 out of 5 and 0 out of 6 C3H, and in 0 out of 10 and 0 out of 10 C57BL/10. Neither was there any significant difference in the rate of growth of these tumors.

While the average mortality from tumors taken from different types of donor was the same, there was considerable variation in tumors taken from individual donors. Thus tumor from enhanced B10.D2 ♂34 killed 1 out of 10 untreated B10.D2's, while a comparable passage from ♂55 killed 8 out of 10. This illustrates the importance of pooling the results from multiple donors.

Test of Hypothesis 2: Blockage by Antiserum of Cellular Immunity.—

In one series of experiments, B10.D2 mice, some of which had and some of which had not received antibody, were immunized with various strain A tissues implanted subcutaneously on both sides, and the nodes assayed for immunity. The tissues used for immunization were SaI-A, thymus, and embryo. The assay was performed in (A × B10.D2)F_1's. Mice of this genotype offer the advantage that SaI-A is compatible, so that any inhibition of its growth must be due entirely to immunity of the transferred nodes, but the disadvantage that the node cells can react against the A component in the assay hosts as well as against the tumor. The results of these experiments are shown in Table I.

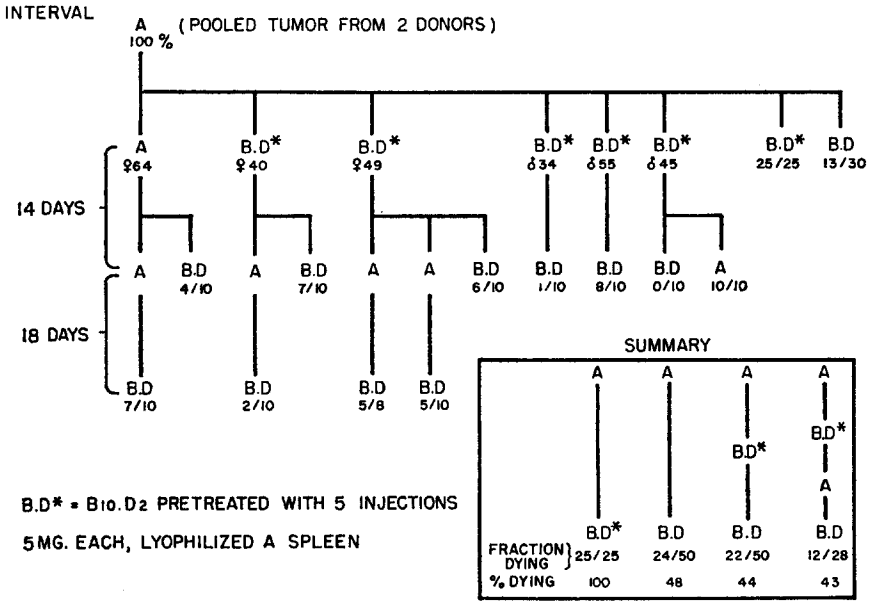

FIG. 2. Diagram showing the various passages of SaI used to test hypothesis 1. The result for each terminal group is given as a fraction in which the numerator is the number dying from the tumor and the denominator the total number inoculated.

The mean tumor weights in 5 groups of controls, which received normal B10.D2 node cells mixed with SaI-A, were 1.56, 0.57, 0.68, 0.74, and 0.63 gm. The group with a mean weight of 1.56 was killed and weighed at 8 days, the others at 7 days. In some of the groups receiving the higher concentrations of presumptively immune B10.D2 node cells, weights much smaller than these were found. We have, somewhat arbitrarily, taken mean weights of 0.27 gm. or less as indicative of inhibition. The difference between these weights and the controls is highly significant ($P < 0.01$). Using these values, we find that node cells from mice receiving tumor alone were active in doses of 1.5 million cells per mouse or greater, and inactive at doses of 0.75 million cells or less. Node cells of

mice receiving tumor plus antiserum were inactive in all doses up to and including 12 million cells per mouse. The activity generated in the absence of antiserum is thus at least 16 times greater than the activity generated in its presence. After immunization with thymus, node cells from unenhanced mice were active at the 4 million cell level and inactive at the 2 million cell level, whereas node cells from enhanced mice showed no activity at the maximum attainable dose, 10.5 million cells per mouse. There is not less than a 5-fold difference. A

TABLE I

Assay of Draining Lymph Nodes of B10.D2 Mice Immunized with Various Strain A Tissues, with and without B10.D2 Anti-A

Immunizing tissue*	Antiserum per mouse	Interval to assay	Mean tumor weight (10 mice) at different lymphocyte dose levels (in millions)					
			12	6	3	1.5	0.75	0.38
		days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Tumor	None	7			**.02**	**.27**	.78	.66
Tumor	0.3 ml.	7	.88	1.23	.74	.79	1.13	
None	None	—			1.56			
			>8	8	4	2	1	0.5
Thymus	None	5		**.08**	**.12**	.48	.43	.65
Thymus	0.3 ml.	5	.59		.68	.90	.84	
None	None	—			.74	.63		
Embryo	None	8		**.01**	**0**	**.12**	**.05**	.31
Embryo	0.3 ml.	8	**.22**	.39	.36	.44		
None	None	—	.57		.68			

Assays performed in (A × B10.D2)F_1 mice, each mouse receiving 1 million cells SaI-A mixed with various lymphocyte dilutions. Tumors weighed at 7 (immunization with embryo or thymus) or 8 (immunization with tumor) days. Tumor weights set in bold face are regarded as indicative of inhibition.

* Tumor (SaI-A), 3 million cells per mouse; thymus, 15 million cells per mouse; embryo given by trocar.

repetition of this experiment gave essentially identical results except that there was not less than an 8-fold difference. Embryo given by trocar produced a particularly strong immunity; activity was found at the one million cell level for unenhanced mice; it appeared in this experiment also in the enhanced mice at the maximum dose level, 13.9 million cells per mouse. The difference in activity is 14-fold.

The selection of intervals of 7, 5, and 8 days for node assay after stimulation with tumor, thymus, and embryo respectively was based on earlier, published data on homograft immunity (8, 16), on pilot assays following immunization with thymus, and on determinations of the cell yield of draining lymph nodes

obtained at different intervals after a subcutaneous injection of foreign strain thymus. For the last named series of observations we are indebted to Mr. Meyer Gottlieb.

Effect of Interval between Immunization and Node Assay.—Mitchison (8) found that the draining nodes of mice immunized with a tumor homograft could transfer immunity if removed at 5 or 10 days after the tumor graft, but not if removed at 3, 15, or 20 days. In the experiments described in the preceding section, we used intervals of 5, 7, and 8 days. Two experiments were also performed in which a much wider range of intervals was used.

Unenhanced and passively enhanced mice were immunized with SaI-A, subcutaneously on the right side, at intervals of 3½, 4, 6, 9, 14, and 22 days prior to assay of the draining nodes. The assay was performed in strain B10.D2. Mice were killed at 1 week and the tumors weighed. The results are presented in Table II and Fig. 3. Besides the mean tumor weight, there is given, as a decimal in parentheses, the proportion of mice in each assay group with a detectable tumor; the number 1 means that 10 out of 10 mice had a tumor.

Because SaI-A is incompatible with the B10.D2 mice used for the assay, tumors were smaller than in the experiments in which the assay was performed in hybrids. (Current work shows that larger tumors can be obtained with larger tumor doses.) However, immunity was demonstrable with fewer node cells, presumably because none of the cells was diverted from the tumor by an A component in the tissues of the host. The mean weights of tumors in 7 control groups were 0.24, 0.28, 0.07, 0.11, 0.31, 0.13 and 0.19 gm. The two low values of 0.07 and 0.11 obtained in Experiment 1 in the normal lymphocyte control, are perhaps partly accounted for by the fact that these groups of mice were inoculated towards the end of a long experiment; the tumor preparation was 7 or 8 hours old. However, other groups done late in the same experiment were of more nearly typical size. As indicative of immunity in the node cells, a mean tumor weight of 0.05 gm. or less was selected. Again the selection was somewhat arbitrary. These inhibited groups always included a number of entirely negative mice; the proportion with tumor was never greater than 0.6, and was 0 in 17 cases, indicating complete inhibition. Only 1 group failed to fall in the sequence expected for a titer test. Among the 10 mice receiving 8 million cells each of 4-day nodes, there were 9 negatives and one mouse with a tumor weighing 0.56 gm. This gave a mean tumor weight of 0.06 gm. and hence above the level selected as indicative of inhibition. Mice receiving the same node suspension at the 4 million and 2 million cell levels were all negative.

In plotting the points in Fig. 3, it was usually possible to interpolate (by inspection; no mathematical treatment was attempted) between two adjacent lymph node dilutions, one of which produced and one of which did not produce, a reduction of mean tumor weight to 0.05 gm. or less. In a few cases the endpoint fell outside the range of dilutions used; *e.g.*, in Experiment 2, assay with 6-day nodes, inhibition was found at all dose levels from 2 million to 0.25 million

node cells. This point in the figure is placed above 0.25 but its exact position is obviously indeterminate.

TABLE II

Assay of Draining Nodes of B10.D2 Mice That Received SaI-A, 3½, 4, 6, 9, 14, or 22 Days Previously, with (Enhanced) or without (Unenhanced) B10.D2 Anti-A

Treatment, and interval to assay	Experiment No.	Mean tumor weight (10 mice), and (in parenthesis) proportion of mice with tumor, at different lymphocyte dose levels (in millions)					
		11.7–6.3	4	2	1	0.5	0.25
days		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Unenhanced							
3½	2		.08 (.7)	.18 (1)	.19 (1)	.24 (1)	
4	1	.06 (.1)	**0** (0)	**0** (0)	**.03** (.4)	.14 (.9)	
6	2			**0** (0)	**0** (0)	**.00** (.1)	**0** (0)
6	1		**0** (0)	**0** (0)	**0** (0)	**.01** (.1)	**.04** (.6)
9	2			**0** (0)	**0** (0)	**.03** (.3)	**.04** (.6)
9	1			**0** (0)	**0** (0)	**0** (0)	.15 (.8)
14	1		**0** (0)	**.01** (.2)	.06 (.5)	.25 (.8)	.17 (.9)
22	1		**0** (0)	**0** (0)	**.01** (.1)	.13 (.6)	.22 (.9)
Enhanced							
3½	2	.19 (1)	.27 (1)	.18 (1)	.27 (1)		
4	1	.13 (.9)	.19 (1)	.21 (.9)	.22 (1)	.12 (.7)	
6	2	**.05** (.4)	.10 (.9)	.08 (.8)	.18 (.9)		
6	1	**.02** (.5)	.31 (1)	.26 (.9)	.39 (1)		
9	2	**.00** (.1)	**.05** (.3)	**.05** (.5)	.18 (1)		
9	1	**.01** (.2)	**.03** (.3)	.13 (1)	.07 (.7)		
14	1	**.05** (.2)	.19 (.8)	.13 (.6)	.27 (.9)		
22	1		**0** (0)	**.04** (.3)	.25 (.9)	.27 (1)	
Control A	2			.24* (1)	.28 (1)		
	1			.07‡ (.7)	.11 (.8)		
Control B	2			.31 (1)			
	1			.13 (.8)	.19 (.9)		

Assay performed in B10.D2's, each mouse receiving 1 million cells SaI-A mixed with various lymphocyte dilutions. Mice killed and tumors weighed at 7 days. Mean tumor weights of 0.05 gm. or less are regarded as indicative of inhibition and are set in bold face. Control A: normal node control; Control B: control receiving tumor only without node cells.

* 3 million lymphocytes.

‡ 3.4 million lymphocytes.

Several points of interest are apparent from the table and the figure. First, while this sensitive assay revealed immunity in the node cells of enhanced mice at 6, 9, 14, and 22 days after tumor implantation, but not at 3½ or 4 days, the level was always lower than in unenhanced mice. The difference was greatest at 6 days; here there was about a 24-fold difference in Experiment 1 and more than a 32-fold difference in Experiment 2. The difference thereafter became pro-

gressively less. Second, these experiments show that immunity can be detected over a longer interval than was found by Mitchison (8). In the unenhanced group, there was no immunity demonstrable in the nodes removed at 3½ days, but there was immunity at 4, 6, 9, 14, and 22 days. Third, in both experiments the unenhanced group showed a peak at 6 days, the enhanced group at 9 days. Fourth, in both groups there is a low point at 14 days and a subsequent rise at 22 days. Since only one of the two experiments was extended beyond 9 days, this rise may be due to chance fluctuations. If real, it is perhaps related to the

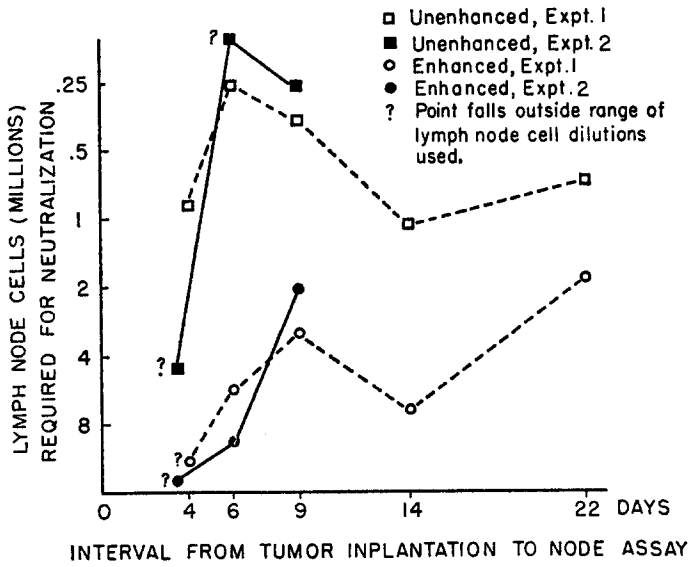

FIG. 3. Graph showing the level of cellular immunity in the draining nodes of C57BL/6Ks mice at different intervals after the implantation of SaI given with and without accompanying antiserum.

second immune peak at about 24 days demonstrated by Barrett and Hansen (17). The enhanced mice of course had large tumors at 22 days whereas the tumors in the unenhanced mice, after temporary growth, had disappeared.

Number of Cells from Stimulated Nodes.—In the experiments described in the two preceding sections, the brachial, axillary, and inguinal lymph nodes from groups of 10 mice, variously treated, were placed in the cytosieve, the "lymphocytes" extracted, and a cell count made. This makes it possible to calculate the "cells per side per mouse." The values are given in Table III. (This includes 2 experiments not included in previous tables.) It will be seen that in general the lowest counts are given by the control nodes (range: 3,600,000 to 9,800,000), the next highest by the nodes of enhanced and immunized mice (range: 7,900,000 to 34,600,000), and the highest by unenhanced and immunized mice (range: 12,-

000,000 to 41,800,000). All the very high counts, including some as early as 3½ days, were obtained from nodes stimulated by tumor. Since many, perhaps most, tumors carry viruses (18), this may account for the excessive cellular response in these instances. In the experiments in which normal tissue was used for immunization, the order of increasing cell yield was, without exception, control, enhanced and immunized, and unenhanced and immunized. Thus the cells

TABLE III

Number of Cells Extracted from Draining Lymph Nodes of B10.D2 Mice Immunized with Strain A Tissue, with (Enhanced) and without (Unenhanced) B10.D2 Anti-A

Immunization			Interval	Lymphocyte yield, cells/side/mouse (in millions)		
Tissue	Sides	Dose/side		Control	Enhanced	Unenhanced
			days			
Thymus	Both	63 million	5	3.6	12.6	13.6
"	"	15 "	5	5.9	7.9	15.5
"	"	15 "	5	9.1	13.8	14.6
Embryo	"	Trocar	8	9.2	12.7	20.5
Tumor	"	3 million	7	3.9	9.7	12.6
Average				6.3	11.3	15.4
Tumor	Right	3 million	4	9.1	24.7	29.7
"	"	3 "	3½	9.8	21.0	14.9
"	"	3 "	6		20.8	24.5
"	"	3 "	6		26.2	22.6
"	"	3 "	9		34.6	41.8
"	"	3 "	9		19.6	43.4
"	"	3 "	14		22.7	24.0
"	"	3 "	22		12.3	12.0

are not only proportionally more immune in the unenhanced mice, but there are more of them.

Passive Enhancement and Spleen Weights.—Green (19) has noted that the splenic enlargement, normally present in rats bearing a transplantable tumor, is absent or only mildly present in rats which have received enhancing tissue injections prior to the tumor implant. In one of our experiments in which B10.D2's, with and without passive enhancement, were implanted with SaI-A, and killed 7 days later, spleen weights were determined. The mean weights were: untreated control, 0.081 gm.; antiserum and SaI-A, 0.115 gm.; SaI-A without antiserum, 0.153 gm. The differences are significant ($P < 0.01$). This confirms Green's observation. However, in a comparable experiment in which

the immunizing injection consisted of 15 million thymus cells, and the interval to killing was 5 days, the weights were: untreated, 0.090 gm.; antiserum and thymus, 0.113 gm.; thymus alone, 0.080 gm. Since the thymus and the thymocyte have special properties which might influence the spleen in one way or another (20, 21), these results do not necessarily contradict results obtained with tumors.

The Effect of Passive Enhancement on the Formation of Humoral Isoantibody.— The preceding experiments have shown that passive enhancement reduces the level of cellular immunity developed in the lymph nodes draining an implant of foreign tissue. Does passive enhancement likewise lower the humoral response to foreign tissue? This question was answered in the affirmative by two experiments.

In each experiment, 36 B10.D2 mice were used. Twenty-four were injected intraabdominally with strain A tissue. Twelve of these, and the 12 otherwise untreated, also received intraabdominally 0.3 cc. B10.D2 anti-A. In the first experiment, each mouse received 61 million cells, mixed A thymus and spleen, in the second, 35 million cells of A thymus. Pairs of mice, usually a male and a female, were bled from the tail at 3, 6, 9, 12, 15, and 18 days after the injections, and the serum of each mouse stored individually in a deep freeze. Each mouse was bled only once. All antisera in each experiment were titered on the same day against the same pool of strain A erythrocytes. The results are shown in Fig. 4, in which each point represents the geometric mean titer for two mice.

As expected, the titer of the serum from mice receiving antiserum alone fell off progressively as the interval to bleeding increased. The curve for mice receiving injections of both antiserum and A tissue was surprisingly similar. In the first experiment, the curve showed a rise above the controls at 6 days, probably indicating antibody formation, but thereafter was below the controls; in the second experiment it was below the controls till the last day. Evidently in the presence of antiserum, the injected antigen induced only limited antibody formation. In the absence of passively transferred antiserum, on the contrary, the antigen stimulated a generous outpouring of antibody; in the first experiment the titer reached 1 in 10,240 at 9 days and in the second experiment, 1 in 20,480 at 12 days.

It should be noted that while isoantiserum is cytotoxic to spleen cells, it is apparently not cytotoxic to mouse thymus cells (9). The passively transferred antiserum may have modified the antigen in the first experiment through the destruction of that portion of the A cells which came from the spleen, but it should not have produced this type of modification in the second experiment. It is also noteworthy that in these experiments the "blocking" effect of antibody was obtained when antigen was injected intraabdominally rather than by the usual subcutaneous route.

Kaliss (5) has reported that when C57BL/Ks mice are given SaI, bled at varying intervals, and the serum tested for enhancing activity, the peak comes

at 21 days. Probably the tardiness of this peak results from the prolonged stimulus provided by a tumor homograft.

The Effect of Varying the Dose and the Kind of Immunizing Tissue.—The preceding experiments have shown that while passive enhancement reduces both the cellular and humoral response to foreign tissue, it does not eliminate them

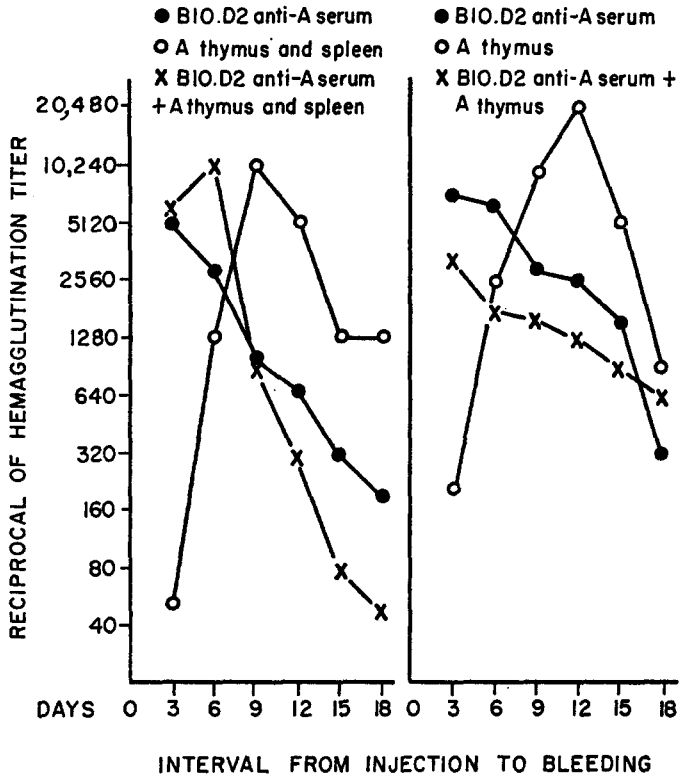

FIG. 4. Graph showing the level of humoral antibody in B10.D2 mice at different intervals after an immunizing injection of strain A tissue given with and without antiserum, and in B10.D2 controls receiving antiserum alone. The results of two separate experiments are shown.

entirely. Whatever the nature of the "block" that antiserum erects, some antigen breaks through it. We have not investigated in detail the conditions that favor a bypassing of the antiserum block, but some evidence has been obtained that both the dose and the kind of tissue are important.

Table IV shows the results of an experiment in which different doses of strain A thymus were given to B10.D2's, with and without passive enhancement, followed by an assay at 7 days of the draining nodes in strain A mice. Serial dilutions were not used; nodes from each group of 10 B10.D2's were pooled and the

cell suspension assayed undiluted in 10 A's. There was tumor growth in all mice, but immune activity in some groups was indicated by a reduction in mean tumor size as compared with the controls.

As expected, the activity of the nodes from the enhanced mice was less than the activity of the nodes from the unenhanced mice. The point of interest here is the effect of dosage. Strain A thymus given at the rate of 1 donor per 10 B10.D2 recipients, or of 1 donor per 2 recipients, did not produce a detectable level of immunity in the enhanced mice. However, the nodes from the enhanced group receiving 2 thymuses each reduced the mean tumor weight from the con-

TABLE IV

Immunity Induced by Various Doses of A Thymus in Draining Nodes of B10.D2 Hosts, with and without Passive Enhancement

A thymus, donors:host	Results of assay			
	Enhanced*		Unenhanced	
	Mean weight	P‡	Mean weight	P‡
	gm.		*gm.*	
2:1	0.75	<0.02	0.63	<0.01
1:2	1.36	0.5	0.31	<0.01
1:10	1.45	0.5	1.00	0.10
None			1.44	

B10.D2's killed and nodes assayed 7 days after injection of thymus. Nodes from each group of B10.D2 mice (10 mice in a group) pooled, and assayed, without serial dilutions, in 10 strain A mice. Tumor dosage for assay: 5,000 cells SaI-A per strain A mouse. Tumors removed and weighed at 19 days.

* 0.5 ml. B10.D2 anti-A given intraperitoneally; thymus given about 1 hour later subcutaneously on the right flank.

‡ Significance of difference as compared with controls.

trol value of 1.44 gm. to 0.75 gm. The difference is probably significant ($P <$ 0.02). A large dose of tissue thus seemed to partly overcome the block imposed by the antiserum.

We have previously reported (11) that the addition of minced, normal strain A lymph nodes to SaI-A prior to implantation in actively enhanced mice will partly overcome the effect of the enhancement. This was tested by growth of the tumor implant, not by a node assay. The A node cells, in this case acting as antigen, are apparently able to break through the block imposed by the antiserum and to induce at least some degree of cellular immunity. As a result there is reduced tumor growth as compared with actively enhanced mice receiving either tumor alone or tumor plus isologous nodes. We have now repeated this experiment under a variety of conditions, including the use of other lymphoid

tissues, and found it usually present though quite variable in degree. It has not been seen in passively enhanced mice, at least at the dose levels used in this particular type of experiment. The difference between passively and actively enhanced mice is perhaps not surprising, since in passively enhanced mice the tumor and nodes constitute a primary stimulus, whereas in actively enhanced mice they are a secondary stimulus.

In a few experiments this phenomenon was checked by a node assay. In one test, the draining nodes of groups of 10 or 20 actively enhanced B10.D2's receiving SaI-A mixed with either B10.D2 (isologous) or A (homologous) nodes, were assayed at 3, 5, and 7 days in A mice. Mean tumor weights for the two groups

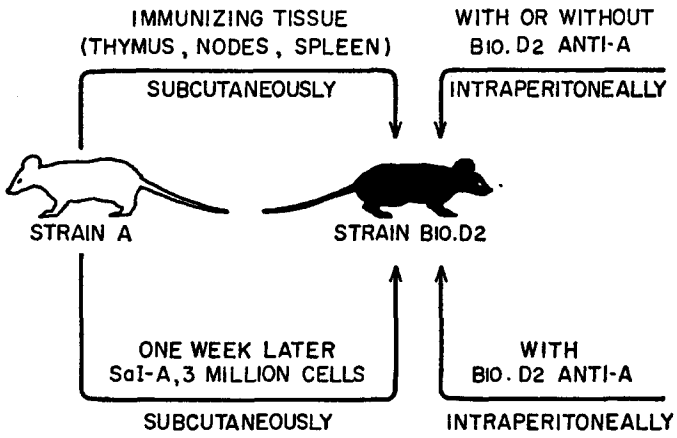

FIG. 5. Diagram showing the method used to estimate the level of cellular immunity in B10.D2 mice given an immunizing injection of strain A tissue, with B10.D2 anti-A either simultaneously or subsequently.

were, respectively, 0.72 gm. and 0.70 gm. at 3 days, 0.63 gm. and 0.21 gm. at 5 days, 0.10 gm. and 0.02 gm. at 7 days. Where A nodes were added to the original tumor inoculum, the assay weights were less, indicating that the added A tissue was effective, despite enhancement of the hosts, in stimulating immunity. The effect was negligible at 3 days, but very clear at 5 days. In another, somewhat similar experiment, there was no significant difference at 7 days.

The Effect of Antiserum Given after a Homograft.—Kaliss has reported that enhanced growth of SaI can be produced in C57BL/Ks mice by the injection of C57BL/Ks anti-A *after* as well as before or at the time of tumor implantation (4, 22). When the antiserum was given 2 days after SaI, all mice succumbed to the tumor; as the interval was increased beyond 2 days, the effect decreased, but some enhancement was still obtained at 10 days. Enhancement was absent at 14 days.

If the role of antiserum in enhancing tumor growth is accomplished through a

"blocking" or depression of cellular immunity, it follows from the above that a block or depression can be initiated *after* the administration of antigen. The direct test of this assumption would be a node assay performed on mice given antigen first and antiserum afterward. As of the present, the only tests we have performed are of a technically simpler and somewhat less conclusive nature. The plan of the experiments is shown in Fig. 5.

B10.D2 mice were given 10 million cells each of strain A thymus subcutaneously, and either on the same day (day 0), or on day 1, 5, or 6, an injection of B10.D2 anti-A intraperitoneally. On day 7 they were given SaI-A, and another injection of B10.D2 anti-A. The pooled results of 3 experiments are shown in Table V. The first two and the last lines are control groups. Of 33 mice receiving tumor alone, 3, or 9 per cent, succumbed; of 29 mice receiving tumor and

TABLE V

Blockage by Antiserum of the Protective (Immunizing) Effect of Strain A Thymus in B10.D2 Hosts, as Tested by a Subsequent Transplant of Strain A Tumor SaI-A

Strain A thymus, cells/ mouse, day 0*	B10.D2 anti-A, intraperitoneally, ml./mouse					SaI-A cells/ mouse, day 7*	Per cent dying (No. of mice in parentheses)
	Day 0	Day 1	Day 5	Day 6	Day 7		
None	—	—	—	—	—	3 million	9 (33)
None	0 or 0.3	—	—	—	0.3 or 0.2	3 "	100 (29)
10 million	0.3	—	—	—	0.2	3 "	67 (30)
10 "	—	0.3	—	—	0.2	3 "	79 (29)
10 "	—	—	0.3	—	0.2	3 "	40 (10)
10 "	—	—	—	0.3	0.2	3 "	30 (10)
10 "	—	—	—	—	0.2 or 0.3	3 "	3 (29)

* Given subcutaneously on the right flank.

antiserum (passive enhancement), all succumbed. When A thymus was given 1 week before tumor and antiserum, an effective (presumably cellular) immunity was induced which overrode the enhancing treatment given with the tumor; only 1 of 29 mice succumbed. The remaining 4 groups test the effect of antiserum given with or after the immunizing injection of thymus. The per cent deaths were 67, 79, 40, and 30 for antiserum given on days 0, 1, 5, and 6. By this test, antiserum was at least as effective in blocking the immunity (presumably cellular) induced by foreign tissue when given 1 day after the tissue as when given on the same day as the tissue. It was also effective to a limited extent on days 5 and 6, even though the test of immunity (SaI implant) was given on day 7, allowing an interval of only 1 or 2 days for the antiserum to produce its effect. It may be noted that nodes assayed by the method of Winn on day 5 after an injection of thymus (15 million cells) show an appreciable level of immunity (Table I), probably quite sufficient to inhibit the growth of some millions of tumor cells when the two types of cells are mixed and implanted together, and immunity has also been found on day 4. Why, we may ask, does the injection of antiserum after this high level of immunity has been reached, result in the growth of SaI in at least some of the mice subsequently implanted therewith? While we cannot give a conclusive answer to this question at the present time, the following would seem to provide a plausible interpretation of this type of result.

Following the injection of A thymus into B10.D2 recipients, the immune mechanism of the recipients is set in motion, and beginning on day 3 or 4 there is a generous and active production of immune lymphocytes. This reaches a peak in the draining nodes on about day 5, but in the absence of antiserum would continue at a high level in these nodes and in the spleen (unpublished data) for some days longer. This production is terminated abruptly by the injection of antiserum. The immune lymphocytes as they are released from the nodes and spleen then have two targets to attack, first, the 10 million strain A thymus cells which constituted the immunizing injection and many of which are probably still distributed through the tissues of the host, and second, the 3 million tumor cells which were administered on day 7. Much of their energy is diverted from the tumor, a diversion that would not occur if the nodes were removed for assay. When operating *in vivo*, also, the immune cells must move from the nodes or spleen to the tumor, a small target in comparison with the total body mass. In the *in vitro* test, the cells are directly mixed with the target. These three factors, the termination by antiserum of the immune process, the diversion of immune cells from the tumor by the remnants of the immunizing tissue, and the need of searching out the target, are sufficient to account for the occasional progressive tumor growth that occurs under conditions in which an *in vitro* test would show sufficient immunity in the sum total of the lymphoid tissues to cause regular inhibition.

DISCUSSION

This paper is concerned with two hypotheses that have been proposed to explain why antiserum causes enhanced growth of tumor homografts in certain tumor-host combinations. According to the first hypothesis (4), the antiserum modifies the tumor, according to the second (11), it in some way blocks the development of cellular immunity (which is the kind of immunity necessary for the destruction of most homografts) in the draining lymph nodes and other lymphoid organs, thereby permitting the tumor to grow.

Kaliss (4) has reviewed the evidence for the first hypothesis. Several investigators (4, 23, 24) have observed improved growth, usually limited in extent, when tumors are retransplanted from mice that have been actively or passively enhanced. In testing this hypothesis, we obtained negative results; tumors homografted from the native strain grew just as well, on the average, as tumors homografted from enhanced mice. The fact that modification of the tumor is not a consistent phenomenon throws considerable doubt on the modification hypothesis. However, it is pertinent to consider why alteration, or apparent alteration, does sometimes occur.

Tumors are complex, biological entities, and changes, fluctuating or permanent, due to a variety of causes, are possible. However, there is a very simple possible explanation of the effect of successful passage through a mouse of foreign strain that should be eliminated before more indirect explanations are invoked. This may be briefly stated as follows. An A tumor grown in A's usually contains a considerable population of strain A leukocytes. Leukocytes are particularly rich in isoantigens (3, and Winn, unpublished data), and they are mobile cells which tend to leave the site of a graft and enter the lymphatic circulation from which they can reach the lymph nodes or spleen. They are thus par-

ticularly effective, as antigen, in evoking homograft immunity (3, 11, 16). When the same tumor is passed through a foreign strain, its population of leukocytes becomes that of the foreign strain. SaI-A grown in enhanced B10.D2's acquires a B10.D2 leukocyte population. This tumor will evoke a lower immunity in B10.D2's than a tumor taken directly from A's, and if the possibility for growth is close to a threshold for this particular tumor-host combination, some progressive growth may be expected. Some evidence pertinent to the role of lymphoid cells as antigen is presented in the section of this paper dealing with the effect of varying the dose and the kind of immunizing tissue. One of a number of other unpublished experiments is pertinent and may be cited. SaI-A, with and without added A thymus, was transplanted in B10.D2's. The SaI-A without added A thymus gave palpable tumors which reached a size of ½ to 1 cm. in diameter and then regressed. The SaI-A with added thymus gave tiny tumors in 3 out of 10 mice, none in the others. This illustrates the effect of added lymphoid cells isologous with the graft. Experiments from other laboratories, which we have reviewed elsewhere (3), indicate that removal of lymphocytes from a graft will increase its chance of survival. Passage of a tumor through enhanced mice of a foreign strain is a simple method of accomplishing this removal, or at least the removal of lymphoid cells with the antigens necessary for inciting a homograft reaction in the particular foreign strain.

The observation of Kaliss (4) that small pieces of SaI (solid form) immersed overnight in appropriate media show enhanced growth in C57BL/Ks can be explained in the same way. Lymphoid cells are relatively mobile, and would tend to migrate from the graft. This would reduce the immunizing potential of the graft.

This effect of lymphoid cells as antigen is not necessarily the correct explanation of the results under discussion, but at least it should be ruled out before more complex explanations are invoked.

A way in which an anti-tumor antiserum might change the growth characteristics of a tumor is through changing its bacterial or viral flora. Many, perhaps most, tumors carry viruses as passengers. There are reports of using antisera to eliminate these. In general, passenger viruses depress the growth potential of a tumor (18), so treatment with an antiserum might improve its growth. Such improvement would be irrelevant to the enhancing phenomenon.

Evidence presented in this paper shows that the administration of antiserum to B10.D2 mice regularly and consistently lowers the level of immunity which the mice develop following a homograft. B10.D2 anti-A given simultaneously with an SaI-A homograft depressed the cellular immunity in the draining nodes at 6 days after tumor implantation to 1/24th that of the controls in one experiment, and to less than 1/32nd that of the controls in another. The difference was present, though somewhat less, at several other intervals tested. The im-

munizing stimulus need not be tumor; the same sort of depressing effect of antiserum on cellular immunity was found when the immunizing tissue was strain A thymus or embryo. The production of humoral antibody was also partly blocked by injected antibody, and so was the increased cell yield from draining nodes and the increased spleen weight which are normal consequences of a homograft.

Since cellular immunity is responsible for the rejection of SaI and most other non-leukotic homografts, the effect of antiserum on this type of immunity is quite sufficient to explain the enhancing phenomenon. It also accounts for the fact that successful enhancement of normal tissue grafts has sometimes been achieved (see reviews in references 3 and 4). It should be emphasized that the "blockage" produced by antiserum is not complete; in particular a rather high level of cellular immunity was found 9 days after an SaI homotransplant to enhanced B10.D2's. A complex balance between antibody, cellular immunity, and the nature and current state of a particular homograft must determine its eventual outcome.

It remains to be determined where the "block" set up by the antiserum occurs. At least three possibilities must be considered. (*a*) The antiserum may prevent, or partially prevent, the antigen from reaching the regional nodes. An apparent obstacle to this hypothesis is that antiserum is effective when given *after* the homograft. This may not be as serious an objection as at first appears. This effect can be explained if we assume that cellular immunity develops only so long as antigen is feeding into the nodes, (or spleen), and terminates abruptly when the stimulus terminates. Humoral antibody production would presumably continue for a much greater interval, and this would be favorable to enhancement. We must also assume, when the graft is a normal tissue such as thymus, that a deposit remains for some time at the implantation site and, in the absence of antiserum, continues to feed antigen into the nodes. A variant of this alternative would be the assumption that, in the presence of antibody, the antigen does not reach the lymph nodes, or does not reach them in effective form, because antibody is able to accomplish the destruction or degradation of phagocytized isoantigens. (*b*) The antigen may reach the nodes in combination with antibody and in this form may provide an ineffective stimulus. Published evidence on antigen-antibody complexes might be cited either for or against this hypothesis. (*c*) The block may be of some more central and as yet entirely undetermined nature. It would be premature to attempt a choice between these hypotheses.

Finally, this study emphasizes the potency and importance of specifically immune cells in the homograft reaction. It seems probable that both immune and non-immune cells accumulate in and around a homograft. Antibody, by touching off an "alarm" system, might actually accelerate the accumulation of non-immune cells. Histiocytes, presumably non-immune, are present early and in

large numbers in enhanced mice bearing certain tumors (25). Destruction of the graft presumably depends on the development and arrival of immune cells. This is blocked by the enhancing treatments.

SUMMARY

This paper reports tests of two hypotheses that have been proposed to account for the enhanced growth of tumor homografts in the presence of antiserum reactive with the graft (immunological enhancement). According to the first hypothesis, enhancement is due to some "physiological" alteration in the tumor, induced by its contact with antiserum, which insures its survival despite the hostile response of the host. According to the second hypothesis, antiserum alters the response of the host. By blocking the development of the cellular type of immunity, which is the main agent in graft destruction, it permits the graft to survive.

To test hypothesis 1, strain A tumor SaI was passed from A's, and from enhanced B10.D2's, into untreated B10.D2's. The per cent of deaths was essentially the same in both groups (48 and 44 per cent, respectively); there was no evidence that passage through enhanced B10.D2's altered the capacity of the tumor to grow in the foreign strain. Several other groups of mice included in the experiment all confirmed this conclusion. The experiment failed to confirm hypothesis 1.

In the tests of hypothesis 2, the effect of isoantiserum on immune responses of both the humoral and cellular type was measured. When antiserum was given together with foreign strain lymphoid cells (antigen), almost no additional antibody was manufactured; in contrast with this, controls receiving foreign cells only produced red cell agglutinating antibody in high titer.

The effect of antiserum on the development of immunity of the cellular type was tested by the method of Winn. In this assay, presumptively immune node cells, in various dilutions, are mixed with tumor cells and injected into appropriate mice. Immunity is indicated by inhibited tumor growth. Antiserum given at the same time as a tumor homograft greatly depressed the immunity of the cells expressed from the draining nodes. At 6 days after the graft, the level of immunity of cells from treated mice was 1/24th to 1/32nd that of cells from controls receiving tumor alone. The same sort of depressing effect was noted when the immunizing tissue was foreign thymus or embryo. Antiserum given 1 or more days after the immunizing tissue also resulted in a lower level of cellular immunity (but the assay used in this case was a less critical one).

These results provide an adequate explanation of the phenomenon of immunological enhancement, at least as it occurs in the particular test system used in these experiments. Since it is cellular immunity rather than humoral antibody that inhibits the growth of most grafts (transplantable leukemias are an excep-

tion), the depression of this immunity by antibody is favorable to the growth of a homograft.

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