totoxic isoantibody *in vitro*. Similar results may also be obtained with small inocula of Sa 1 *in vivo*. In view of these results, it no longer seems possible to retain the past distinctions based on differences in isoantibody sensitivity between tumors of different origins.

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Passive Transfer of Immunity to Sarcoma I with Serum.* (27763)

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It has not previously been possible to demonstrate passive serum transfer of immunity to Sarcoma I, a transplantable tumor derived from the inbred A strain of mice. When transplanted into these mice or into F_1 hybrids of this strain, this tumor grows progressively and kills the animals, but when transplanted as an allogeneic homograft into mice of other strains it grows only for a limited time and then is rejected by a typical homograft reaction(1,2). Mice which have rejected this tumor are generally found to be specifically immune to subsequent implantation(3). Although the rejection is accompanied by a brisk humoral antibody response (4), previous attempts to demonstrate an *in vivo* or *in vitro* cytotoxic effect of these antibodies on the tumor cells have been negative; in fact, antisera obtained from mice immune to Sarcoma I frequently have been found to produce "enhancement" of growth of this tumor in passive transfer experiments(1-5). This has led to the concept that cells of Sarcoma I are qualitatively "antibody-resistant" and that the homograft reaction against such cells must be mediated by some aspect of the immune response other than isoantibody(1-7).

Improvements in technic, however, have permitted the recent demonstration(8-10) that some cell lines previously thought to be resistant to the cytotoxic effect of isoantibody and complement are, in fact, fully susceptible. It has indeed become of some consequence to determine whether any cell is truly "antibody-resistant," and in the present study the effect of isoantibody on the cells of Sarcoma I has been reinvestigated.

Materials and methods. Male mice of the strains A/Jax, A/HeJ and of the cross (C57Bl/6 × DBA/2), and female mice of the C57Bl/6 strain, were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor. All mice were vaccinated against ectromelia one to 2 weeks before use and were caged in groups of 10 or less before and during experiments. Mice of approximately 25 g in weight were used after randomization into experimental and control groups; all were individually numbered by coded ear punches.

The Sarcoma I was obtained in the ascites form from Dr. George Snell, and was main-

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tained by transfer (every 4 to 6 days) of 3 × 10^6 cells in a volume of 0.5 ml physiological saline solution containing 2 units of heparin per ml, given intraperitoneally to A/Jax or A/HeJ mice. Subcutaneous tumors for experimental observation were made by subcutaneous inoculation of 0.05 ml heparinized physiological saline solution containing 10^6 cells. These inoculations resulted in "takes" in over 95% of the (C57Bl/6 × DBA/2) hybrids used throughout as test animals; the tumors were inspected and measured daily and recordings were made of the greatest diameter (in mm) of each tumor and of the diameter at right angles to this.

For production of antisera, (C57Bl/6 × DBA/2) mice were inoculated with 10^6 Sarcoma I cells as above and those which showed growth of tumor followed by complete rejection subsequently received 3 or 4 intraperitoneal booster injections at intervals of 3 weeks. The first booster injection contained 3-5 × 10^6 cells, the second consisted of 8-10 × 10^6 cells, the third of approximately 20 × 10^6 cells and, when a fourth booster injection was given, a dose of 20-30 × 10^6 Sarcoma I cells was used. Mice were bled from the retro-orbital plexus at intervals, usually on the eighth day after each booster injection, and sera were stored frozen until used.

Target cells for *in vitro* demonstration of the cytotoxic effect of antisera were either lymph node cells obtained from A/Jax or A/HeJ mice or Sarcoma I cells from ascites fluid. Cells were suspended in the tissue culture medium 199 (Microbiological Associates, Bethesda) to a concentration of about 5 × 10^3 cells/cu mm and 0.1-ml quantities of the cell suspension were incubated with 0.1 ml aliquots of the antisera dilution for 30 minutes at 37°C. Guinea pig complement (Vacseal, Probio, Nyack) in a volume of 0.1 ml was then added to each tube and incubation continued for an additional 30 minutes with shaking. Then 0.3 ml trypan blue solution(8) was added to each tube, aliquots of the mixture were transferred to glass slides and the percentage of killed (*i.e.*, stained) cells was estimated under the microscope. Serum samples of comparable cytotoxic activity were combined to provide pools showing strong, moderate or weak activity.

For passive immunization experiments, antiserum from these pools was administered both intraperitoneally and locally with the inoculum of Sarcoma I cells. Each mouse received an inoculum of 0.1 ml consisting of 0.05 ml antiserum plus 0.05 ml saline solution containing 10^6 tumor cells, given subcutaneously, followed immediately by an intraperitoneal injection of 0.45 ml of the same antiserum. Control animals received similar injections in which the immune serum was replaced by normal serum or by physiological saline solution.

Results. Preliminary experiments established that subcutaneous inoculation of 10^6 Sarcoma I cells was required to produce palpable, visible subcutaneous growths of tumor in approximately 95% of recipient hybrids of the strain used. These inoculations were made in the flank close to the costal margin. Irregular edematous reactions were often visible at the site of inoculation on the first and second days thereafter. These early reactions, presumably inflammatory in nature, subsided quickly, and definite tumor growth was then usually apparent by inspection and palpation on the third or fourth day after inoculation. The tumors then grew progressively until the 6th to 10th day, by which time most had begun to regress. In about 70% of our animals, complete regression of the tumor had occurred by the 17th day; however, in the remaining 30% of animals the tumors showed only partial regression and resumed progressive growth by the beginning of the third week. Fig. 1 illustrates these two patterns of response; the existence of a powerful immune mechanism, operating during the second week after tumor inoculation, is apparent in both groups of animals. Mice which had accomplished complete rejection of Sarcoma I were generally immune to reinoculation with this tumor. The intraperitoneal booster inoculations, given 21 days after the initial subcutaneous inoculation and about a week after the subcutaneous tumor had regressed, usually resulted in no visible ascites tumor development although normal mice of this strain were fully susceptible to inoculation by this route.

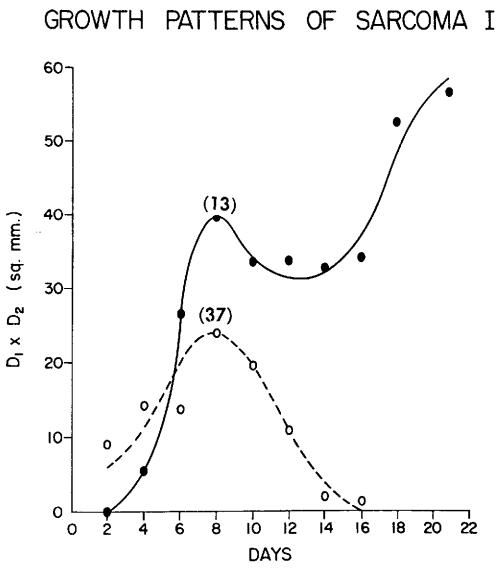

FIG. 1. Of 50 mice given inocula of Sarcoma I, 37 showed complete regression of tumor while the remainder showed persistent or progressive growth of tumor by the 21st day. Avg tumor size (expressed by $D_1 \times D_2$, the product of largest diameter of the tumor and diameter at right angles to this) throughout this period is shown for these 2 groups of animals.

It is known that isoantibodies, capable of agglutinating donor strain erythrocytes, appear in the serum of mice rejecting Sarcoma I (4,6,7). Recent evidence indicates that hemagglutinating mouse isoantibodies are also capable of sensitizing donor strain lymphocytes or other nucleated cells for lysis by complement(11). Table I indicates that sera of mice which have rejected Sarcoma I homografts are indeed cytotoxic for lymphocytes of A strain mice. Serum samples obtained during the first 2 weeks after primary implantation of Sarcoma I showed no cytotoxic activity by the method used, but definite cytotoxicity was demonstrated in more than half of the samples obtained during the third and fourth weeks. It was frequently noted that, within a given experiment, those animals showing persistent or progressive growth of tumor during the third and fourth weeks after inoculation tended to have appreciable cytotoxic antibody levels in their sera at that time, while those animals which had completely rejected their tumors usually had little or no cytotoxic antibody. It is possible

TABLE I. Cytotoxic Antibody Response to Inoculation of Sarcoma I.

Serum No.	Antigenic stimulus	Interval* (days)	Cytotoxic effect at serum dilution 1:5	1:10	1:50	1:250	1:1,250
48	Primary	6	B†	B	—	—	—
55	"	9	B	B	—	—	—
57	"	14	B	B	—	—	—
19	"	20	15	B	—	—	—
21	"	20	25	B	—	—	—
22	"	20	85	—	—	—	—
12	"	27	B	B	—	—	—
13	"	27	95	24	24	B	—
14	"	27	40	B	—	—	—
59	1st booster	10	90	100	100	50	—
60	"	10	85	100	50	20	B
34	"	14	35	B	—	—	—
28	"	8	B	—	—	—	—
31	2nd booster	8	50	—	—	—	—
4	"	8	100	100	65	B	—
47	"	8	—	95	85	20	B
54	3rd booster	8	—	100	100	95	B
58	"	17	—	100	100	100	—
67	"	30	—	100	100	100	—
80	"	39	—	100	100	90	—
83	"	43	—	100	95	85	—
10	4th booster	8	100	100	100	80	—

* Interval between last inj. of tumor and day of bleeding.

† Percentage of lymphocytes killed. B: background count, observed in control tubes (usually 5%-15%). — signifies "not done." These are representative titrations chosen to illustrate the range of antibody response observed during courses of immunization with Sarcoma I.

that the considerably larger and prolonged antigenic stimulation experienced by the former mice may account for this difference.

There was an appreciable rise in cytotoxic antibody titer after the first booster injection, and subsequent booster injections usually resulted in progressively higher antibody responses. Some of the sera showing strong cytotoxic activity against A lymphocytes were tested against Sarcoma I cells and were found to kill these cells as well, although much more antibody was required to kill a given number of Sarcoma I cells. This finding was not pursued further, as other investigators have made a detailed study of the sensitivity of Sarcoma I cells to cytotoxic isoantibody(12).

Having shown that high serum levels of cytotoxic antibody could be achieved by repeated antigenic stimulation with Sarcoma I cells, attempts were next made passively to transfer immunity with such serum. The results of 4 experiments are presented in Table II. In view of the negative results of previous experiments along this line(1-5), it was surprising to find that the immune serum consistently afforded a pronounced degree of protection against the transplanted tumor. In each experiment, tumors failed to grow at all in most of the passively immunized hosts. As can be seen in the data from Experiments 2 and 3, smaller amounts of antibody apparently produced the well-known phenomenon of immunological enhancement of tumor growth. In those experiments, most of the tumors in controls showed initial growth followed by regression, while in the animals given small amounts of antibody nearly all of the tumors showed delayed onset of growth but then grew progressively.

In these transfer experiments, only mice of the C57Bl/6 line and (C57Bl/6 $\times$ DBA/2) hybrids were used, and the immune sera were always prepared in the latter strain. In similar experiments, others(12) have used different strain combinations, suggesting that the present findings are not peculiar to a particular set of experimental circumstances. It will be of interest, however, to determine whether the antibodies involved are directed against the H-2 isoantigens and whether the protective effect fulfills immunogenetic predictions (13).

Discussion. Much of the weight of the argument against the central role of antibody in effecting homograft rejection(1,2,4-7) has been based on negative findings: failure to detect antibodies in the sera of homograft-immune animals, failure passively to transfer graft immunity with serum, failure to observe destruction of homografts in cell-impenetrable diffusion chambers, and failure to demonstrate susceptibility of certain cell types to the cytotoxic effect of isoantibody and complement. In particular, much has been made of the failure of isoantisera from mice immune to Sarcoma I to passively confer immunity upon normal hosts. This tumor is usually cited as the prototype of tumors whose growth is "enhanced" rather than inhibited by isoantibody(2-7).

The present study and other current work (12) serve to bring the behavior of Sarcoma I into line with that reported for other transplantable neoplasms: its growth is enhanced by small quantities of isoantibody and inhibited by larger doses. Earlier failures to demonstrate unequivocal inhibition of growth in passive transfer experiments almost certainly have been due to the relatively weak antisera or to the small doses used. Sarcoma I cells are evidently considerably more resistant than normal lymphocytes or leukemia cells to cytotoxic antibody and complement, and quite large doses of potent antisera were required to obtain the results noted in this study. Also, the Sarcoma I line used here was an ascites variant, and it has often been suggested that dissociated cells in suspension may be more readily accessible to antibody and complement than are cells in solid tissues.

The chief significance of these findings does not lie in the demonstration that immunity to a tumor homograft can be passively transferred with serum, as this result has been achieved many times before with other tumors (14); rather, it lies in the demonstration that this tumor, which had been thought to be "antibody-resistant," is apparently only quantitatively less susceptible than, for example, the transplantable leukemias. This suggests that the isoantibody response may be suf-

ficient to account for the rejection of homografts of this tissue, and raises the possibility that the "adoptive" immunization achieved by lymphoid cell transfer(4) may be due to the isoantibody produced by these cells.

Summary. Successful passive transfer of immunity to Sarcoma I has been achieved with immune serum. The effect of isoanti-

TABLE II. Passive Transfer of Immunity to Sarcoma I with Serum.

Exp. No.	Serum No. (Table I)	Mouse No.	Size of tumor (D_1/D_2) on day											
			4	5	6	7	8	9	10	11	12	13	14	21
1	10	1	0	0	0	0	0	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	3	6/4	5/4	6/6	5/4	5/4	5/4	3/3	2/2	2/2	0	0	0
		4	8/2	5/3	7/4	8/5	8/5	8/5	8/5	6/5	6/5	5/5	5/5	8/7
		5	5/5	5/5	5/5	6/6	5/5	4/4	3/3	2/2	2/2	0	0	0
		6	4/2	5/3	5/4	8/5	6/6	8/8	9/6	8/6	8/6	8/6	10/8	22/11
2	10	7	0	0	0	0	0	0	0	0	—	0	0	0
		8	0	0	0	0	0	0	0	0	—	0	0	0
		9	0	4/3	5/4	7/5	7/5	8/5	9/6	8/8	—	11/9	11/9	12/9
	10 (1:5)	10	0	2/2	3/2	4/3	4/3	3/3	3/3	4/3	—	7/5	8/6	12/9
		11	0	0	0	4/4	5/4	7/5	8/6	9/7	—	11/8	12/8	15/11
		12	0	0	0	3/3	4/3	4/4	4/4	5/4	—	5/4	5/4	8/7
	NaCl	13	5/5	6/5	6/6	6/5	7/7	8/7	8/7	8/8	—	7/6	7/6	3/2
		14	5/3	5/3	6/5	5/4	7/5	8/5	8/5	9/7	—	8/6	6/6	5/2
		15	6/3	5/3	6/4	6/5	7/5	8/5	9/6	11/8	—	12/8	12/9	13/12
		16	4/3	5/2	4/4	4/4	5/4	4/3	5/4	4/3	—	3/2	2/2	0
		17	5/4	5/4	5/4	5/5	6/5	7/5	7/5	6/5	—	7/6	6/5	11/9
		18	5/3	6/4	6/4	6/5	7/5	7/5	6/5	6/5	—	5/5	5/4	3/3
3	Pool 11 (4, 47 & 54)	19	—	—	0	0	—	0	—	0	—	0	—	0
		20	—	—	0	0	—	0	—	0	—	0	—	0
		21	—	—	1/1	0	—	0	—	0	—	1/1	—	7/5
	Pool 16 (12, 19, 28, 48, 14, 21, 34 & 57)	22	—	—	0	3/3	—	3/3	—	3/3	—	4/4	—	6/5
		23	—	—	2/2	5/4	—	5/5	—	6/5	—	8/6	—	5/5
		24	—	—	1/1	4/4	—	4/3	—	4/4	—	4/4	—	6/5
		25	—	—	0	4/3	—	4/3	—	4/4	—	5/5	—	9/6
		26	—	—	0	3/2	—	3/2	—	3/3	—	4/4	—	7/7
	Normal serum	27	—	—	1/1	1/1	—	0	—	0	—	0	—	0
		28	—	—	5/4	6/4	—	8/5	—	4/4	—	13/10	—	16/13
		29	—	—	4/3	5/4	—	4/3	—	2/2	—	0	—	0
		30	—	—	2/2	1/1	—	0	—	0	—	0	—	0
		31	—	—	3/3	3/3	—	2/2	—	1/1	—	0	—	0
4	Pool 17 (58, 67, 80 & 83)	32	0	—	0	0	0	0	—	0	0	0	0	0
		33	0	—	0	0	0	0	—	0	0	0	0	0
		34	0	—	0	0	0	0	—	0	0	0	0	0
	Pool 18 (13, 22, 31)	35	5/4	—	7/5	9/5	9/6	7/6	—	4/3	0	0	0	0
		36	0	—	0	0	0	0	—	0	0	0	0	0
		37	0	—	0	0	0	0	—	0	0	0	0	0
		38	3/2	—	5/5	5/5	4/4	4/4	—	0	0	0	0	0
	Pool 16 (as above)	39	5/4	—	6/5	6/5	5/5	5/5	—	0	0	0	0	0
		40	3/3	—	5/4	5/5	4/3	4/3	—	1/1	0	0	0	0
		41	6/5	—	6/6	7/6	6/6	5/5	—	0	0	0	0	0
		42	4/3	—	4/4	5/3	4/3	2/2	—	0	0	0	0	0
	Normal serum	43	5/3	—	6/4	6/4	6/3	4/2	—	0	0	0	0	0
		44	2/2	—	4/3	4/3	5/3	5/2	—	0	0	0	0	0
		45	4/4	—	5/4	5/4	5/4	4/4	—	0	0	0	0	0
		46	4/4	—	5/5	5/5	5/4	4/3	—	0	0	0	0	0
	NaCl	47	4/4	—	5/5	5/5	4/3	4/3	—	0	0	0	0	0
		48	4/4	—	7/6	7/6	6/5	5/5	—	4/3	4/3	0	0	0

Serum 10 and pool 17 (containing the sera indicated) were of high cytotoxic potency and pool 11 was only slightly less cytotoxic *in vitro*. Pool 18, however, was strongly cytotoxic at dilutions of 1:5 or less and pool 16 showed only weak cytotoxicity at this dilution. Mice 1-31 were (C57Bl/6 × DBA/2) male hybrids, while mice 32-48 were C57Bl/6 females.

body on this tumor appears to be qualitatively similar to that reported for other transplantable tumors, and may account for the rejection of Sarcoma I homografts.

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Separation of Bound and Free Vit. B_{12} on Sephadex G-25 Column.* (27764)

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In the study of Vit. B_{12} metabolism and intrinsic factor the determination of free and bound Vit. B_{12} is of primary importance. Several methods have been developed for separating free from bound Vit. B_{12}, among the most popular of which are those of dialysis (1,2) and ultrafiltration(3) with the use of radioactive Vit. B_{12}. There are some disadvantages, however, to the use of these technics, such as dissociation of bound Vit. B_{12} (4) and partial denaturation of the binder during the procedure, as well as variability of results, depending on the variant of the technic used.

Gel filtration on Sephadex columns has recently been introduced(5-7) to achieve a 'molecular sieving' of materials of different molecular size. This has been used in our laboratory for fractionation of large molecular weight materials in gastric juice for the last 2 years(8). The molecular weight of Vit. B_{12} is about 1,300, and the molecular weight of Vit. B_{12} binders has consistently been found to be above 5,000(9). It occurred to us therefore that Sephadex G-25, which excludes materials of molecular weight over 3,500-4,500, would be well suited for separation of free from bound Vit. B_{12}. While this work was in progress, Daisley reported the applicability of Sephadex to the study of Vit. B_{12} in ocean water(10). A preliminary report of our work has been presented and published in abstract form(11).

Materials and methods. Sephadex G-25 columns were prepared according to instructions of Porath and Flodin(5-7). 6-20 g of dry Sephadex was suspended in borate buffer, pH 9.0, $\Gamma/2$ ranging from 0.06 to 0.24, and poured into the columns. The column sizes ranged from 0.8-1.7 by 32.0-50.0 cm and their total bed volumes were from 32.0-114.0 ml. The "void volumes" (Vo)(7) were from 11.0-43.0 ml and the 'inner volumes' (Vi) from 13.0-46 ml. Various amounts of dialyzed and lyophilized normal human gastric juice, ranging from 10 mg dissolved in 1.0 ml of buffer, to 100 mg dissolved in 14.0 ml of buffer, were applied on top of the column and eluted with the same buffer in which it was dissolved. The rate of elution was from 3.0-10.0 ml per hour, and the size of fractions collected was from 1.0-3.0 ml, depending on

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