STUDIES ON THE IMMUNOLOGICAL RESPONSE TO FOREIGN TUMOR TRANSPLANTS IN THE MOUSE

II. The Relation between Hemagglutinating Antibody and Graft Resistance in the Normal Mouse and Mice Pretreated with Tissue Preparations*

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In preceding papers (1–3) it has been shown that heightened resistance to certain tumor homografts in mice can be transferred to secondary hosts by lymph node cells, but not by serum. Similar results have been reported by Billingham, Brent, and Medawar (4), with resistance to homografts of skin. Nevertheless antibodies against the foreign tissue have been repeatedly demonstrated in the serum of mice in which homografts have regressed. Gorer (5–7) has shown that erythrocytes which share antigens in common with a regressed tumor are agglutinated by antibody in the serum, and similar hemagglutinins have been recently reported after sloughing of skin homografts (8). Gorer (9) working with leukemic cells, and Billingham and Sparrow (10) working with skin epithelial cells, have also shown that serum antibody can combine with these cells *in vitro* and inhibit their growth on subsequent transplantation into susceptible hosts.

The relation between serum antibody and graft resistance is the subject of further investigation in the present work. Particular attention has been paid to the hemagglutinating antibody produced in C57BR/a mice in response to homografts of a sarcoma, Sarcoma 1 (SA1). The production of this antibody has been followed in actively immunized mice, and also in mice which have received lymph node and spleen cells from immunized donors. Comparisons have been made between antibody titres and resistance to test grafts of tumor.

The experiments have been extended to a study of the immunological response in mice which have been treated with lyophilized or frozen tissue preparations. The growth of tumor homografts is enhanced by this pretreat-

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ment, in certain combinations of tumor and host. Previous findings on this subject have been reviewed by Snell (11, 12). The report of Kaliss and Molomut (13) that hemagglutinating antibody is elicited by this pretreatment, together with findings of remarkably high titres of hemagglutinin in pretreated mice bearing progressively growing tumor homografts (6, 14), suggested that use could be made of the pretreatment to separate hemagglutinin production from graft resistance. The production of hemagglutinin, the response of the regional lymph nodes to homografts, and the protection conferred by non-pretreated lymph node cells, have accordingly been investigated in pretreated mice.

Materials and Methods

The strains of mice, the transplantable tumors, and the procedures used for transferring lymph nodes have been described in the previous paper (3).

Serological Methods.—For agglutination of erythrocytes the technique of Gorer and Mikulska (8) was followed. The sera were diluted in saline containing 2 per cent dextran (average molecular weight 80,000). The dextran was dissolved directly in the saline and stored at 4°C. without being autoclaved. The red cells were suspended in suitably absorbed human serum. In view of the variation in potency noted by Gorer, a single sample of serum was used throughout the present work. In other respects the agglutination technique is the same as that used in *rhesus* typing.

Methods Used for Enhancing the Growth of Tumor Homografts.—Two procedures were used for enhancing the growth of tumor homografts by pretreatment of the host with tissue preparations. Following Kaliss and Snell (15), lyophilized A splenic tissue was suspended in saline and injected intraperitoneally in 6 equal amounts at intervals of 2, 2, 3, 2, and 2 days, each mouse receiving a total of 6 mg. lyophilized tissue. Following Snell (16), frozen Sarcoma 1 (SA1) was homogenized, suspended in saline, and centrifuged for 1 hour at 16,000 *g*. Four equal amounts of the supernatant fluid were injected intraperitoneally, at intervals of 3, 4, and 3 days, each mouse receiving the equivalent of 40 mg. lyophilized tissue. The pretreated mice were of the C57BL/6Ks, C57BR/a, and B10.D2 strains. SA1 was employed as the test tumor after pretreatment. Thus an $H\text{-}2^a$ tumor was tested in $H\text{-}2^d$ or $H\text{-}2^k$ hosts; this is the combination which has been most fully investigated (12), and in which pretreatment has been found to be most effective.

Experimental

Serum Cytotoxin against SA1

An isoantibody against SA1 was demonstrated by a modification of the method of Gorer (9). An example of cytotoxic activity is shown in Table I in which serum taken from C57BR/a mice 5 days after a second injection of SA1 was under test.

Portions of a suspension of SA1, containing 180,000 cells 0.1 ml., were incubated with equal volumes of normal C57BR/a serum or the serum under test, for 30 minutes at 37°C. The suspensions of cells in normal and test serum were implanted subcutaneously in the opposite flanks of 5 C57BR/a and 5 A hosts, at a dosage of 0.1 ml./implantation. Eight days later the tumor growths were excised and weighed, and the paired weights of those from each host are shown in the table.

Greater growth of both antiserum-treated and control cells were found in the susceptible hosts. Growths from cells treated with antiserum were smaller than the controls treated with normal serum, although the difference was slight. The effect has been observed repeatedly, but with findings that were not strictly quantitative. Sera from mice grafted only once with tumor were particularly variable.

Hemagglutinin Production in C57BR/a Mice Receiving SA1

Sera from C57BR/a mice immunized with SA1 regularly and repeatedly agglutinated the red blood cells of the strain of origin of the tumor, so attention was centered on the role of this type of antibody in graft breakdown. Curves of hemagglutinin production following trocar implantation of SA1 are given in Fig. 1, the geometric means of the titres of individual sera being shown.

TABLE I

The Effect on Their Subsequent Growth of Incubation of SA1 Cells with Serum from Immunized C57BR/a Mice

Weight of tumor growths after 8 days in milligrams.

In Susceptible (A) Hosts					
Cells incubated with antiserum (right flank)	130	100	64	49	50
Cells incubated with normal serum (left flank)	152	101	108	66	69
In Non-Susceptible (C57BR/a) Hosts					
Cells incubated with antiserum (right flank)	0	7	0	8	2
Cells incubated with normal serum (left flank)	2	49	7	11	7

The response shown in previously immunized mice was obtained 17 weeks after a first implantation of SA1. The peak of hemagglutinin production was reached at 15 days, or later, after the first implantation of tumor, and at 7 days after the second. The peaks were thus reached after the immunizing homografts had all died: the median survival time of a first graft of SA1 in C57BR/a mice was 10.8 days, and of a second 5.5 days (3).

Hemagglutinin Production by the Regional Lymph Nodes and Spleen

The ability of the lymph node and spleen cells of immunized mice to transfer hemagglutinin production into secondary hosts was next tested.

In two series of experiments regional lymph nodes and spleens were taken at 5 day intervals after implantation of C57BR/a donors with SA1 by trocar. At each interval the lymph nodes were minced and transferred to 4 C57BR/a hosts, at a dosage of 4 donors per host, and the spleens were transferred to 4 hosts at a dosage of 1 donor per host. The hosts were bled at intervals of 1, 4, 8, 15, and 30 days after transfer, and the sera titrated for hemagglutinin.

Antibody was duly found, the geometric means of the individual titres being shown in Fig. 2.

The appearance of antibody in the serum of the hosts can hardly be due to transfer of preformed antibody in the lymph node or spleen cells, in view of the course of the rise in titre, and the height reached. Titres rose consistently between 1 and 4 days, and occasionally between 4 and 8 days after transfer of cells. Indeed the fact is shown below in Fig. 5 that after transfer of cells from "hyperimmunized" donors, antibody titres in the serum of the host did not begin to decline for more than 8 days. This may be compared with the

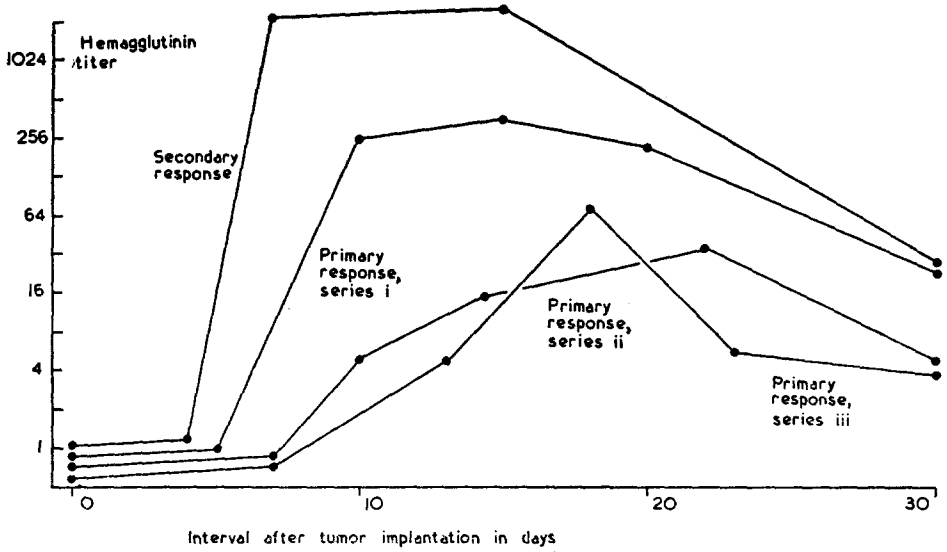

FIG. 1. Hemagglutinin production in C57BR/a mice in response to implantation of SA1. Geometric mean titres: Primary response (*a*) repeated bleedings from 6 individuals; (*b*) repeated bleedings from 6 individuals, another series; (*c*) samples of 6 individuals killed at intervals. Secondary response from repeated bleedings of 6 individuals.

half-life of isohemagglutinin in the mouse, which is approximately 2 days (17). On the other hand, antibody appeared more rapidly than in actively immunized mice. As with transfer of heightened resistance to homografts (3), the evidence therefore indicates that the transferred cells continued to function in their host, or possibly that an antibody producing mechanism was transferred into the cells of the host.

Peak activity was reached earlier by the regional lymph nodes than by the spleen, in both series. This can be accounted for by the direct lymphatic drainage from the homograft to the regional lymph nodes. Greater activity in the regional lymph nodes than in the spleen is also evident.

The highest titres of hemagglutinin were produced by lymph nodes transferred 10 to 15 days after the donors had been implanted with tumor. This

is later than the period (5 to 10 days), reported in the previous paper (3), during which the nodes are most effective in conferring heightened resistance to homografts.

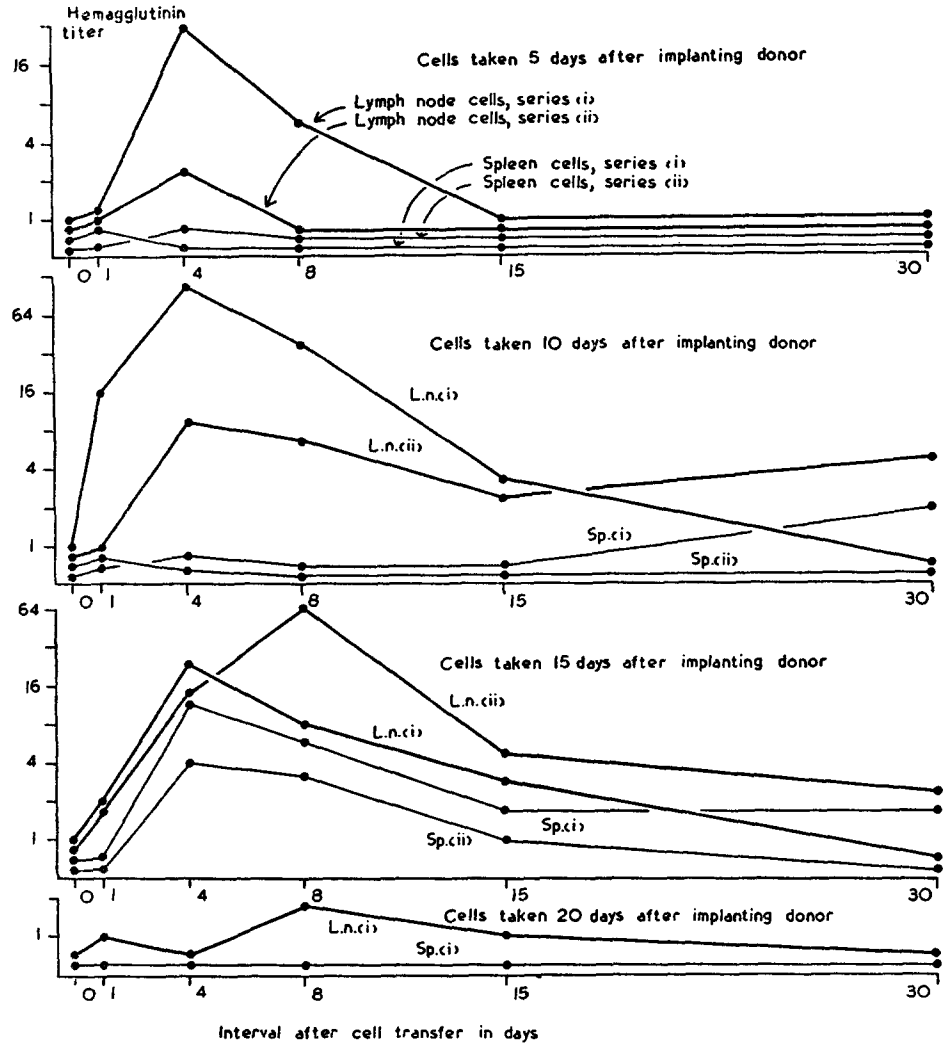

FIG. 2. Hemagglutinin production in C57BR/a hosts after receiving lymph node and spleen cells transferred from immunized donors.

Comparison of the Power of Lymph Node Cells to Transfer Heightened Graft Resistance and Hemagglutinin Production

The separation in time of graft resistance and hemagglutinin production in the regional lymph nodes is shown more clearly in the following experiment.

A single group of C57BR/a mice were implanted by trocar with SA1. 6 days later the regional lymph nodes were taken from half the group, and transferred to mice of the same strain, at a dosage of 4 donors/host. Lymph nodes were later transferred from the second half of the group, 14 days after tumor implantation. Both groups of hosts were bled 4 days after the transfer, and their serum hemagglutinin titrated. After bleeding they were implanted subcutaneously with 1×10^6 ascitic cells of SA1 suspended in 0.1 ml. Ringer, and the tumors weighed 8 days later. An equal number of untreated mice were implanted with each group as a control.

The mean titres of hemagglutinin and the mean weights of the test implantations are shown in Table II. The nodes transferred 14 days after the immunizing tumor transfer produced slightly more hemagglutinin than those transferred after 6 days. Although conferring some heightened resistance to the test grafts of tumor, they conferred much less than did the 6 day nodes,

TABLE II

Comparison of Power of Lymph Node Cells to Transfer Heightened Graft Resistance and Hemagglutinin Production

Implantation of SA1 into C57BR/a mice.

Interval between implantation of donors and transfer of cells.	No. of hosts	Log^2 hemagglutinin titre* in hosts after 4 days (mean and standard deviation)	8 day old test tumor grafts: mean weight and standard deviation in mg.
days			
6	10	4.6 ± 1.7	2.3 ± 4.3
(Control)	10		102.6 ± 29.3
14	10	5.7 ± 2.0	127.5 ± 87.3
(Control)	10		254.1 ± 81.5

* Serial number of last tube showing agglutination.

as judged either by the absolute weights of the test grafts, or by comparison with the weight of the grafts in the controls. The conclusion follows that more than one factor is produced by the regional lymph nodes in response to implantation of SA1. The agent which is mainly responsible for graft breakdown must be produced earlier than the hemagglutinating antibody.

The Secondary Hemagglutinin Response in Hosts of Transferred Lymph Node Cells

An experiment was carried out to test for more prolonged effects of the transferred cells. At 9 to 12 weeks after transfer of minced regional lymph nodes and spleens from donors implanted by trocar 15 days before transfer, groups of C57BR/a hosts were implanted by trocar with SA1. They were bled before implantation, and at 7 and 17 days postimplantation. The individual hemagglutinin responses are shown in Fig. 3, together with the normal primary and secondary responses to SA1 taken from Fig. 1. If the transferred

material had survived for this length of time in the hosts, or if the hosts had been actively immunized, responses similar to the normal secondary response were to be expected. However, if the material had disappeared without immunizing the hosts, a normal primary response was to be expected. The observed responses resembled for the most part the primary type, indicating that the transferred material had been lost. But certain individuals showed the high titres at 7 days postimplantations, characteristic of the secondary response, and others showed an intermediate response. Some transferred antibody-producing mechanism may have persisted in these mice, or more likely, active immunity may have been produced by transferred antigen. Similar prolonged

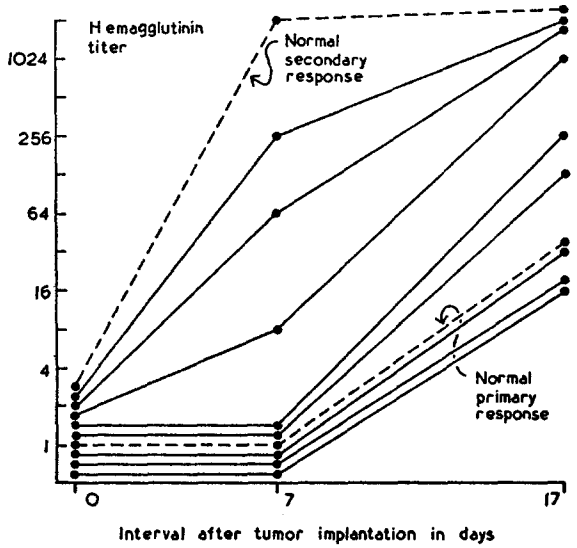

FIG. 3. Responses to a challenging implantation of SA1, of individual C57BR/a mice which have previously received lymph node cells from immunized donors.

effects of transferred lymph node cells have been reported in the previous paper (3), as judged by heightened graft resistance.

Hemagglutinin Production during Tumor Growth in Pretreated Mice

The course of hemagglutinin production was followed in mice pretreated with frozen or lyophilized tissue before receiving a homograft.

Six C57BR/a mice were pretreated by injection of lyophilized spleen, and implanted with SA1 by trocar subsequently. Six mice similarly treated with the tissue preparation were retained as controls and not injected; and a further six C57BR/a controls were implanted with the tumor, but not pretreated. The three groups were bled together at 10 day intervals, commencing immediately before tumor implantation. Hemagglutinin against red blood cells was titrated in the individual sera.

The efficacy of the pretreatment was demonstrated by an additional control group which was pretreated and implanted together with these experimental mice: out of 12 mice tested, all succumbed to the tumor.

An additional series of hemagglutinin titrations was carried out with C57-BL/6Ks mice. The same procedure was followed, except that the pretreatment was carried out with SA1 supernatant instead of A spleen; pretreated and implanted controls showed a mortality of 6 out of 6.

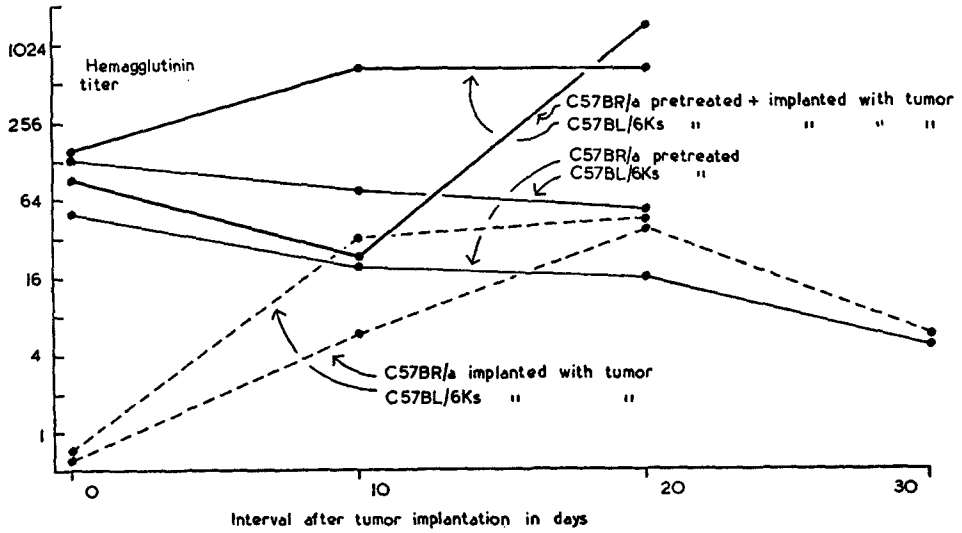

FIG. 4. Hemagglutinin production in response to implantation of SA1, in C57BR/a mice after pretreatment with lyophilized A spleen, and in C57BL/6Ks mice after pretreatment with frozen-thawed SA1.

The geometric means of the individual titres of hemagglutinin are shown in Fig. 4. Pretreated mice bearing tumors were not bled later than 20 days after tumor implantation, since most had died before the 13th day. The groups which were implanted but not treated with tissue preparations gave the expected hemagglutinin response, maximum titres being reached 20 days after implantation. When given alone, the lyophilized and frozen tissue preparations elicited a marked production of hemagglutinin, which declined slowly after the completion of the injections. Consequently at the time when the pretreated mice were implanted with tumor, hemagglutinating antibody was circulating in their blood, at a titre similar to that found in normal mice giving the sloughing off of a homograft. Furthermore, as the tumor grew progressively the titre of hemagglutinin rose, and finally reached a far higher

level than that normally found during the sloughing of a first graft of tumor. The pretreatment therefore certainly does not impair the ability to produce hemagglutinating antibody.

Hemagglutinin Production after Transfer of Lymph Node Cells from Pretreated Mice Bearing a Tumor

The regional lymph nodes can be shown to participate in hemagglutinin production in pretreated mice bearing a tumor.

C57BR/a mice were pretreated with lyophilized A spleen and implanted with SA1. 18 days later the regional lymph nodes and spleens were transferred to mice of the same strain,

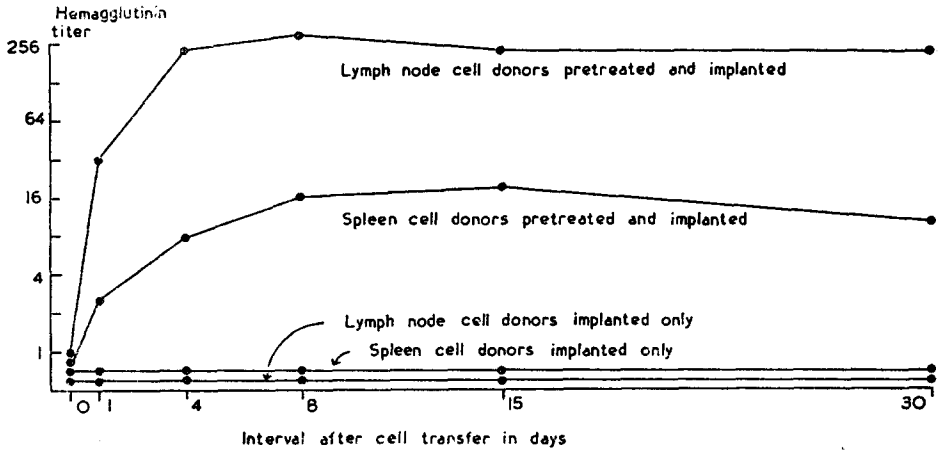

FIG. 5. The effect of pretreatment of the donors with lyophilized tissue, in increasing hemagglutinin production by cells transferred into secondary hosts (B10.D2 mice implanted with SA1).

the lymph nodes to 6 hosts at a dosage of 4 donors per host, and the spleens to 6 hosts at a dosage of 1 donor per host. The efficacy of the pretreatment was shown by a mortality of 6 out of 9 control mice pretreated with the donors and implanted at the same time. Nodes and spleens were also transferred as a control out of normal C57BR/a donors bearing 18 day old growths of SA1, at the same dosage. The hosts were bled at intervals of 1, 4, 8, 15, and 30 days after transfer, and the individual sera titrated for hemagglutinin.

The geometric means of the individual titres are shown in Fig. 5. No hemagglutinin production was detected in the hosts of the transferred control cells, possibly because of weak implants of immunizing SA1 that had given rise to only slight growths in the donors. But both the nodes and spleens from the pretreated and implanted donors gave rise to high and persistent titres of hemagglutinin.

The Duration of Hemagglutinin Production after Transfer of Lymph Node Cells to Foreign Hosts

A test of the duration of hemagglutinin production after transfer of cells into foreign hosts was made possible by the high activity of cells from pretreated and implanted donors. The purpose of this experiment was similar to that of the tests of the duration of heightened graft resistance after transfer of cells into foreign hosts, reported in the accompanying paper (3): to test whether the transferred material can be destroyed by the homograft reaction of the host.

B10.D2 donors were pretreated with lyophilized A spleen and inoculated with SA1. 17 days later the regional lymph nodes were transferred to 6 B10.D2 hosts, and also to

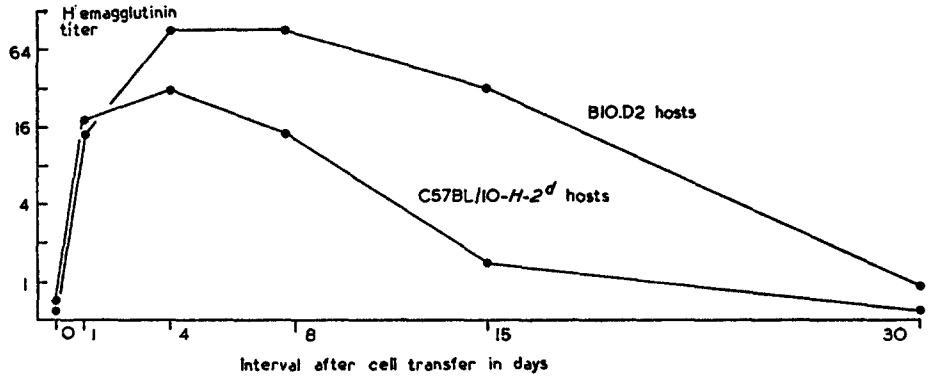

FIG. 6. Hemagglutinin production in B10.D2 and C57BL/10-*H-2*^{d} hosts after transfer of lymph node cells from pretreated and implanted B10.D2 donors.

6 C57BL/10-*H-2*^{d} hosts, at a dosage of 4 donors per host. The hosts were bled after the same intervals as in the previous experiment, and the geometric means of the individual titres are shown in Fig. 6.

As has been shown for heightened graft resistance, the production of hemagglutinin declined more rapidly, following transfer of lymph node cells into foreign hosts. The hypothesis that the transferred material is itself susceptible to a homograft reaction was therefore confirmed.

Failure of Lymph Node Cells Donated by Pretreated Mice Bearing a Tumor to Confer Heightened Resistance

A group of C57BL/6Ks donors were pretreated with SA1 supernatant and implanted by trocar with SA1. These mice were pretreated and implanted together with a group of controls already referred to, which showed a mortality of 6 out of 9. A control group of normal C57BL/6Ks donors were also implanted with SA1 at the same time. 10 days after implantation the regional lymph nodes were transferred from both groups of donors into fresh mice of the same strain, at dosages of 4, 2, and 1 donor per host. The hosts were then in-

jected subcutaneously with 1×10^6 ascitic cells of SA1, suspended in 0.1 ml. Ringer, and the tumor growths were weighed after 8 days. Untreated mice of the same strain, and mice actively immunized with SA1 were also implanted, as controls.

The weights of the test tumor growths, shown in Table III, indicate that the larger doses of cells from non-pretreated and implanted donors conferred heightened resistance to the tumor, as expected. The smaller doses of cells from the pretreated implanted donors had no significant effect, while at the largest dose of 4 donors per host they appeared to enhance the growth of the tumor. This enhanced growth can be accounted for: nodes from pretreated and implanted donors have been shown to produce hemagglutinin after trans-

TABLE III

Failure of Lymph Node Cells from Pretreated and Implanted Donors to Confer Immunity
Implantation of SA1 into C57BL/6Ks mice; pretreatment with SA1 supernatant.

Treatment of donors	No. of donors per host	No. of hosts	8 day old SA1 test tumors: mean weight and standard deviation in mg.
Pretreated:			
Implanted with SA1	4	5	124.8 ± 64.6
" " "	2	5	42.2 ± 27.5
" " "	1	5	88.9 ± 87.3
Not pretreated:			
Implanted with SA1	4	5	1.8 ± 2.8
" " "	2	5	10.3 ± 8.4
" " "	1	5	80.9 ± 86.1
Untreated controls		6	46.0 ± 12.1
Controls actively immunized with SA1		5	0.2 ± 0.1

fer; and hemagglutinating serum has been shown to enhance growth of the tumor (3).

Protection of Pretreated Mice by Lymph Node Cells from Immunized Donors

The failure of lymph node cells from pretreated and implanted mice to confer heightened resistance to the tumor indicated that the normal ability of lymphoid tissue to protect against a tumor homograft is blocked in pretreated mice. The ability of lymph nodes from immunized donors to protect pretreated mice against the tumor was next tested.

Use was made of the experimental design employed by Billingham, Brent, and Medawar (18), in their demonstration of homograft breakdown in mice with actively acquired tolerance after transfer of lymph node cells. Groups of normal C57BR/a and B10.D2 mice

were implanted with SA1, and after 9 days their regional lymph nodes were transferred to pretreated mice of the same strain. The hosts were implanted on the same day with test grafts of SA1 by trocar, together with control groups which had also been pretreated, but which had not received lymph node cells.

The results of this experiment are shown in the first and second rows of Table IV. In all series tested, the lymph node cells transferred out of immunized donors conferred protection on their pretreated hosts.

A gradual loss of the protection conferred by transferred node cells could be demonstrated, parallel to the gradual loss of adoptive immunity described in the previous paper (3). Some of the protected mice were left for an in-

TABLE IV

Immunity Conferred by Transferred Lymph Node Cells on Pretreated Mice

Mortality from test inoculation of SA1.

Mice received pretreatment plus:	C57BR/a	C57BR/a	C57BR/a	B10.D2	C57BL/6Ks
Test implantation alone	12/12	9/10	6/9	5/5	6/6
Lymph node cells from immunized donors + test implantation	0/10	0/6	0/5	0/5	—
Lymph node cells from normal donors + test implantation	—	6/6	—	—	—
Lymph node cells from normal donors + prior tail implantation + test implantation	—	1/5	0/6	—	4/5
Prior tail implantation + test implantation	—	4/6	4/6	—	4/5
Lymph node cells from pretreated donors + prior tail implantation + test implantation	—	—	5/5	—	4/5

All host strains not susceptible to SA1; all hosts pretreated with lyophilized A spleen. Each column refers to a group of mice pretreated at the same time; a separate control was included in each group, since the control mortalities were not homogeneous.

terval after their test implantation, and were then reimplanted with SA1. The second graft of tumor grew progressively and killed the host in a proportion of cases, as shown in Table V. The data show signs of an irregular increase in mortality as the interval between the first and second tests is increased. This experiment is made possible by the very long duration of susceptibility to SA1 after pretreatment, noted by Kaliss and Day (19): the mortalities observed in the control groups are nearly as high after the second test graft as after the first. The control and experimental groups were slightly different in that different control mice were implanted in the first and second tests, while the hosts of transferred nodes which were implanted in the second test were the survivors of the first test.

The susceptibility of a progressively growing tumor to destruction by immunological means was then tested, making use of the protection con-

ferred by transferred lymph node cells on pretreated mice. A single group of C57BR/a mice were pretreated with lyophilized A spleen, and implanted with SA1 by trocar. At intervals after implantation minced regional lymph nodes were transferred to these mice from donors of the same strain immunized with 9 day old grafts of SA1 at a dosage of 4 donors per host. The mortalities in this experiment are shown in Table VI. They show that as the tumor

TABLE V

Duration of Immunity Conferred by Transferred Lymph Node Cells on Pretreated Mice

Implantation of SA1 in hosts pretreated with lyophilized A spleen.

Host strain	Interval from pretreatment to transfer + 1st test	Interval between 1st and 2nd tests	Mortality from test implantation of SA1			
			Hosts with transferred cells		Hosts without transferred cells (controls)	
			1st test	2nd test	1st test	2nd test
	days	*days*				
C57BR/a	8	48	0/10	3/9	12/12	
C57BR/a	13	25	0/6	0/6	9/10	4/6
C57BR/a	13	90	0/5	1/5	6/9	
B10.D2	6	96	0/5	4/6	5/5	4/6

TABLE VI

Decrease in Vulnerability of SA1 during Progressive Growth in Pretreated Hosts

C57BR/a hosts pretreated with lyophilized A spleen.

Interval between tumor implantation and receipt of lymph node cells	Mortality from progressive growth of SA1	Approximate tumor weight at time of receipt of lymph node cells
days		*mg*
0	0/6	2
2	0/6	2
7	4/6	800
17	6/6	3000
(No cells transferred)	12/12	

increased in size it became progressively less susceptible to destruction by transferred cells.

Protection of Pretreated Mice by Lymph Node Cells from Normal Donors

Following again the experimental design of Billingham, Brent, and Medawar (18), limited success has been attained with protection of pretreated mice by transferred normal lymph node cells. This "replacement therapy" experiment is a test of the possibility that the transferred cells can be acti-

vated immunologically by the tumor homograft, and can in turn bring about destruction of the tumor, although the host's own lymphoid tissue is paralyzed.

Normal lymph node cells, transferred immediately before inoculation of SA1, failed to protect pretreated mice, as shown in the third row of Table IV. The mortality following transfer of normal lymph node cells and implantation was no lower than the mortality following implantation alone. This failure can be accounted for: an interval must have elapsed after implantation before the transferred cells could have been activated; during this interval the tumor was growing, and therefore becoming progressively less vulnerable.

With the aim of avoiding the difficulty outlined, normal lymph node cells were transferred to the peritoneum of mice of the same strain, and a graft of SA1 was immediately implanted in the tail. In spite of the remoteness of the two sites, it was hoped that antigen from the tumor would reach the transferred lymph node cells. 7 or 8 days later, just before the tumor spread into the body, the tail was cut off. A test graft of tumor was then implanted in the flank, to test whether the transferred cells had become capable of destroying the tumor. Since the prior tail implantation alone might have conferred some protection on the mice, controls received the prior implantation but no transferred lymph node cells. Additional controls were treated in the same way as the experimental group, except that the transferred cells were donated by pretreated instead of normal mice.

The results of three such series are shown in Table IV, fourth, fifth, and sixth rows. In two series the normal cells appeared to confer marked protection, with little or no protection in either of the control groups receiving prior tail implantations. However the protection could not be repeated in C57BL/6Ks mice, as shown in the last column. The results taken together, though not statistically significant, are suggestive of a protective effect of normal lymph node cells.

Failure of Treatment with Lyophilized Tissue to Enhance Tumor Growth in Previously Immunized Mice

Evidence has been presented that graft resistance is blocked by pretreatment. The possibility of blocking resistance once it has been developed was then investigated by testing the effect of treatment with lyophilized tissue on previously immunized mice.

Ten C57BR/a mice were immunized with SA1 implanted by trocar. Lyophilized A spleen was then injected, commencing 25 days after the immunizing implantation. Test grafts of SA1 were implanted subsequently: none of the mice succumbed to the tumor. These mice were treated and implanted together with the mice of the third column in Table IV, the pretreated and implanted controls showing a mortality of 6 out of 9.

Treatment with lyophilized tissue appears therefore to be unable to block graft resistance in previously immunized mice.

Localization of Transferred Lymph Node Cells

On the hypothesis that homografts are destroyed by cell-bound antibody, lymph node cells from an immunized donor transplanted into the peritoneum of secondary hosts would be expected to localize in a subcutaneous homograft. An attempt was made to follow the fate of transferred lymph node cells by the acriflavine marker technique of De Bruyn, Robertson, and Farr (20). This dye has the property of staining cell nuclei *in vivo*, and of fluorescing in ultraviolet light. Stained cells can therefore be picked out by the use

TABLE VII

The Effect of Acriflavine Staining on the Ability of Lymph Node Cells to Transfer Immunity

Immunity to SA1 in C57BR/a mice.

No. of lymph node cells transferred	Weights of 8 day test tumors	
	Acriflavine stained lymph node cells	Unstained control cells
	mg.	*mg.*
80×10^6	0.0	0.0
	0.0	2.4
40×10^6	3.0	0.9
	25.4	4.7
20×10^6	19.0	30.3
	77.9	42.9
10×10^6	69.3	106.7
	154.9	188.1
None	54.0	
	89.1	
	93.6	
	146.3	

of a suitable optical system. The light source and filters used in the present work were of the type used in the original report of De Bruyn, Robertson, and Farr (20).

Preliminary experiments were carried out to test quantitatively the effect of staining with acriflavine on the capacity of lymph node cells to transfer immunity.

A group of 32 C57BR/a donors were implanted with SA1 by trocar. 9 days later the dye was injected intraperitoneally into half the group, at a dosage of 60 mg./kilo. body weight. This is the approximate median lethal dose for the sample of dye used, in this strain. 2 hours after injection, when staining of the regional lymph node cells was at a maximum, the regional lymph nodes were excised, pooled, and a suspension of cells prepared. The sus-

pension was counted, and injected at various doses into C57BR/a hosts. A similarly prepared unstained cell suspension was transferred from the remaining half of the donor group. Both sets of hosts were then injected subcutaneously with 1×10^6 ascitic cells of SA1 suspended in 0.1 ml. Ringer, and the resulting tumor growths weighed after 8 days. These weights are shown in Table VII.

Staining with acriflavine appeared to have no effect on the capacity of cells to transfer immunity.

Following the same staining procedure, lymph nodes were transferred as mince. Smears of living cells from the abdominal cavity and organs of the hosts were examined at intervals after transfer. 2 hours after transfer stained cells could be seen in the spleen, liver, kidneys, lungs, and mediastinal lymph node, but not in the thymus, axillary, or inguinal lymph nodes, nor in the buffy coat of the blood. Some cells were more heavily stained than others, making quantitative estimates of their number difficult. After transfer of mince into the peritoneum, at a dosage of 12 stained lymph nodes per host, a rough count gave between 1 in 100 and 1 in 1000 stained cells in the spleen. The stain in the transferred cells faded slowly, disappearing overnight. Subcutaneous homografts of SA1 were searched for stained cells, after transfer to their hosts of stained lymph node cells from immunized donors. Marked cells could not be found in the grafts.

This failure indicates either that transferred cells do not reach grafts, or that they do so and are rapidly destroyed, or else that they do so too slowly to be detected by this technique. However it is clear that transferred cells have ample opportunity of passing out of the peritoneum into a graft, or into the lymphoid tissue of the host.

DISCUSSION

Lymph node cells from donors immunized by homografts have been shown to transfer hemagglutinin production to secondary hosts. These cells also confer heightened graft resistance on their hosts. This poses the problem whether the hemagglutinating antibody itself is the cause of heightened graft resistance. The alternative hypothesis is that lymph node cells produce more than one kind of antibody, the antibody effective in combating homografts being distinct from the hemagglutinating antibody. The dual function hypothesis is supported by the different timing in lymph node cells of the ability to transfer heightened graft resistance and hemagglutinin production. It is also supported by the findings with lymph node cells from tumor-bearing mice pretreated with frozen or lyophilized tissue. These cells transferred hemagglutinin production but not graft resistance, indicating that their ability to produce antibody effective against homografts could be blocked without impairing hemagglutinin production. Further evidence for this block is provided by the transplantation of lymph node cells into pretreated mice: cells from immunized and possibly also from normal donors conferred protection against tumor homografts.

The failure of serum to confer heightened graft resistance in passive transfer experiments suggests that the antibody effective against homografts is bound to cells, presumably lymphocytes. On this hypothesis, cells from immunized donors transferred into secondary hosts would be expected to localize in a test homograft. The failure to detect this localization with dye-marked cells is not conclusive evidence against cell-bound antibody because of the rapid fading of the dye. It may be noted that attempts to detect localization of serum isoantibody have also been unsuccessful (21).

Further resemblances between the homograft reaction and sensitization to tuberculin and to simple organic compounds emerge from the present work. *a*) Cells which transfer delayed sensitivity have been shown by Chase also to transfer antibody of the anaphylactic type (22, 23). *b*) The course of serum antibody production during sensitization does not run parallel to the development of delayed sensitivity (24). *c*) Tuberculoprotein, picryl chloride, and even egg albumen injected together with tubercle bacillary wax adjuvant elicit delayed reactivity; but when injected alone these agents induce only serum antibody (25–27). The adjuvant, though not itself antigenic, incites a granulomatous response of the type which has often been compared to the round cell infiltration of homografts (28). There are indications that the preparations of frozen or lyophilized tissue which induce serum antibody without heightened graft resistance do not incite the granulomatous response: Darcy (29) reports that the cellular response to frozen homografts in the rabbit is absent or greatly delayed, although the antigenic specificity of the frozen tissue is retained, as judged by the reaction provoked in previously immunized hosts. This evidence therefore suggests that the failure of lyophilized or frozen tissue to induce heightened graft resistance may be due to a failure to incite the proper granulomatous response. However this can hardly be the full explanation of the action of the tissue preparations: the granulomatous response is probably not the sole basis for the action of bacillary wax; nor can the analogy account for the almost complete block in graft resistance brought about by the tissue preparations.

SUMMARY

The relation between serum antibody and resistance to tumor homografts in the mouse has been investigated. Production of serum antibody in response to homografts of a transplantable sarcoma (Sarcoma 1) was demonstrated, by cytotoxic action on the cells of the tumor, and also by a hemagglutinin test. The simpler and more repeatable hemagglutinin test was further investigated.

Peak hemagglutinin titres were reached after the immunizing homografts underwent breakdown. Following transfer of lymph node cells from immunized mice into hosts of the same strain, hemagglutinin could be detected

in the host serum. The course of its production showed that this secondary antibody was not elicited by transferred antigen, nor could it be due to transfer of preformed antibody. The cells developed the capacity to transfer hemagglutinin production later than the power to transfer heightened graft resistance. Spleen cells also transferred hemagglutinin production, at a later stage after immunization and to a lesser extent than cells from the regional lymph nodes.

Implantation of the sarcoma in mice pretreated with certain preparations of lyophilized or frozen tissue stimulated hemagglutinin production, although the tumor grew progressively. The regional lymph nodes participated in the response: they could transfer hemagglutinin production into secondary hosts, but not graft resistance, and indeed appeared to diminish resistance. Lymph node cells from immunized donors conferred protection against the tumor on pretreated mice. Lymph nodes from normal donors also appeared in some experiments to confer protection although the effect was obscured by the rapidity with which the growing tumor became immunologically invulnerable.

The fate of lymph node cells stained with acriflavine was followed after transfer. No effect of the staining on the power of the cells to confer immunity could be detected. Cells transferred to the peritoneal cavity passed into various host tissues, but were not found in test homografts.

The conclusion is drawn that the hemagglutinating antibody is distinct from the antibody effective in combating homografts. The similarity in this respect between the homograft reaction and sensitization is emphasized in discussion.

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