Transplantation Immunity:

Some Properties of Induction and Expression *

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EVENTS leading up to the breakdown of homografts of skin are clouded by a lack of observable phenomena early in the period of survival. The emphasis in past studies of the primary response has been on the establishment of the graft, at one extreme, and on the time of the graft breakdown, as judged by several observed events [1, 13] (see recent review by Brent [5]) at the other. Between these two are several days during which the graft looks and behaves in a fashion similar to that of an autograft. Medawar [7] noted that during this time, the survival of the tissue, the establishment of vascular communications with the host, and even the proliferation of cells in the graft are essentially the same in all "first-set" grafts, regardless of their homologous or autologous origin. In skin grafts between inbred strains of mice it is only on the sixth to eighth day,[1] and often even later, that in the homograft can be seen the earliest changes presaging its eventual destruction. That much has gone on in the host, and possibly also in the graft, prior to this time is obvious. It is toward a better understanding of the events that transpire in this "latent" period that the present experiments were directed.

A study was made of the effects on skin grafts of various degrees of pre-existing immunity up to that sufficient to cause a strong second-set reaction. Inside of this range, it was possible to determine the time required for immunization to take place and for immunity to develop. It will be shown that a definite period of contact is necessary and that the route of immunization has an effect on the time at which immunity appears.

Materials and Methods

The experiments were made by transplanting skin grafts between inbred strains of mice. In all of the experiments mice from four inbred strains were used: A/HeN (to be designated as A), C57BL.10/10ScBsN (B), C3H/HeN (C), C57BL.10/H-2^d BsN (D). The strains used in any individual case are described with the experiment. Donor and recipient animals were of the same sex. The method of grafting was that of Billingham and Medawar.[4] In the selection of donors, only those animals were used that demonstrated "inactive" skin at the time of grafting.[10] In those experiments in which a test for immunity of the host was indicated, this was done in the accepted way,[2] by means of a "second-set" graft, the latter being read at six days after grafting. In some experiments immunization was obtained by the intraperioneal injection of spleen cells at a dose of one-fourth spleen suspended in 0.5 cc. normal saline per injection.[3]

In the grading system used, objectivity was the primary factor and four degrees

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of graft rejection were recorded. Complete acceptance of a graft, but including any graft in which a trace of epithelial destruction was present (up to 95% survival), was represented by a plus (+) sign. Complete destruction, but including those grafts showing a trace (up to 5%) of survival, was represented by a minus (−) sign. The plus-minus (±) sign designated grafts falling between the first two categories. The symbol "0" was the grade assigned to second-set grafts that at six days showed no evidence of vascularization or healing, a phenomenon noted by Billingham, Brent, and Medawar.[2] Throughout this paper the term "healing" is used in its most general sense, no reference being made to the many properties of this complex process.

Experiment I. Following the simultaneous placement of two or more grafts from the same donor to different sites on a single host, the grafts break down at essentially the same time.[8] In the first experiment three sets of B mice received one D graft each, on the same day. One day later mice of the first group received a second D graft on the other side. The second group of mice received their test grafts two days after the first graft, and the third group theirs three days after the first. As a result of this, each mouse had two grafts, separated by from one to three days in their times of placement. These grafts were all inspected at eight, ten, and 11 days, and the results are shown in Table 1. At eight days all grafts were in good condition. At ten days, however, most of the grafts in each group showed complete destruction, and only very few of those remaining appeared to be in good condition. It is of note that while the grafts did not all break down at exactly the same time, the two grafts on any individual ran quite parallel courses.

Experiment II. In the second experiment two strain combinations were used to determine the time of continuous contact required for a homograft to render its host immune as evidenced by the early rejection of a second graft from the same donor strain. The strains used were D → A and D → C. Grafts were placed on the right flank of the host animals and remained in place for two, three, four, and six days as

TABLE 1. *Experiment I: Breakdown Times of Overlapping First Set Homografts*

Number of Days Separating Placement of Control and Test Grafts	Day of Reading					
	Day 8		Day 10		Day 11	
	Control	Test	Control	Test	Control	Test
1 day	+	+	±	±		
	+	+	−	−		
	+	+	−	−		
	+	+	±	+	−	−
	+	+	−	−		
	+	+	−	−		
	+	+	−	−		
2 days	+	+	+	±	−	−
	+	+	+	±		
	+	+	−	−		
	+	+	−	−		
	+	+	−	−		
	+	+	±	−		
	+	+	−	−		
	+	+	−	−		
3 days	+	+	+	+	±	±
	+	+	−	−		
	+	+	−	−		
	+	+	−	−		
	+	+	±	±	±	±
	+	+	−	−		
	+	+	−	−		
	+	+	−	±		

TABLE 2. *Experiment II: Time Required for Immunization*

Mouse Strain Combinations	Time Immunizing Graft Left in Place (Days)	Scores of Test Grafts, Read at 6 Days
D → A	2	+ + + + + +
	4	± ± − − − −
	6	− − − − − −
	8	± − −
D → C	2	+ + +
	3	+ + − −
	4	+ − − − − −

TABLE 3. *Experiment III: Appearance of Immunity Following Adequate Stimulus by Skin Graft*

Chronology of Experiment (Strains of Mice Used: D → B)	Scores of Test Grafts
I. Immunizing graft removed 4th day, Test graft placed 4th day, Test graft read 10th day.	+ + + + + + ± ± ± ± ± ± ± ± −
II. Immunizing graft removed 4th day, Test graft placed 6th day, Test graft read 12th day.	0 0 0 0 0 0

shown in Table 2. The animals were rebandaged following the removal of the first graft. At ten days from the initial grafting a second graft from the same donor strain was placed on a fresh bed prepared on the left flank. This graft was read six days later for a second-set reaction. It was felt that if the initial contact with the foreign graft had been long enough to produce immunity, the second graft should show a second-set type of breakdown at six days. If the initial contact was of too short duration the second graft would be on a nonimmune animal, and being essentially a first-set graft, should appear healthy at six days. The data in Table 2 indicate that some mice are immunized by three days, and virtually all by four days of continuous contact with a homograft, as tested by the acceptance or rejection of a second graft from the same foreign strain a few days later.

Experiment III. In the above experiment the second, or test graft was placed on the animal ten days after the first graft was put in place, regardless of when the first graft was removed. Strictly speaking, the animal was immunized by four days of contact with a foreign graft followed by an additional six-day interval before the immunity was challenged and demonstrated. While it was suggested that immunization was completed in four days, it is possible that immunity was only initiated in that period and that some of the remaining time was required for its maturation. To differentiate between these possibilities, the design of Experiment I was extended to include intervals between the placement of the first and second grafts of four and six days.

Homografts from D were placed on B animals, and in the first group of animals (I) the immunizing graft was removed and the second or test graft was placed at the same time, on the fourth day. This graft was read for a second-set response on the tenth day of the experiment. In the second group (II) the immunizing graft was removed on the fourth day and the test graft was placed two days later, on the sixth day. This graft was read for a second-set reaction on the twelfth day of the experiment.

The results given in Table 3 show that in Group I most of the grafts showed only partial breakdown or none at all at the time of reading. In Group II in which an interval of two days elapsed between the removal of the immunizing stimulus and the placement of the test graft, these grafts failed to establish themselves at all (grade "0").

Experiment IV. In the design of the foregoing experiments it was emphasized that the immunizing or first graft was in "continuous contact" with the host. In the next experiment an attempt was made to better define the events of the first four days of immunization by breaking down the continuity of contact between graft and host. In all cases the host was exposed to the graft for a total of four days. In the further breakdown of this period several variables were tested. In Group I the graft was removed at two days, rotated 180° and replaced on the same graft bed. In Group

II the graft was removed at two days and a new graft from the same foreign strain was placed on the old graft bed. In Group III the graft was removed at two days and was transferred to a *new* graft bed on the opposite side of the animal. In Group IV the graft was removed at two days and a *new* graft placed on a fresh bed on the opposite side of the animal. In Group V the graft was removed and replaced by a new graft *every* day to a total of four days. Group VI comprised controls in which the graft was left undisturbed for four days. The results of this experiment, shown in Table 4, indicate that interruption of the continuity of contact between host and graft in *any* of the several ways tested did not prevent complete immunization of the host.

Experiment V. In this experiment an attempt was made to assess the role of regional nodes in the first few days of immunization. Skin from D donors was grafted to B recipients. In the first group the graft *and* regional nodes—axillary and inguinal—were removed at three days. In the second group, these tissues were removed on the fourth day. On the tenth day of the experiment all animals received a test graft from D donors, the results of these being read six days later. Included with the results in Table 5 are the graft scores from comparable groups of animals from Experiment II, in which the grafts alone were removed at three and four days, respectively. It will be seen that graft scores in the corresponding groups, particularly in those from which the tissues were removed at four days, do not differ greatly.

TABLE 4. *Experiment IV: Variations Within Four-Day Period of Stimulation*

Group	Variables in Immunizing Graft	Strains of Mice Used: D → C Survival Scores of Test Grafts
I.	Reverse orientation, same site at 2 days	− − − − − −
II.	New graft, same site at 2 days	− − − − −
III.	Same graft, new site at 2 days	− − − − −
IV.	New graft, new site at 2 days	− − − −
V.	New graft, same site every day to total of 4 days	− − − −
VI.	Control: same graft, same site 4 days	+ − − − − −

TABLE 5. *Experiment V: Effect of Lymph Node Removal on Immunization by Minimal Exposure to Skin Graft*

	Strains of Mice Used: D → B Scores of Test Grafts
I. Graft and nodes removed on third day	+ + + + + ± −
(From Experiment II, graft alone removed at three days, strain D → C)	+ + ± ± ± − −
II. Graft and nodes removed on fourth day	+ − − − − −
(From Experiment II, graft alone removed at four days, strains D → C, D → A)	± ± − − − −

Experiment VI. In this experiment a study was made of the effect of intraperitoneal immunization superimposed on the reaction seen in an orthotopic skin graft. Six sets of A mice received skin grafts from D donors. At the same day as the grafting, or at various intervals before or after the grafting as shown in Table 6, different sets of the mice also received a single intraperitoneal injection of spleen cells from D-strain donors. A control set of mice was grafted but received no spleen cell injections. All of the grafts were then inspected at six, eight, and ten days after grafting. In the results, shown in Table 6, it will be noted by the four grades used that a distinction was made between those grafts whose epithelium was completely destroyed, but that were adherent to the graft bed, showed signs of healing and of having been vascularized (graded −), and those that appeared to have been rejected from the very moment

TABLE 6. *Experiment VI: Graft Scores Showing Effect of Rapid Immunization on Skin Grafts*

Day of i.p. Cell Injection, Relative to Day of Test Graft		Day of Reading of Test Graft		
		6th Day	8th Day	10th Day
Group I.	−3	0 0 0 0 0 0		
Group II.	−1	± ± 0 0 0 0		
Group III.	0 (day of grafting)	+ + + + ± 0	− − −	
Group IV.	+1	+ + ± ± 0 0	− − − −	
Group V.	+3	+ + + + ± ±	± ± ± − − −	± − −
Group VI.	Control	+ + + + + +	+ + + + + +	− − − − − −

cf their placement, evidencing neither vascularization nor healing (graded 0).

In the mice receiving cell injections three days (− 3), and even as late as one day (− 1) prior to grafting, most of the grafts had undergone what was interpreted to be a most complete rejection when inspected at six days. In mice that received the cell injection on the same day (0), or one day (+ 1) after they were grafted, most of the grafts showed some degree of survival, and some showed complete survival when inspected at six days. Even in these groups, three mice had "0" grafts. When the surviving grafts from these groups were inspected again two days later, eight days after grafting, all were broken down, but all showed signs of transient survival. In the mice who received their cell injection three days after grafting (+ 3), most of the grafts were in good condition when examined at six days, but half were completely broken down, and none was in perfect condition at eight days. This can be compared to the control groups that received grafts but no cell injections, in which all of the grafts were in good condition at eight days.

Discussion

This study was directed at some aspects of the induction of immunity, and at the requirements, in terms of immunity for graft destruction. In the strains of mice used, it was demonstrated that three to four days of contact with a foreign graft are necessary to produce immunity. This indicates that the induction of immunity is not a chance phenomenon, requiring only transient exposure during which time a few cells escape into the host's vascular or lymphatic vessels, but implies that a quantitative transfer of antigen is necessary and that this transfer may be a continuous process. To further break down this time and possibly disrupt the activities taking place, the graft was shifted during the four-day period. With all of the variables tested, immunization was as complete as if the graft had been undisturbed for the entire time. Within this time no special properties were attached to the particular graft, in that it could be replaced by a new graft from the same foreign strain. The graft site itself did not appear to be endowed with special properties during immunization, and through the same experiment it was shown that it is not necessary for the entire immunizing stimulus to go to the same regional nodes. The fact that the graft could be replaced daily suggests that antigenic material is leaving the graft continuously, even right after transfer.

Scothorne [12] showed that continuity of lymphatics between graft and host is not established until the fifth day after grafting, and concludes from this that restoration of this continuity is not necessary for immunization. Taylor and Lehrfeld [13] showed that vascularization of skin grafts takes place between 24 and 48 hours and that ac-

tive circulation is even further delayed. The finding that the replacement of grafts every 24 hours does not lessen their ability to immunize the host, suggests that the restoration of vascular continuity is not necessary for immunization either. Scothorne [11] demonstrated morphologic changes in regional nodes as early as three days after grafting and Mitchison [9] and Billingham, Brent, and Medawar [2] showed that immunity could be transferred by the nodes at five days. Immunization can take place following the surgical removal of regional nodes, said immunity requiring a slightly longer time to be achieved.[2] The demonstration that immunization is little affected by the removal of regional nodes along with the immunizing graft at four days suggests that by then the immune reaction is not confined to these structures. Moreover, the observation that a minimal immunizing stimulus (in terms of time) can be divided between two sets of nodes on opposite sides of the animal is compatible with the impression of others [2] that the reaction to foreign tissue is widespread at an early stage.

It was noted that in instances where the interval between immunizing and test graft was small, from 0 to 4 days, the second graft was handled by the host essentially as was the first. The results found here with inbred mice were remarkably similar to those of Lehrfeld, Taylor, and Converse [6] using a more heterogeneous population of rats. When both grafts were left in place, both became vascularized and healed in a normal fashion, and both broke down at the same time, this time corresponding to the expected first-set survival time of the earlier graft. The survival of a homograft appears to be dependent in part on a critical level of immunity in the host and not on a finite period of time that the graft is in place.

In Experiment III immunization was minimized by removing the first graft at four days. A difference, both quantitative and qualitative, was seen in the breakdown of the test grafts, a result that was related to the time after immunization that the test graft challenged the host. In the group where two days elapsed between the removal of the immunizing and the placement of the test grafts, the second graft showed no evidence of having established itself even for a short time on the host. Of test grafts that were placed on the recipients at four days, when their immunizing grafts were removed, many showed complete survival on the tenth day. Here the recipients had ten consecutive days of contact with foreign grafts (i.e., the normal breakdown time of first-set grafts in the strain combination used). At the same time, the test grafts survived for six days on recipients having previously received what was shown to be an adequate immunizing stimulus. Despite both of these conditions, the fact that the grafts showed more than minimal survival on the tenth day suggests that while four days of contact with a foreign graft was enough to *induce* immunity, it was not enough time for the immunity to reach a level sufficient to initiate the destruction of a fresh graft.

The rate at which immunity developed in the host was shown to be affected by the route of administration of the antigenic stimulus. Six days must follow the placement of a skin graft before immunity will prevent the establishment of a second graft. In contrast, a second-set reaction of equal violence was initiated by only one day of exposure to spleen cells given intraperitoneally. Use was made of this difference to further study the effects on skin grafts of variations in their immune environment. The types of graft rejection were seen to differ in the presence of different levels of immunity. If immunity preceeded the physiological establishment of the graft on the host, such establishment never took place and the graft was rejected with no signs of vascularization or healing. The picture was essentially the same regardless of whether the immunity was provoked by six days of

exposure to a skin graft or a single day of exposure to spleen cells. If, on the other hand, the graft had time to establish itself prior to its assault by host immunity, it was able to survive for a short time under conditions that would not support a new graft. Technically, these were first-set grafts, in that they represented the first encounter of the host with foreign tissue. Rapid immunization, however, can reduce the lifetime of such a graft until it overlaps considerably the area previously designated as a "second-set" reaction.

A less violent form of second-set graft reaction is well known,[2] in which the graft is broken down prematurely, but evidences some degree of healing and possibly of vascularization. This usually is the case when the interval between immunization and test graft is long. In light of what has been shown here, it appears that the lapse of time brings an attenuation of immunity to levels insufficient to prevent some small degree of healing from taking place.

Summary

1. It has been shown that in inbred strains of mice three to four days of contact with an orthotopic homograft of skin are necessary to induce immunity, and that up to two additional days may be necessary for an effective level to be achieved.

2. The continuity of contact between immunizing graft and host could be broken in any of several ways tested without lessening immunization. Development of immunity was not found to be contingent on the establishment of vascular continuity, nor was it necessary that immunization be directed at a single set of regional nodes.

3. The time at which immunity appears is controlled in part by the route of immunization. The time of graft breakdown is related to the level of immunity and presumably of antibody in the host, and the degree of healing that has taken place prior to exposure to this antibody.

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