THE VASCULAR BED AS THE PRIMARY TARGET IN THE DESTRUCTION OF SKIN GRAFTS BY ANTISERUM

II. Loss of Sensitivity to Antiserum in Long-Term Xenografts of Skin*

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In our studies on the effects of humoral antibodies on xenografts and allografts of rat skin, we have shown that the interval between placement of the grafts and administration of the serum is crucial in determining the fate of the transplanted skin (1, 2). Antiserum injected at the time of transplantation or within a week thereafter has little or no detectable effects on the skin, but during the ensuing week the grafts become increasingly sensitive to the serum, and for a period of 2–3 wk they can be acutely damaged by this agent in routine fashion. Grafts that survive beyond 3 wk gradually lose their sensitivity to antiserum and at about 5–6 wk after transplantation they fail to respond to treatment or do so only with mild and transient inflammation and, rarely, some focal tissue damage.

The period of insensitivity that occurs immediately after transplantation appears to depend on the nonspecific resistance of new and regenerating blood vessels to inflammatory stimuli, and we have described our studies on this phenomenon in the preceding paper (3). We present here the results of our studies on the loss of resistance that occurs in long-standing grafts and appears to depend on the replacement of donor endothelium by host cells.

Materials and Methods

Animals. Pure strain and F_1 hybrid mice of the following kinds were obtained from The Jackson Laboratory, Bar Harbor, Maine: A/J, B10.D2/SgSn, $AKD2F_1$, $B6D2F_1$, $B6AF_1$, CAF_1, and LAF_1. Fisher (Fi), Lewis (Le), and (Lewis × Brown Norwary) F_1 hybrid (LBN) rats were purchased from Microbiological Associates, Walkersville, Md. CD rats were purchased from local dealers.

Antisera. Rabbit anti-mouse thymocyte serum (RAMTS),[1] rabbit anti-rat serum (RARS), and mouse anti-rat serum (MARS) were prepared as previously described (4). Rat anti-mouse serum (RAMS) was prepared by injecting LBN rats with a mixture of cells prepared from B10.D2 lymph nodes, spleens, and thymuses (10^8 cells per rat, intraperitoneally). The rats received three injections of cells at weekly intervals and 7 d after the last injection they were bled for the purpose of preparing a single pool of antiserum.

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[1] *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; MARS, mouse anti-rat serum; RAMS, rat anti-mouse serum; RAMTS, rabbit anti-mouse thymocyte serum; RARS, rabbit anti-rat serum.

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Experimental System. Mice were thymectomized 2–3 wk before receiving rat skin grafts, and they were injected intraperitoneally with 0.25 ml of RAMTS 2 d before grafting, on the day of grafting, and on the 2nd and 4th d after placement of the grafts. In one experiment, treatment with RAMTS was continued for 8 wk after grafting at a dose of 0.2 ml administered three times weekly.

Grafting was carried out as described previously using 15- × 20-mm pieces of rat ear skin (4). At various intervals after transplantation, the recipients were injected with RARS and the effects of these injections on the grafts were studied.

Immunofluorescent Studies. Grafts were excised from their hosts and prepared for studies with fluorescent antisera as described previously (3). In some experiments, the hosts had been injected with anti-graft serum before removal of the grafts, and in these cases sections of tissue were stained directly with fluoresceinated anti-immunoglobulin or anti-C3 reagents. In other cases, sections of frozen skin were treated in vitro with MARS or RAMS and then with fluoresceinated anti-immunoglobulin serum of appropriate specificity.

Results

Effect of MARS on Long-Term Rat Skin Grafts That Have Been Retransplanted to New Immunosuppressed Mice. Skin grafts that had been maintained in a state of excellent condition for 45–99 d on immunosuppressed $B6AF_1$ mice were removed from their primary hosts and retransplanted to new immunosuppressed $B6AF_1$ mice. In some cases the secondary recipients were grafted simultaneously with freshly prepared rat skin; in other cases the secondary hosts received only the retransplanted skin and freshly prepared rat skin grafts were placed on separate mice that served as controls. All of the graft recipients were injected with MARS 14 d later. The results of these experiments are summarized in Table I. In none of the 12 retransplanted grafts was there any sign of inflammation or tissue damage after the administration of MARS. In contrast, 14 of 15 primary grafts were destroyed by antiserum. Eight of these primary grafts had been placed simultaneously with, and contralaterally to, retransplanted rat skin, and seven of these eight grafts were destroyed as a result of the injection of MARS.

TABLE I

*Sensitivity to MARS of Long-Surviving Rat Skin Grafts That Had Been Retransplanted to New Immunosuppressed Mice**

Number of mice	Age of old grafts‡	Effects of MARS on grafts			
		Inflammation§		Destruction	
		Old	New	Old	New
	d				
	Old and new grafts on same recipients				
8	66–99	0	7	0	7
	Old and new grafts on separate recipients				
4	45	0	—	0	—
7	—	—	7	—	7

* Le skin grafts were regrafted to immunosuppressed mice syngeneic with the primary recipients, and MARS (0.5 ml intraperitoneally) was administered on day 14 after retransplantation.

‡ Age at the time of regrafting.

§ Edema, erythema, and local hemorrhage.

Retransplantation alone does not make skin grafts resistant to antiserum at 14 d, as we have shown in an earlier report (3). Moreover, we have, as part of the present study, transplanted Le skin to five LBN rats and allowed these grafts to remain in place for 67 d. The grafts were then removed and transplanted onto immunosuppressed $B6AF_1$ mice. 14 d after this regrafting, the secondary recipients were injected with MARS. All five of the grafts were destroyed within 24 h. Thus the continued resistance to antiserum of retransplanted rat skin is not an artifact of technique, and it points clearly to alterations in the long-standing grafts themselves, rather than in their hosts, as the major cause for the development of resistance to antiserum.

Rejection of Long-Term Rat Skin Grafts on Retransplantation to New Untreated Recipients. Long-standing xenografts of rat skin are completely insensitive to antisera and their resistance is maintained even when they are regrafted to new immunosuppressed hosts. They are, however, frequently rejected by these secondary hosts several weeks or months after transplantation. Hence they are vulnerable to other, presumably cell-mediated, forms of immune attack. To examine such vulnerability more precisely, we have removed long-standing grafts and retransplanted them to untreated mice syngeneic to the first hosts. The patterns of rejection of these grafts were compared with fresh grafts of rat skin place also on untreated mice. In some instances both fresh and regrafted skin were placed simultaneously on the same recipients. The results of these experiments are shown in Table II. Five mice received grafts of skin that had previously been in place on immunosuppressed hosts for 50 d, and all of the grafts were rejected acutely 7.5–10.5 d after regrafting (mean survival time 9.5 ± 1.5) 11 mice that received fresh rat skin rejected their grafts acutely 6.5–8.5 d after grafting (mean survival time 7.25 ± 0.6). Thus, the retransplanted skin survived for a slightly but significantly longer period of time than did skin taken directly from donor rats. When four mice were grafted with both fresh and previously grafted rat skin, the new graft was in every case rejected 1–3 d before the retransplanted skin, an observation that indicates that the longer survival of regrafted skin is traceable, at least in part, to its lower susceptibility to an established state of transplantation immunity. Micro-

TABLE II

*Fate of Long-Surviving Rat Skin Grafts after Retransplantation to New Untreated Mice**

Number of mice	Time on first host	Graft survival on second host
	d	*d*
5	50	9.5 ± 1.5
11	0‡	7.25 ± 0.6

Old and new grafts on same recipients

	Old	New‡
280	11	10
213	11	8
102	11	8
72	12	10

* Rat skin was initially grafted onto immunosuppressed B10.D2 mice, and at various times thereafter they were removed and placed on new nonsuppressed B10.D2 hosts.

‡ Control grafts of skin taken directly from rats.

scopic examination of sections of fixed specimens of grafts of both kinds showed that rejection was accompanied by a marked infiltration of the graft bed by mononuclear cells.

Effect of Placing New Grafts of Rat Skin on the Bearers of Long-Standing Grafts. The elevated resistance of long-standing grafts of rat skin is not dependent on their being regrafted, as shown in the following experiment. Eight mice bearing Le rat skin grafts of 54–152 d duration received second grafts of Le skin. The survival times of these grafts are indicated in Table III. In six cases, the second grafts were rejected much earlier (11 to >150 d) than the original grafts on the same recipient. In one case both grafts were rejected 19 d after placement of the second graft and one mouse died with both grafts intact 21 d after application of the second graft. Again, there is decisive evidence that long-standing grafts are susceptible to immunologically mediated rejection, presumably of a cellular nature, but they are clearly less susceptible than recently placed grafts.

Deposition of Injected Immunoglobulin in Long-Standing Grafts after the Administration of Anti-Graft Sera. We have shown that beginning at about the 4th d after transplantation, the injection of antiserum leads to the deposition of the injected immunoglobulin and endogenous C3 on the luminal surfaces of graft vessels (3), and we have suggested that these deposits play a crucial role in antiserum-mediated destruction of grafts. We have looked, therefore, for evidence that anti-graft antibodies react with endothelial antigens of long-standing grafts. Anti-graft sera (rabbit or chicken anti-rat sera that had been absorbed with mouse tissues) were injected into mice bearing grafts of 50–150 d duration, and 1–2 h later the grafts were removed and frozen sections of them were treated with fluorescein isothiocyanate (FITC) conjugates of rabbit anti-immunoglobulin. In the case of four grafts whose hosts had been injected with rabbit anti-rat serum, no detectable deposits of immunoglobulin were detected; grafts taken from four of five mice that received chicken anti-rat serum similarly lacked detectable deposits of immunoglobulin, whereas the fifth graft had numerous vessels that showed deposition of chicken immunoglobulin, though staining in this

TABLE III

*Effect of Transplanting Fresh Rat Skin to Hosts Bearing Long-Term Grafts**

Age of old grafts‡	Serum titer§	Graft survival	
		Old	New
d		*d*	
152	0	23	12
150	1:16	>21‖	>21
150	0	>42‖	10
117	1:4	>163‖	9
54	0	19	19
54	1:8	20	9
54	0	39	22
54	0	23	9

* Le skin was transplanted contralaterally to established long-term Le grafts. No additional immunosuppression was applied.

‡ Age at application of the second graft.

§ Activity of recipient serum as assayed in cytotoxicity tests with rat spleen cells on day of placement of second grafts.

‖ Mice died with intact grafts.

instance was less intense than was observed in younger grafts that were still sensitive to antiserum. The insensitivity of long-standing skin grafts to antiserum is clearly associated with alterations in graft vessels that prevent significant reactivity of injected antibodies with endothelial cell surfaces.

Reactivity In Vitro of Long-Standing Skin Grafts with MARS and RAMS. The failure of vessels in long-standing skin grafts to react with MARS in vivo indicates that graft antigens have been altered or that the endothelium of the graft vessels has been replaced by host cells. To distinguish between these two possibilities, grafts were removed from their suppressed hosts at various times after transplantation and tested in vitro for reactivity with MARS and RAMS. The grafts were frozen immediately after removal and cut at a thickness of 2 μm. They were treated with either MARS or RAMS for 30 min, washed with phosphate-buffered saline, and then treated for 30 min with fluorescein-conjugated rabbit anti-mouse or rat IgG, reactive with both light and heavy chains. The unreacted anti-IgG was removed by washing again with saline and the grafts were examined by fluorescence microscopy and scored arbitrarily on the basis of the numbers of fluorescent vessels observed. These observations are summarized in Table IV, and representative examples of the appearances of stained grafts are presented in Figs. 1–3.

In all of 10 grafts removed and tested 4–14 d after transplantation, there were numerous small vessels within the graft that displayed bright fluorescence after treatment with MARS and FITC-anti-immunoglobulin. An example of these reactions is shown in Fig. 1 B. In 8 of these 10 grafts, no fluorescent vessels were detected after treatment with RAMS and FITC-anti-immunoglobulin (Fig. 1 A); in two of them there were a few positive vessels, one near the base of the graft and the other in the upper dermis. Vessels in the beds or around the edges of the grafts were never observed to fluoresce after treatment with MARS and fluoresceinated anti-immunoglobulin (Fig. 1 B), although they showed variable degrees of staining when treated with RAMS and FITC-anti-immunoglobulin (Fig. 1 A and Table IV). These patterns of immunofluorescent staining of graft and host vessels are completely consistent with those observed when MARS was injected into graft recipients before the grafts were removed and treated with FITC-anti-immunoglobulin (3).

Four long-standing rat skin grafts displayed quite different patterns of staining. In

TABLE IV

*Reactivity In Vitro of Rat Skin Grafts with MARS and RAMS**

Number of grafts	Age of grafts	Vessels in graft staining with		Vessels in bed/edge staining with	
		MARS	RAMS	MARS	RAMS
	d				
2	4	++++	−	−	+
2	6	++++	−	−	++++
1	8	++++	−	−	+
5	14	++/+++	−/+	−	+/++++
3	73	+/++	++++	−	++++
1	109	−	++++	−	++++

* Rat skin was grafted onto immunosuppressed B6D2F$_1$ mice, and the grafts were removed at various intervals and tested with MARS or RAMS followed by FITC-anti-immunoglobulin.

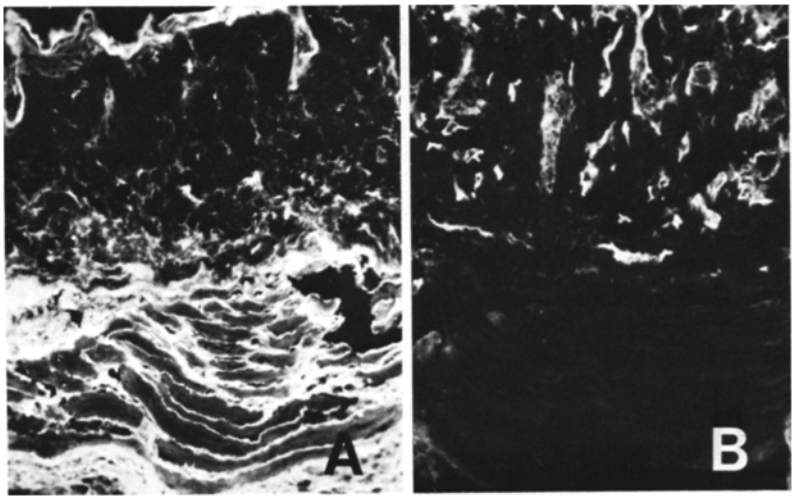

FIG. 1. Immunofluorescence photomicrographs of a rat skin xenograft sampled 4 d after grafting onto an immunosuppressed mouse. Cryostat sections were stained in vitro with (A) RAMS and fluorescein-conjugated anti-rat IgG or (B) MARS and fluorescein-conjugated anti-mouse IgG. The vessels in the graft bed, but not the graft itself, stain in (A); in contrast, the vessels in the graft, but not the graft bed, stain in (B).

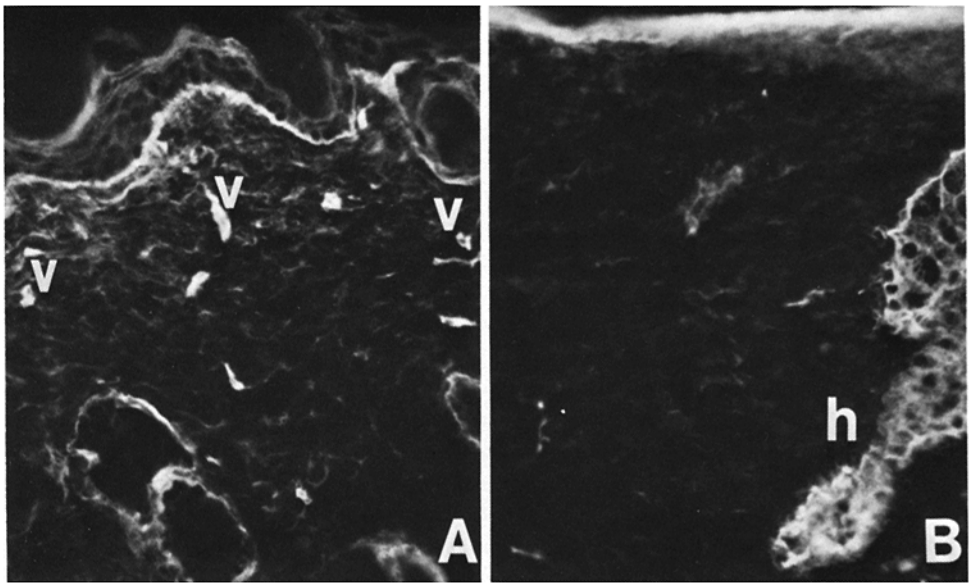

FIG. 2. Immunofluorescence photomicrographs of a rat skin xenograft sampled 3 mo after grafting and stained as in Fig. 1. Almost all graft vessels, e.g., the three marked V strain with RAMS (A), but only rare vessels stain with MARS (B). The graft epidermis at the top, and the hair follicle (h) strain with MARS but not with RAMS.

the case of one removed from its host at 109 d after grafting, there was widespread staining of graft vessels when RAMS was used but no staining when MARS was used in the initial step in vitro (Fig. 2 A and B). In three grafts removed after 73 d residence on mice, there were again numerous graft vessels fluorescing when RAMS was used.

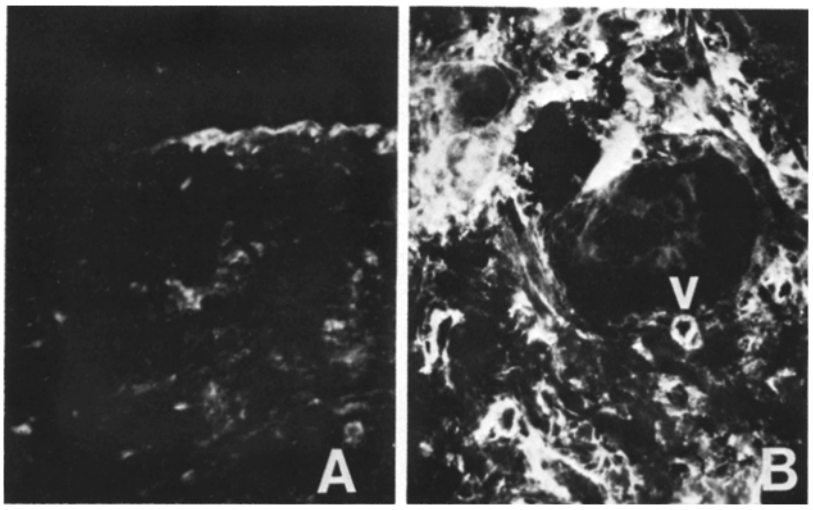

FIG. 3. Immunofluorescence photomicrograph of (A) an old (3 mo) and (B) a new (14 d) rat skin xenograft on mice given MARS intraperitoneally 3–4 h earlier. Cryostat sections stained with fluorescein-conjugated anti-mouse IgG show bright endothelial staining of the graft vessels (v) in (B) but not in (A).

In these grafts, however, there were still some vessels that reacted with MARS; in two of the grafts there was only a small number of such vessels, whereas one graft contained many weakly staining vessels in the upper dermis. Again, these results are in complete accord with the observations, reported above, that MARS administered to the bearers of long-term rat skin grafts does not react detectably with graft vessels. Both the short- and long-term grafts showed faint but definite diffuse staining of the graft epidermis and connective tissue with MARS but not with RAMS.

It is evident from these observations that in long-surviving xenografts of skin there is gradual replacement of donor endothelium by host cells, and that this process is associated with the acquisition of resistance to antisera by long-term skin grafts. With respect to other constituents of skin grafts, it should be noted, as reported above, that long-standing grafts of rat skin are acutely rejected shortly after retransplantation to new recipients syngeneic to the primary hosts. Furthermore, observations on long-term grafts that have been removed and treated in vitro with MARS and FITC-anti-immunoglobulin reveal fluorescence of both dermal and epidermal elements of the grafts (Fig. 2 B).

Fate of Long-Term Rat Skin Xenografts after Retransplantation to Untreated Rats of the Initial Donor Strain. Rat skin that has survived for several weeks or months on immunosuppressed mice is a composite of rat epidermal and dermal cells and mouse endothelial cells. It is resistant to MARS but is rejected when regrafted to nonimmunosuppressed mice. It was of interest, therefore, to determine its fate when retransplanted to rats of the original donor strain. Five LBN skin grafts that had survived in excellent condition for 50 d on B10.D2 mice, and eight Fisher skin grafts that had survived for 70 d on B6D2F_1 mice were removed with a narrow cuff of surrounding host skin and placed on rats of the LBN and Fisher types, respectively. Eight Fisher grafts that had resided on B6D2F_1 mice for 14 d were removed and regrafted to Fisher rats. The fate of the grafts is indicated in Table V. 10 of the 11 long-term grafts were acutely rejected 7–

TABLE V

*Fate of Rat Skin Regrafted to Rats after Residing for Various Periods of Time on Immunosuppressed Mice**

	Number of grafts	Number rejected
LBN → B10.D2 − 50d → LBN	5	4
Fi → B6D2F$_1$ − 70d → Fi	8	8
Fi → B6D2F$_1$ − 15d → Fi	8	0

* Rat (LBN or Fi) skin was transplanted to immunosuppressed mice (B10.D2 or B6D2F$_1$) and, at the intervals indicated, regrafted to rats of the original donor strain.

9 d after secondary transplantation. The remaining long-term graft survived indefinitely, although its surrounding cuff of mouse skin was acutely rejected at 9 d. None of the eight grafts that were retransplanted after 14 d in residence on mice were rejected, although, as expected, the surrounding mouse skin was acutely destroyed at 8–10 d after grafting. In other experiments, we have retransplanted skin that had been in place for 14–16 d to animals syngeneic to the initial donors; and in none of 22 instances were the grafts rejected. Evidently, the rejection of mouse endothelial cells by the secondary recipients of the long-term grafts leads to destruction of the syngeneic elements of the graft as well. The survival of one retransplanted long-term graft may be attributable to persistence of a sufficient number of vessels lined with rat endothelium, for as we have shown above, variable amounts of donor endothelium persist in such grafts.

Discussion

It has been recognized for several decades, largely on the basis of morphologic studies carried out directly on living tissues or on sections of fixed tissues that had been removed at various times after grafting, that the small blood vessels of tissue grafts constitute a primary focus of attack by immune reactants. This is easily understood because the vascular bed of the graft is the major interface between host and donor tissues, and vascular changes are the hallmark of all inflammatory responses. What is surprising about the findings reported here is their implication that humoral antibody can initiate damage to grafts only if it reacts with antigens of the donor vessels. It cannot, of course, be formally proved that the replacement of graft endothelium by host cells is the major cause of resistance of long-surviving grafts to antisera, but the evidence weights heavily in favor of such a view. All of the gross and microscopic features of antiserum-induced damage of skin grafts can be attributed to vascular damage, and anti-graft antibodies and endogenous C3 are detectable on the luminal surfaces of graft vessels just before the development of grossly detectable damage. The gradual loss of sensitivity to antiserum begins at about 2–5 wk after grafting just at a time when mouse cell surface antigens become detectable in graft vessels, and when the grafts attain complete resistance to antiserum, all but a few of their vessels are lined with cells of host origin. Furthermore, the state of resistance develops and persists even though other elements of the graft are of donor origin. This is clear from the acute rejection of the grafts that occurs when they are retransplanted to mice syngeneic with the primary recipients, and from the staining of extravascular structures that is observed when long-surviving grafts are excised and treated in vitro

with MARS and fluoresceinated anti-mouse immunoglobulin. In experiments in which similar grafts are excised after the injection of their hosts with antiserum and then treated with anti-immunoglobulin, there is no staining of graft elements, indicating that antibodies had not reached extravascular cells. Evidently, grafts are acutely damaged by humoral antibodies only if these agents react with endothelium, though it is not clear whether this is due to the inability of antibodies to reach extravascular elements in sufficient concentration to incite damage or to a requirement for a direct reaction of antibodies with endothelial cells. In either case there are important implications for other types of antibody-mediated damage to vascularized tissues, including tumors.

In addition to providing a mechanism for the resistance of long-surviving skin grafts to antiserum, the observations we report here bear on an issue of recurring interest in the study of tissue transplantation. The notion that host cells might gradually replace the endothelium or other parts of grafted tissues has been put forth on a number of occasions, and it has been suggested that such replacement could be partly responsible for the lowered state of host reactivity toward long-standing grafts. However, previous efforts to demonstrate replacement of donor cells by those of the host have been unsuccessful, owing perhaps to the use of primarily vascularized organ grafts in which the phenomenon takes place slowly or not at all. The unequivocal demonstration of the replacement of donor endothelium by host cells that we describe here has been facilitated by the use of skin grafts, in which only small segments of donor vessels are preserved, and by the use of inter-species grafts that provides for sharp distinction between host and donor cells, a feature that has been especially valuable in carrying out immunofluorescent studies. Although we have not demonstrated replacement of endothelium in allografts by direct methods, its occurrence can be inferred from the observations that long-standing allografts lose their sensitivity to antisera, and they are rejected on regrafting to animals syngeneic to the original donor.

Whereas the replacement of graft endothelium by host cells leads to a high level of resistance to humoral antibodies, its effect on sensitivity to T cell-mediated damage is far less dramatic. Grafts that have survived for long periods of time on immunosuppressed hosts survive longer than expected when they are regrafted to new nonsuppressed recipients syngeneic to the primary hosts, but the increase in survival time is small and not clearly traceable to the changes in vascular endothelium. Furthermore, the grafts are very acutely rejected in every case in spite of their complete resistance to anti-graft serum. T cells are obviously more versatile and more devastating in their attacks on organized tissues and organs than are humoral antibodies and their action is only mildly attenuated when donor endothelium has been replaced by host cells. The destruction of tissues by T cells is associated with vascular phenomena and frequently involves vessels of the host, but the involvement of vessels obviously does not require direct reaction of the T cells with endothelial antigens, as is the case for antiserum-mediated damage. That long-surviving grafts of rat skin are rejected when retransplanted to rats of the original donor strain that respond primarily to the included mouse endothelium, as well as when they are grafted to new mouse recipients that respond primarily to nonvascular elements, provides experimental systems that will be useful in determining the importance of vascular damage in cell-mediated, as well as humorally mediated, damage to tissues.

Summary

Rat skin that survives for long periods of time on immunosuppressed mice becomes resistant to anti-graft serum and remains so for as long as it survives. When long-standing grafts are removed and placed on new immunosuppressed mice, they remain resistant to antiserum for as long as they survive. The acquired resistance to antiserum seems, therefore, to be due to changes in the grafts rather than to changes in their hosts. Furthermore, it was found that the acquisition of resistance is correlated with replacement of graft endothelium by host cells, as demonstrated by the use of immunofluorescent techniques in conjunction with mouse anti-rat serum and rat anti-mouse serum. Evidently, humoral antibodies are able to cause acute damage to skin grafts, and presumably to grafts to other organized tissues, only if they react with antigens of graft endothelium.

Long-term grafts that are retransplanted to their original donors or to rats syngeneic with those donors are in most cases rejected, whereas 14-d-old grafts similarly regrafted are in no case rejected. Apparently, the responses of the secondary recipients to the mouse endothelial antigens in long-term grafts lead to destruction of the entire grafts. When long-standing rat skin xenografts are removed and placed on untreated mice syngeneic with the primary hosts, they are in every case rejected, although they survive slightly longer than skin taken directly from rat donors. Rejection is accompanied by a mononuclear infiltrate and is qualitatively indistinguishable from the rejection of freshly prepared rat skin. Clearly, sensitized cells are more efficient than humoral antibody in destroying grafted tissues.

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