Prolonged Retention of Glutaraldehyde-treated Skin Allografts and Xenografts:

Immunological and Histological Studies

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Glutaraldehyde (GA)-treated skin allografts and xenografts (from mice, rats and guinea-pigs) behave in the same way as judged from retention time, gross inspection, microscopic examination, and assays for graft antigenicity. The GA-treated grafts are retained for long periods of time (an increase by more than 6-fold as compared to untreated grafts), they are tightly bound to the recipient, they are initially soft but become progressively stiffer with minimal shrinkage in size, and remain free from infection. The histology shows that the grafts are nonviable and fixed by the GA, they are avascularized but the general structure of the skin (epidermis, adnexa and dermis) is preserved for about 3 months. The antigenicity of the GA-treated grafts is very poor, actually it is undetectable. They do not elicit the formation of cytotoxic antibodies, and animals sensitized by untreated allografts retain the GA-treated allografts similarly to normal unsensitized recipients. The lack of transplantation immunity is also indicated by the fact that GA-treated isografts behave and are rejected similarly to GA-treated allografts and xenografts. Microscopic examination suggests that the mechanism of rejection of GA-treated grafts is similar to that operating in the rejection of an inert foreign body. The marked prolongation in the retention of Ga-treated skin grafts and their properties justify investigations on the applicability of these grafts in clinical practice.

IN A PREVIOUS COMMUNICATION it was shown that mice allografts treated in vitro with glutaraldehyde (GA) were retained for long periods of time with minimal shrinkage in size of the graft.[14] These observations were confirmed by Im and Simmons.[8] In both studies,[8,14] however, the mice used shared identical major histocompatibility loci and they differed at minor histocompatibility loci. The present study was designed to investigate the generality of these findings by grafting GA-treated skins across the strong H-2 histocompatibility barriers and across species. Histological features and immunological parameters were also studied in order to evaluate the mechanism for the prolonged retention of GA-treated grafts. The properties of the GA-treated grafts (retention for about two months, preservation of the skin structure, and the lack of infection) justify investigation of the clinical use of these grafts.

Materials and Methods

Aqueous solution of 25% (w/v) of glutaraldehyde (GA)* and sterile solutions of 0.01 M sodium phosphate buffer (pH 7.4) - 0.15 M sodium chloride (PBS) were used.

The inbred strains employed in grafting experiments were C57BL/6 (H-2^b) and BALB/c (H-2^d) mice weighing 25-30 g, Lewis (150-200 g) and Brown Norway (BN) (100-120 g) rats which differ at major and minor histocompatibility loci,[10] and guinea pigs of the N13 strain (300-350 g). The animals were obtained from the Experimental Animal Unit, The Weizmann Institute of Science.

Full-thickness skin grafts were performed according to Billingham and Medawar.[4] Grafts were protected by vaseline gauze and plaster jackets, which were removed 10 days after transplantation. Grafts were inspected daily until they were rejected. When grafts were still retained on the host at the last inspection, the retention time was indicated with an "over" symbol (>). In the control (untreated grafts) and experimental (GA-treated grafts) groups the course of graft rejection was different. In the

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*From Sigma, St. Louis, Missouri.

TABLE 1. *Prolonged Retention of Glutaraldehyde-treated Skin Grafts.*

Group	Donor*	Recipient	Graft treatment	No. of animals	Retention time (days)	
					Average	Range
Allografts						
A	BA	C57	none	26	12.9	(11- 16)
			GA	22	>108	(29->135)
B	C57	BA	none	17	13.6	(11- 17)
			GA	16	> 63	(37->70)
C	BN	Le	none	13	14.3	(13- 16)
			GA	16	> 91	(55->130)
Xenografts						
D	C57	Le	none	17	10.5	(10- 12)
			GA	19	>106	(55->130)
E	GP	Le	none	9	15.3	(14- 18)
			GA	12	>105	(62->130)
F	BN	C57	none	9	16.1	(15- 18)
			GA	13	> 82	(28->120)
Isografts						
G	C57	C57	none	12	>130	(>130)
			GA	13	>120	(88->130)
H	BA	BA	none	11	>130	(>130)
			GA	9	>104	(58->130)
I	Le	Le	none	7	>130	(>130)
			GA	10	>103	(62->130)

*Abbreviation for animals are: BA, BALB/c mice; C57, C57BL/6 mice; BN, Brown Norway rats; Le, Lewis rats; GP, guinea pigs of the N13 strain.

controls, survival end point was considered when about 80% of the graft was necrotic and macerated. In the experimental groups rejection was considered when about 70% of the graft was detached from the recipient bed.

Skin grafts (about 1.5 cm in diameter) were excised from the donor animal, treated in vitro, and then applied to the dorsum of the recipient. Twelve skin patches were placed in a glass vessel containing 30 ml of 3 mg/ml of GA in PBS, and kept at room temperature for 20 min with gentle manual shaking to prevent clumping. The grafts were freed from the GA by washing 4 times in 50 ml PBS. In every experiment fresh GA solution was prepared by adding 0.36 ml of GA (25% w/v) to 29.6 ml of PBS. In the control groups the grafts were exposed only to PBS.

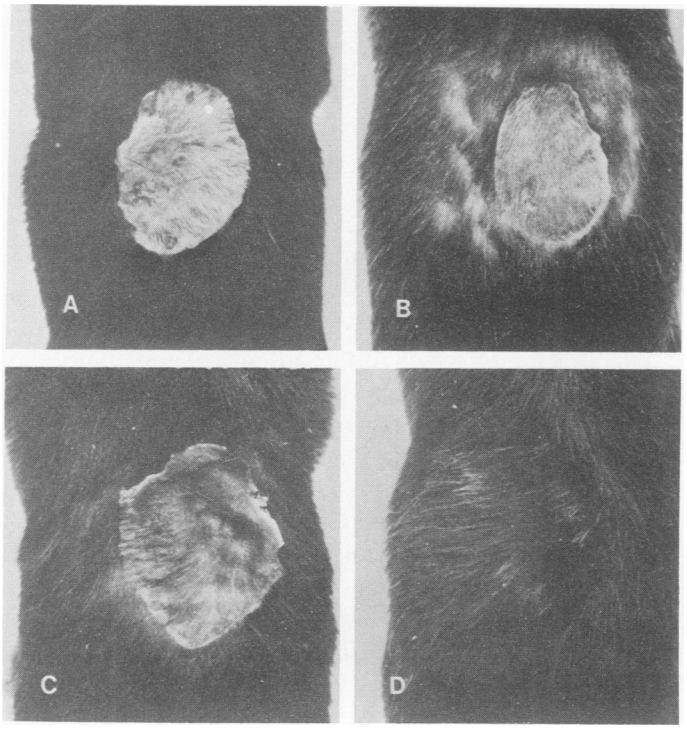

FIG. 1. Skin grafts on C57BL/6 mice at 100 days. (A), GA-treated allograft from BALB/c mouse; (B), GA-treated xenograft from BN rat; (C), GA-treated isograft; (D) untreated isograft.

The complement dependent cytotoxic antibodies in the recipients of C57BL/6 grafts were measured by the ^{51}Cr release assay (13). The ascites tumor cell EL-4, maintained in the syngeneic C57BL/6 mice, served as target cells.[5] The ^{51}Cr released by 4 times freeze-thaw of the labeled EL-4 target cells was taken as the maximum released (100%). In control experiments the sera without added complement were not cytotoxic, and they did not have anticomplementary activity.

Histological sections were stained with hematoxylin and eosin.

Results

The rejection of untreated allografts and xenografts in 6 different control groups (Table 1) occurred within a short period of time (11 to 16 days). It involved shrinkage of the skin-graft, maceration and serum exudation with slough formation.

The pattern of rejection of GA-treated skin grafts was the same in three allogeneic and three xenogeneic combinations. About half of the GA-treated grafts were retained on the host for over 100 days (Table 1, Fig. 1). After removal of the cast (day 10) all grafts were soft and

pliable. However, 2-6 days later they began to stiffen, but small soft areas persisted for a long period of time. The GA-treated grafts were firmly adherent to the bed (Fig. 2). In contrast to the untreated grafts, the GA-treated grafts retained their original size (Fig. 1), and only towards the end showed some diminution in size. The rejection process was characterized by progressive desiccation of the graft, and undermining of the GA-treated graft by the host epidermis. Separation started at peripheral portions of the graft and proceeded in a continuous fashion. Occasionally, by the end of this process a dry graft having its original dimension was detached in one piece from the host. Concurrently with the graft separation, the recipient bed was found to be healed and did not expose a denuded surface. At all stages neither infection nor inflammation was observed.

In order to gain some information on the mechanism of rejection of GA-treated grafts, the effect of GA on isografts was studied. The untreated isografts in two strains of mice and in Lewis rats were soft, and survived indefinitely with normal hair growth, as expected. On the other hand, the GA-treated isografts became stiff and were rejected in a pattern which was indistinguishable from that of GA-treated allografts and xenografts (Table 1, Fig. 1). These results indicated that an immune process was not involved in the rejection of GA-treated grafts.

The sera of BALB/c mice receiving C57BL/6 allografts contained cytotoxic antibodies to C57BL/6 cells, as expected.[5] On the other hand, these antibodies were undetectable in the sera of BALB/c mice grafted with GA-treated skins of C57BL/6 mice (Fig. 3). Similar results were obtained with Lewis rats receiving untreated and GA-treated xenografts from C57BL/6 mice. These experiments show that the humoral immune response towards allografts and xenografts was not elicited upon challenging the recipients with GA-treated skins.

It is known that in addition to cytotoxic antibodies, sensitized lymphocytes are also involved in graft rejection.[5] To gain information on the cellular component we grafted 12 Lewis rats simultaneously with untreated and GA-treated skin allografts from BN rats. It was found that the average retention time of the untreated grafts was 13.6 days (range 12-15 days), while with the GA-treated grafts it was > 98 days (range 66—> 135 days). These values are similar to those determined in Lewis rats receiving either allograft separately (see Table 1). The pattern of allograft rejection was also identical in the doubly and singly grafted rats. Similar results were obtained with mice. In C57BL/6 recipients (16 mice) the first set of untreated allografts from BALB/c donors was rejected after 12.1 days (range, 10-15 days). At 20 days (counting from the first grafting) the C57BL/6 mice received a second set of GA-treated BALB/c allografts which had an average retention time of >99 days (range, 35—>130 days). Thus, in rats and mice sensitized by untreated allografts there was no demonstrable change in the retention time and in the pattern of rejection of GA-treated allografts.

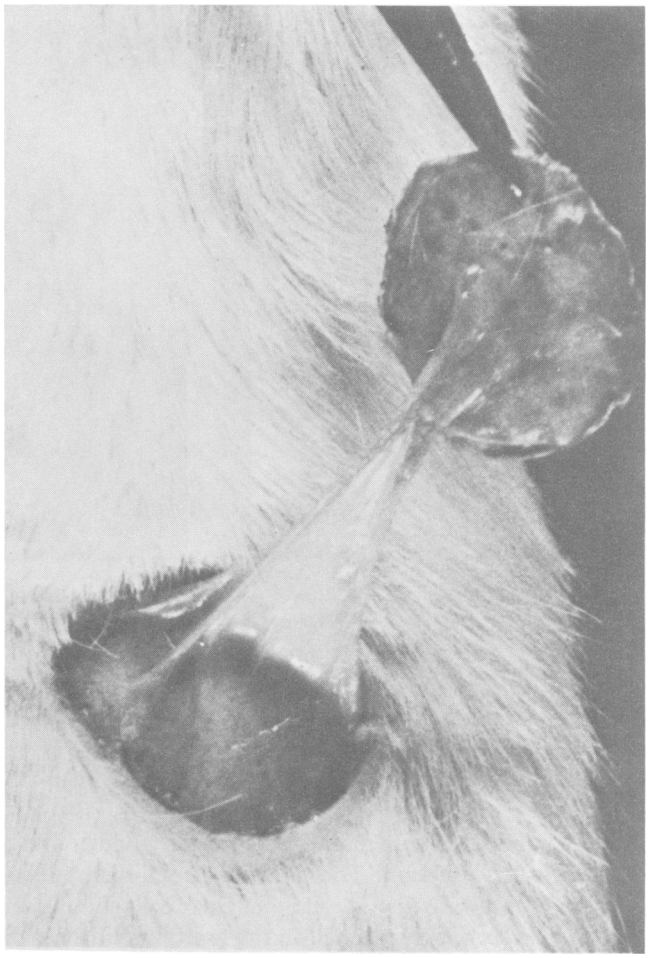

FIG. 2. The attachment of GA-treated N13 guinea pig xenograft to the bed of Lewis rat at 62 days. The xenograft was removed mechanically to demonstrate the adherence by connective tissue elements. The graft bed was free from infection.

By microscopic examination the GA-treated isografts, allografts and xenografts showed the same histological features. The characteristic findings were the preservation of the general structure of the skin over a long period of time, cellular infiltrate which subsided in the course of time, adherence of the graft to the bed by fibrillar elements, and the proliferation of blood vessels in the bed of the graft with the lack of vascularization within the graft. After 2 weeks the untreated allografts and xenografts were necrotic (epidermis and adnexa were not discernible) and heavily infiltrated by cells; subsequently the open wound healed and was covered by host epidermis. On the other hand, at 20-38 days, in the GA-treated grafts the general architecture of the epidermis and adnexa was preserved, yet the nuclei were pyknotic in many cells.

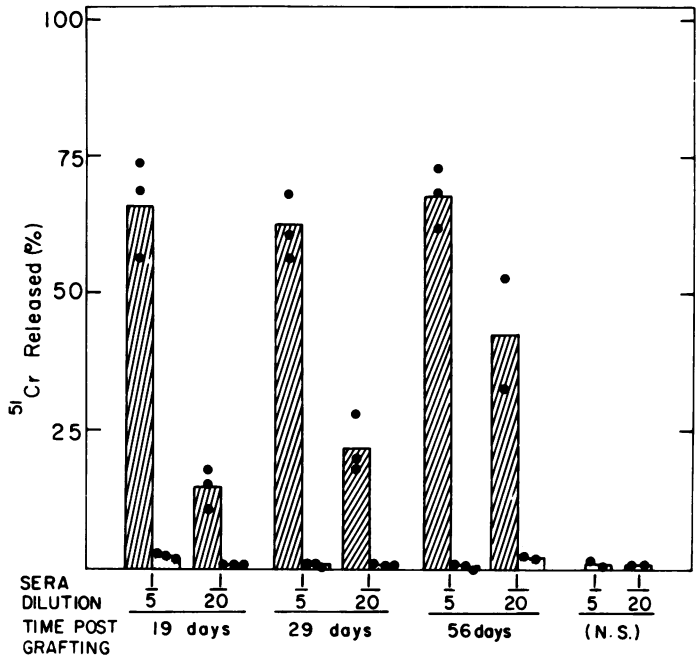

FIG. 3. Cytotoxic antibodies in the sera of BALB/c mice receiving untreated (hatched columns) and GA-treated (open columns) skin allografts from C57BL/6 mice donors. (N.S.) represent normal sera from BALC/c mice without any graft. Each filled circle represents serum from a single mouse. The target cells were the ^{51}Cr-labeled EL-4 cells.

Cellular infiltrate was mainly around the adnexa and at the graft-bed junction. The graft bed contained varying degree of infiltrate (mainly mononuclear cells) with many blood vessels which reached to the graft junction but did not penetrate it. At 90-130 days the infiltrate within the graft and bed was considerably reduced. The general structure of the graft was retained in many places, in fact it was more prominent than at earlier stages because of paucity of cellular infiltrate. The graft proper remained avascular and connection to the bed was still intact in many places (Fig. 4). In Lewis recipients, mononuclear and multinucleated giant-cells surrounded remnants of GA-treated allografts and xenografts in a manner reminiscent of the reaction towards inert foreign bodies.

The GA-treated grafts are nonviable. This is evident from the gross appearance of the grafts, their hard consistency and from the absence of vascularization.

Discussion

The studies aimed to prolong graft retention by *in vitro* treatment with GA were based on the hypothesis that GA may directly and covalently bind to the histocompatibility antigen molecules or in their close vicinity. In consequence the histocompatibility antigens would be masked and become inaccessible to the immune apparatus of the recipient.[14] This may be analogous to the situation where the coupling of long polyalanine chains to protein carrier have led to a considerable diminution in the immune response towards the protein moiety.[3] Experiments with mice which differed at minor histocompatibility loci showed that GA-treated allografts had prolonged retention time.[8,14] The data presented here demonstrated that this is also true for GA-treated allografts applied across major histocompatibility barriers and for GA-treated xenografts (Table 1).

The antigenicity of the GA-treated allografts and xenografts is markedly diminished. They did not elicit the formation of cytotoxic antibodies (Fig. 3) and sensitization of the hosts with untreated allografts did not affect the course and the prolonged retention of the GA- treated allografts (see Results). These findings confirm previous observations on the reduced antigenicity of grafts rendered nonviable by cyanide,[7] by formalin[11] or by freeze-drying.[1]

The rejection of the GA-treated grafts was characterized by progressive dessication of the graft with slow separation from the host bed. The data quoted above indicate that an immune response against the GA-treated grafts was not involved in the rejection process. The abundance of mononuclear cells and of giant-cells in the graft's bed suggest that the mechanism of rejection is similar to that operating in the reaction towards an inert foreign body.

Glutaraldehyde is a bifunctional reagent capable of reacting rapidly in aqueous solutions with the ϵ-amino group of lysine and with the α-amino groups of amino acids. These properties make it an efficient reagent for the cross-linking of proteins[2,12] which can be hardly degraded by proteolytic enzymes. It seems therefore that the GA-treated grafts remained free from infection throughout the long period of observation because the skin constituents were cross-linked by the GA. To explore this point we are currently testing the susceptibility of GA-treated skins to infestation by bacteria. The preliminary results show that GA kills bacteria commonly present on skin. Furthermore, when exposed to increasing concentrations of different bacteria (e.g. staphylococci, pseudomonas), the bacterial concentration required to initiate minor infestation of GA-treated skins is 10,000-fold higher than the concentration capable of inducing massive infestation of untreated skins.[8]

The GA-treated grafts are nonviable, yet not all dead grafts are alike. The retention of freeze-dried grafts was extended from 10 to 13 days.[1] No extention was observed in cyanide-treated allografts, and they shrank more rapidly than viable untreated allografts.[7] On the other hand, with GA-treated grafts the retention time was much more prolonged (Table 1) with minimal diminution in size (Fig. 1). The fixation by the GA is such that the general structure of the skin (epidermis, adnexa and dermis) is preserved for long periods (Fig. 4). It is avascularized but serves as a good substrate for the penetration of connec-

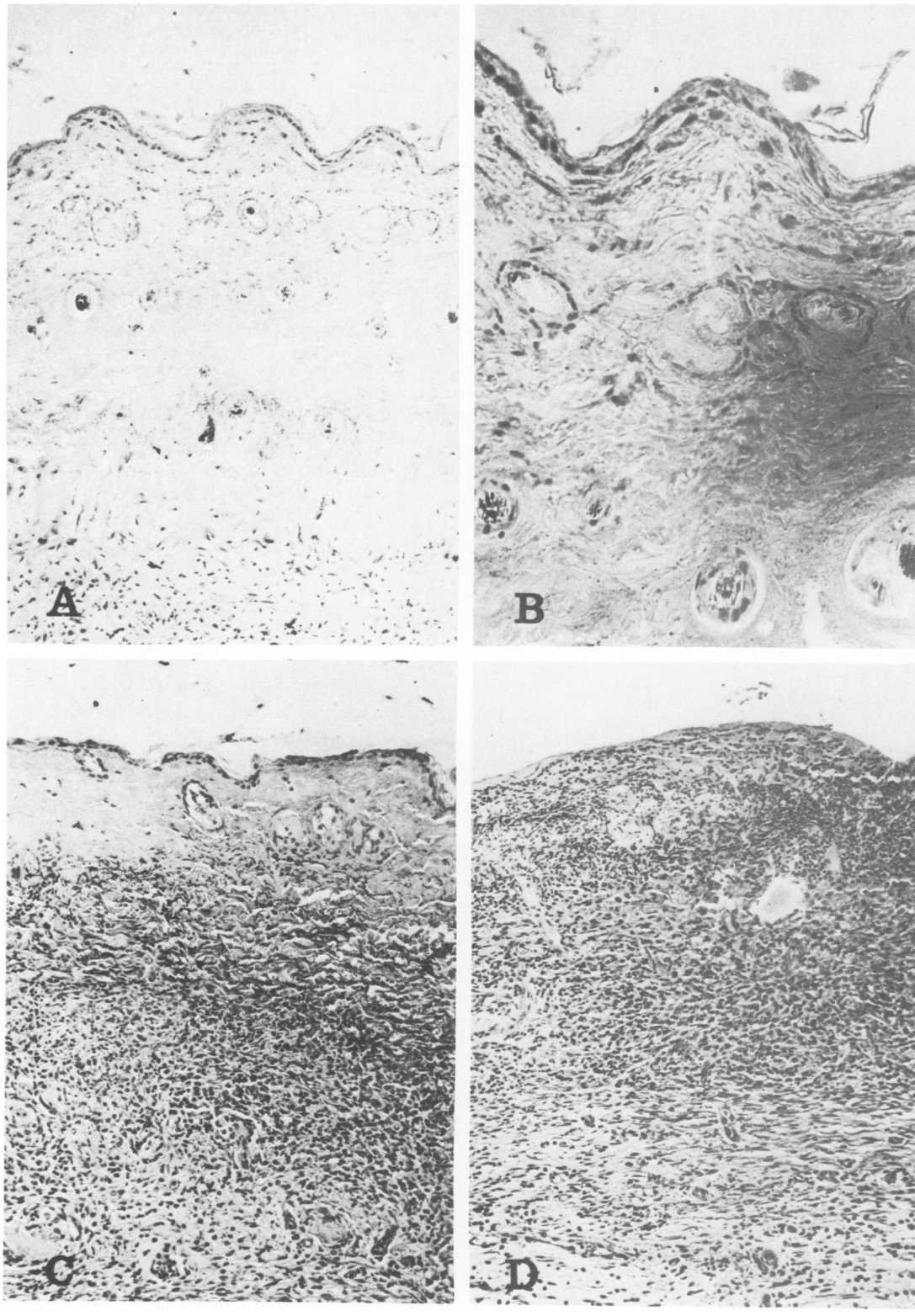

FIG. 4. Histology of xenografts. GA-treated skin from BN rat donor onto C57BL/6 mouse recipient at 92 days: A (x40); B (x100). Skin grafts from C57BL/6 mice onto Lewis rats: GA-treated at 37 days (C, x40); untreated at 14 days (D, x40). The GA-treated xenografts (A,B,C) are avascularized, the epithelial elements are preserved, and they contain cellular infiltrates (mainly mononuclear cells) which decrease with time. At 37 days the bed contains many small and medium sized blood vessels. The untreated xenograft (D) is destroyed and heavily infiltrated.

tive tissue fibers which keep it closely adherent to the recipient (Fig. 2), and granulation tissue develops at the wound bed.

The marked prolongation in retention times and the properties of GA-treated grafts prompted us to evaluate their clinical applicability. Indeed, the use of GA-treated skin allografts for the treatment of burned patients,[15] and of GA-treated umbilical cord veins for arterial substitutions in humans[6] have given encouraging initial results. The findings that in animals GA-treated allografts and

xenografts behave in the same way are relevant for the clinical trials. They suggest that it would be possible to overcome the difficulties encountered in the limited supply of skin (or other organs) from human sources by using GA-treated xenografts in clinical practice.

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