Antibody Response to Homografts VI. *In vitro* Cytotoxins Produced by Skin Homografts in Rabbits.* (26261)

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It is generally agreed that antibodies play a minor role in rejection of normal tissue homografts(1-4). Much of this conclusion is based on the failure of early attempts to demonstrate humoral cytotoxins following homografts of normal tissues(5-9). In contrast to these results, evidence suggesting the existence of cytotoxic antibodies to homografts has appeared in the past few years in various *in vivo* experiments(10-13). Moreover, direct evidence has been obtained of an *in vitro* toxicity of serum from animals injected with splenic cells(14-17). In view of these recent demonstrations of cytotoxins following grafts of homologous lymphoid tissues, the question of whether a simple skin homograft would elicit a similar antibody response was reexamined. The conditions under which these cytotoxins can be revealed and the time when they appear in the serum are reported.

Methods. Intra-breed or inter-breed skin homografts (4 × 6 cm) were applied on Dutch (D) or New Zealand (NZ) rabbits by the technic of Billingham and Medawar(18), and the recipient rabbits were bled at various intervals after grafting. Cytotoxicity of the serum was determined by incubating lymph node cells of the skin donor rabbit in serum dilutions of the skin graft recipient for 2 hours at 37°C, then examining the cells for viability by eosin dye exclusion as described by Hanks and Wallace(19). A phase contrast microscope with a 40× objective was employed to count 200 cells for each determination. All tests were performed in a plastic tray containing 5/8 × 5/16 inch wells, using a moist blotter on the under surface of the cover to prevent evaporation. Cell suspensions were prepared by teasing out cells of the lymph node in Hank's balanced salt solution (B.S.S.) which contained 10% normal rabbit serum, allowing clumps to settle, and adjusting the count to 50 × 10^6 lymphocytes per ml. To a total of 0.2 ml of normal or "immune" serum dilution in B.S.S., 500,000 lymphocytes were added per well. In most instances, fresh sera taken 2-6 hours prior to the test were used. For tests of sera stored at −10°C (sera No. 219, 220, 221, after first and second graft), following 1 hour incubation at 37°C, 0.2 ml of fresh normal rabbit serum was added to each dilution and incubation continued for 1 hour.

Results. Toxicity of sera from animals which have rejected skin homografts is summarized in Table I. Breakdown of all homografts was complete by the 20th day. Since the base level of viability of lymph node cells in normal serum varied somewhat from experiment to experiment (60-80%), a cytotoxic index (C.I.) was calculated as the difference between percentage of viable cells in experimental and control serum divided by the percent viable in control serum. Thus a maximal effect is shown by an index of 1.0 and a minimal effect by 0. Taking the viability in autologous serum as a control, the cytotoxic index of normal homologous sera and sera of autografted rabbits remained within narrow limits of −.17 to .09. It is evident that the index of sera from homografted rabbits was in most instances well above the control values. The mean cytotoxic index of the experimental sera diluted 1:2 was 0.30, as compared to control serum mean of −.01. The serum of rabbits 848 and 926 caused almost total destruction of donor lymph node cells during the 2-hour incubation period. If an index of 0.2 is selected as indicating a positive reduction in viable cells, the sera of 10 rabbits out of 18 can be considered to have contained toxic factors after a single graft. A 1:2 dilution of sera was more destructive than straight serum in a few samples, though the reason for this is

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TABLE I. Cytotoxic Effect of Serum from Normal and Homografted Rabbits on Lymph Node Cells of the Skin Graft Donor.

Donor of lymph node cells	Serum tested: Rabbit No.	Treatment	Time after grafting (days)	Cytotoxic Index (C.I.)* at serum dilutions: 1:1	1:2	1:4
842 NZ	848 NZ	Homografted with skin once	25		1.0	.92
" "	926 NZ		"		.92	.92
" "	849 NZ		"		.07	.19
752 D	840 NZ		21	.79	.17	.10
" "	839 D		"	.17	.14	
" "	748 D		"	.08	.14	
959 NZ	962 NZ		23	.06	.25	.29
" "	930 NZ		"	.43	.46	.35
" "	929 NZ		"	.03	.01	.03
960 NZ	955 NZ		38	.66	.68	.09
" "	928 NZ		"	.24	.19	.15
" "	971 NZ		"	.09	.19	.02
740 NZ	226 D		11	.21	.44	.21
" "	228 D		15	−.08	.08	.17
218 NZ	221 D		19	.15	.11	
" "	220 D		"	.24	−.06	
" "	219 D		25	−.03	.02	
		Mean of sera after first graft			.30	
		Mean of 19 controls before homograft or autograft; or after autograft			−.01	
218 NZ	221 D	Homografted with skin twice	23	.47	.43	
" "	220 D		"	.77	.75	
" "	219 D		30	.63	.44	
" "	221 D	Homografted with skin 3	32	.67	.81	.59
" "	220 D	times	"	.96	.38	.45
" "	219 D		"	.55	.34	.29
959 NZ	930 NZ		"	.84	.70	.78
" "	929 NZ		"	.27	.25	.15
959 NZ	221 D	Homografted 3 times, tested	"	.37	.12	.29
" "	220 D	with lymph node cells from	"	−.01	0	
" "	219 D	a rabbit other than the	"	−.03	−.10	
218 NZ	930 NZ	skin donor	"	.03	.05	−.16
" "	929 NZ		"	−.07	−.08	.05
960 NZ	221 D		"	.02	−.07	−.03
" "	220 D		"	.55	−.07	−.17
" "	219 D		"	−.19	−.08	
" "	930 NZ		"	.31	.44	.47
" "	929 NZ		"	−.02	−.11	

* Cytotoxic index $= \frac{C - E}{C}$, where C = % viable lymph node cells in control serum and E = % viable lymph node cells in experimental serum.

not immediately clear. Regrafting for a second or third time can be seen significantly to increase the toxicity of serum. Also, the sera of 3 rabbits which were inactive following the first graft became toxic after a second or third graft (No. 219, 221, and 929). Some degree of non-specific toxicity was noted when the sera were tested with lymph node cells of rabbits other than the original skin donor (Table I). However, it is important to note that the sera were always more deleterious to cells of the skin donor than to cells of any other rabbits. Moreover, non-specific toxicity was not found to the same degree against cells of 2 different individuals; thus the serum of rabbit 221 was slightly toxic to cells of rabbit 959, but not of rabbit 960, while the serum of rabbit 220 acted in the reverse fashion upon cells of the same 2 animals. Similarly, while the serum of rabbit

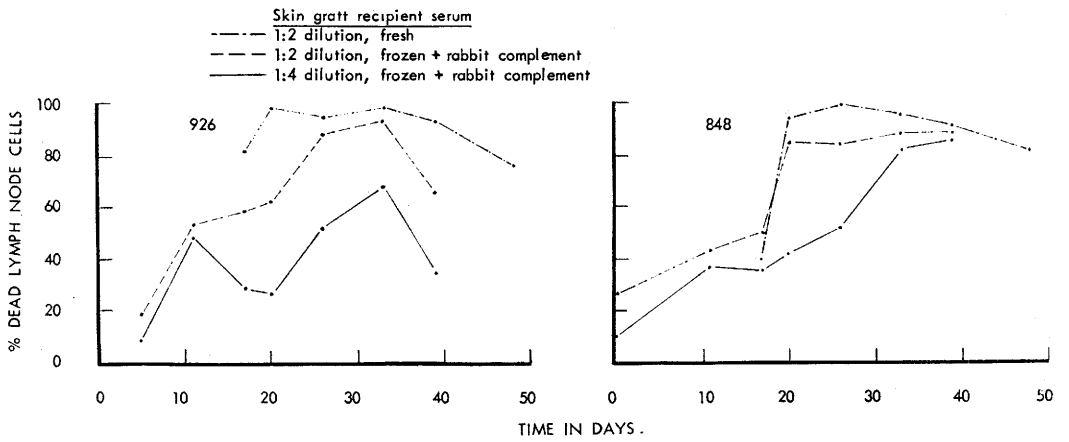

FIG. 1. Appearance of cytotoxins in sera of skin grafted rabbits to the skin donor lymph node cells.

930 was somewhat lethal to lymph node cells of No. 960, it was completely innocuous to cells of No. 218.

Time of appearance of lymphotoxins in the blood is illustrated in Fig. 1. Rejection of the graft had started in both rabbits by the 11th day and was complete by the 17th day in rabbit 926 and by the 20th day in rabbit 848. It is apparent that cytotoxins appeared in the serum when the graft began to show signs of degeneration, and reached a peak upon complete breakdown of the graft.

Discussion. One argument against the vital participation of antibodies in the homograft reaction to normal tissues is the inability to demonstrate any *in vitro* cytotoxicity of serum taken from a homografted animal. In the first extensive attempt, Medawar found that sera taken from rabbits homografted with skin did not affect survival, frequency of cell division, or migratory activity of tissue cultured skin cells from the donor animal(6). With similar tissue culture technics, Allgower, Blocker and Engley were also unable to find any deleterious effect of "immune serum" on cultured skin cells(7). The difficulty that all cells of the explant may not have been exposed to the serum was overcome in later experiments by Billingham using dissociated epidermal cells(20). Although no evidence of direct cytotoxicity of immune serum was found, a "protective" effect was shown by retransplantation of the epidermal cells after *in vitro* incubation with immune serum. It was thought that the application of immune serum to the Malphghian cells *in vitro* may have caused them to provoke and succumb to nonspecific vascular inflammatory reactions on subsequent transplantation.

In the present experiments, it has been possible to demonstrate *in vitro* cytotoxins following a simple skin homograft by using lymph node cells as the target cell. Cytotoxicity was quite marked in most instances; the sera of 2 animals killed almost all the lymph node cells of the skin donor within 2 hours. Though the sera of some animals were not toxic, an average 30% depression in viability of cells was produced by the sera of 18 rabbits. The following evidence indicates that this toxicity is probably caused by factors having general characteristics of antibodies and not by nonspecific toxins. 1). Normal homologous sera were innocuous. Following the application of skin homografts, peak activity was reached in 20-30 days. 2). Cytotoxicity of the sera increased following a second or third skin graft from the original doner. 3). Heating sera at 56°C for 30 min abolished its activity and addition of fresh normal rabbit serum restored toxicity (unpublished). 4). To a large extent, toxicity was specifically directed against the cells of the skin donor rabbit. Some cross reactivity occurred with cells of some non-specific rabbits, but not with others. This could be the result of

sharing of common antigens by the cells tested.

Although it was shown that lymphoid cells are susceptible to serum factors of a skin-grafted rabbit, it remains to be seen whether other types of cells are also affected. Preliminary tests have shown that polymorphonuclear leucocytes of the skin donor are agglutinated by active sera and that epidermal cells are killed by such sera.

Summary. The sera of rabbits which were homografted with skin are shown to be toxic to lymph node cells of the skin donor rabbit. Cytotoxicity was demonstrated upon incubation *in vitro* for 2 hours at 37°C. Sera of grafted rabbits became toxic after graft breakdown had commenced (11 days), and reached a peak after the rejection (20-30 days). The sera were in all instances more toxic to lymph node cells of the skin donor than to cells of other rabbits.

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Negative Feedback Mechanism of Cholesterol Synthesis in Experimental Nephrosis.* (26262)

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To date the mechanism for production of hypercholesterolemia in the nephrotic syndrome is unknown. Various postulates have been offered including hypothyroidism(1), increased intestinal cholesterol absorption (2), defective lipid transport(3), etc. Recently the existence of a negative feedback mechanism for hepatic cholesterol synthesis has been demonstrated in normal animals (4,5). It seemed possible that an absence of, or a defect in this mechanism might explain, at least in part, the increase in serum cholesterol in nephrosis. To test this possibility, the effects of cholesterol feeding upon normal and nephrotic animals were studied. Observations were made of C^{14}-acetate incorpora-

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