Demonstration of Cytotoxic Humoral Substances during the Course of First Set Skin Allograft Rejection* (34100)

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Previous studies by the author and co-workers (1) have suggested that humoral factors may play a predominant role in heart allograft rejection since there was increased capillary permeability of the graft before cellular infiltration occurred. Similar results were obtained in experiments in which the isolated heart was perfused with cell-free sensitized plasma (1). Serum electrophoretic analysis done on heart-grafted animals (2) indicated that elevated serum gamma globulin observed after grafting is closely related to allograft rejection and possibly represents cytotoxic antibody. The presence of humoral cytotoxic antibody in skin allograft rejection has also been demonstrated by a number of investigators. Stetson and Demopoulos (3) found that vigorous immunization of rabbits or mice against allogeneic splenic tissue resulted in the development of specific transplantation immunity of the white-graft type; this immunity could be transfered passively with serum from the immunized animals, and such sera, upon local injection, could also cause accelerated breakdown of established test allografts. Similar findings were reported by Chutna (4) and Kretschmer and Perez-Tamayo (5). These experimental results strongly indicate the importance of the humoral factor in destruction of allografted tissue. An experimental technique, using a cell-impenetrable diffusion chamber, first demonstrated by Algire (6), has been utilized by a number of workers. Algire found that cells of plasma-cell neoplasm within such chambers were either killed or inhibited in their growth in hyperimmune allogeneic host by humoral factors without the participation of host cells (7). Similar results were obtained by Amos and Wakefield (8, 9). Another kind of evidence was reported by Najarian and Feldman (10, 11). In their experiments, immune lymphocytes were enclosed in the chamber and implantation near the test grafts caused accelerated destruction of the graft. The present study was undertaken, using diffusion-chamber technique, to demonstrate the presence of humoral cytotoxic substances which appear during the course of first-set skin allograft rejection, and the duration of the presence of such humoral substances.

Materials and Methods. Partly because inbred rats are not commercially available in Japan, and partly because skin-graft survival time is more variable in closed-colony rats, making it possible to observe the relationship between graft rejection and the appearance of cytotoxic humoral substances, closed-colony Wistar female rats weighing from 100 to 130 g were used. All animals were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 5 mg/100 g of body weight. A full-thickness round skin graft measuring 12 mm in diameter was prepared from the abdominal wall of the donor rats. The wound of the donor rat was sutured and the rat was kept alive until it was used as a lymphocyte donor. Grafted skin was covered with tulle gras and sterilized surgical gauze. The entire thorax was then firmly wound with vinyl tape in order to prevent displacement of the graft. Seven days after transplantation, the tulle gras was removed and daily inspection began at this time. The vinyl tape was completely removed on Day 10 of grafting. Diffusion chambers were made by gluing two pore-size 0.1mμ

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Millipore filters[1] to a Plexiglas ring. Sterilization of the chambers was performed by irradiating both sides of the chamber for 30 min with an ultraviolet lamp. Thoracic duct lymph was collected from skin-graft donors or from unrelated rats, depending on the experiment requirement, according to the technique described by Bollman (12). One modification was made; the lymphatic cisterna was punctured with a sterilized tuberculin needle attached to a tuberculin syringe containing 0.02 ml of heparin sodium, instead of cannulating the thoracic duct with polyethylene tube. Approximately 0.2 ml of lymph from rats was collected and emptied into a centrifuge tube containing 2 ml of cold Hanks' balanced salt solution (B.S.S.). It was then centrifuged at 800 rpm for 5 min and the supernatant portion was discarded. The cells at the bottom were resuspended with an appropriate amount of cold Hanks' B.S.S. In most instances, cell concentration was made in a range from 3–6 million per ml. Cell survival was examined by mixing equal amounts of cell suspension and 0.1% erythrosin B solution. Cells which showed staining within 5 min were considered to be dead. In each test, more than 300 cells were examined. Lymphocyte suspensions with red cell contamination of 10% or more, or with cell survival of less than 90% were excluded from the experiment. Lymphocyte suspension thus prepared (0.15 ml) was inserted into each chamber through the access hole using a tuberculin syringe and a needle, and a nylon thread was used to seal the chamber. Chambers containing lymphocytes from the donor rat were then implanted into the peritoneal cavity of the recipient animals. The chambers were removed from the peritoneal cavities 1, 3, or 5 days later, and a total lymphocyte count and cell survival were determined as described previously. Approximately 70–90% of the originally enclosed cells were recovered in the control group. In the skin-grafted animals, on the other hand, the number of recovered cells was usually lower: a mean of approximately 50% with the lowest being 14% in two cases. Cell recovery again became comparable to that of the control group 12 weeks after transplantation. Cell survival ratio was calculated by the following formula:

$$\text{Cell survival ratio} = \frac{\text{Total viable lymphocyte number obtained from chamber}}{\text{Total viable lymphocyte number enclosed}} \times 100.$$

The animals were divided into seven groups as shown in Table I. Chambers inserted in groups I–V contained lymphocytes from the skin donor. The control group received no skin graft prior to chamber implantation; group I received chamber implantation 1 week after skin grafting; group II, with grafts that had been rejected, received chamber implantation 3 weeks after grafting; those bearing viable grafts then made up group III which also received chamber implantation 3 weeks after grafting; group IV had chambers implanted 6 weeks after receiving skin grafting; group V animals were implanted with chambers 12 weeks after receiving skin grafts; group VI received chamber implantation 3 weeks after grafting; lymphocytes enclosed in these chambers, however, had been collected from a rat which was not the skin-graft donor. The condition of the graft of each animal at the time of chamber implantation was recorded using the following criteria: —, graft with healthy normal appearance; +, graft with partly brownish change; + entire surface of the graft is lightly brown; ++ entire graft is dark brown and dry; +++ graft of desiccated scab-like disc or completely severed.

Results. Skin grafts exchanged between two individuals in this closed-colony Wistar female rats were, in most instances, rejected within 2 weeks. Some survived longer than 3 weeks, but none survived longer than 6 weeks.

Control group. Lymphocytes obtained from a rat were enclosed in diffusion chambers and implanted into nongrafted rats. The cell survival ratios on Day 1, 3, and 5 of implantation are shown in Table II. The mean values indicate that 68.4% of enclosed lympho-

[1] Millipore Filter Corporation, Bedford, Massachusetts.

TABLE I. Experimental Design.

Exptl. group	No. of animals	Skin grafting	Condition of grafts	Time of chamber implantation (weeks after grafting)	Lymphocytes from:
Control	30	—			Rat
I	34	+	—~+++	1	Skin donor
II	25	+	++~+++	3	Skin donor
III	14	+	—~+	3	Skin donor
IV	21	+	+++	6	Skin donor
V	6	+	+++	12	Skin donor
VI	24	+	++~+++	3	Unrelated rat

cytes were still alive after 1 day of *in vivo* incubation, 51.6% on Day 3 , and 40.9% on Day 5 of implantation. The cell recovery in this group was quite high; a mean of 87% of the enclosed cells were recovered on Day 1, 74% on Day 3, and 70% on Day 5 of implantation. Morphologically, there were slight changes in the cell size, but most of these lymphocytes retained their original appearance.

Experimental group I. Chambers containing graft donor's lymphocytes were implanted into the recipient's peritoneal cavities 1 week after skin grafting. Condition of the graft at the time of implantation, and the cell survival ratios are shown in Table II. In the

TABLE II. Cell-Survival Ratio in Control and Experimental Groups.

Group	Enclosed cell number (mean) × 10^3		Cell survival ratio (%)[a] Day: 1	3	5
Control	614	*n*	10	10	10
		mean ± SD	68.5 ± 5.7	51.6 ± 5.9	40.9 ± 10.7
Expt. I	655	*n*	—~± 10	—~+ 8	—~± 7
			+~+++ 3	+~+++ 2	+~+++ 4
		mean ± SD	—~+ 52.6 ±17.5 (s, 0.02)[a]	—~+ 35.2 ±15.1 (s, 0.02)	—~+ 39.5 ±5.0 (ns)
			+~+++ 24.9 ±5.7 (s, 0.001)	+~+++ 22.4 ±12.8 (s, 0.001)	+~+++ 17.0 ±14.2 (s, 0.001)
Expt. II	687	*n*	9	8	8
		mean ± SD	24.6 ± 15.4 (s, 0.001)	21.7 ± 12.1 (s, 0.001)	13.8 ± 7.4 (s, 0.001)
Expt. III	652	*n*	5	5	4
		mean ± SD	54.1 ± 8.1 (s, 0.001)	51.1 ± 15.5 (ns)	34.8 ± 2.2 (ns)
Expt. IV	669	*n*	7	7	7
		mean ± SD	21.8 ± 10.4 (s, 0.001)	14.4 ± 7.9 (s, 0.001)	14.3 ± 8.4 (s, 0.001)
Expt. V	762	*n*	2	2	2
		mean ± SD	76.3 ± 1.7 (ns)	46.3 ± 5.9 (ns)	29.1 ± 4.6 (ns)
Expt. VI	699	*n*	8	8	8
		mean ± SD	21.9 ± 11.4 (s, 0.001)	20.7 ± 8.1 (s, 0.001)	13.5 ± 5.2 (s, 0.001)

[a] Statistical comparison of each value with control group on same day; s = significant, ns = not significant.

animals with viable graft (—~+), the mean cell survival ratios were: 52.6% on Day 1, 35.2% on Day 3, and 39.5% on Day 5, whereas that of animals which had rejected the grafts (+~+++) were: 24.9% on Day 1, 22.4% on Day 3, and 17.0% on Day 5 postimplantation.

Experimental group II. Rats in this group were implanted with chambers 3 weeks after transplantation, and at that time their skin grafts showed progressed change classified as ++~+++. Mean cell-survival ratios were, as seen in Table II, 24.6% on Day 1, 21.7% on Day 3, and 13.8% on Day 5 of implantation. The size of lymphocytes recovered from the chamber varied to a great extent, and many deformed cells and ghost-like outlines were observed. These cells stained instantly when mixed with erythrosin B. The cell recovery in this group was generally low; a mean of 51% on Day 1, 56% on Day 3, and 55% on Day 5 of implantation. In two cases, the total lymphocyte count was as low as 14% of the originally enclosed cells even on the first day of implantation.

Experimental group III. Rats bearing viable grafts (—~+) at the time of chamber implantation 3 weeks after transplantation are classified into this group. Results are shown in Table II. Mean cell-survival ratio on Day 1 was 54.1%, 51.1% on Day 3, and 31.2% on Day 5 of chamber implantation. Severe morphologic changes as described in the group II animals were not observed in this group.

Experimental group IV. The recipient rats were kept until the sixth week of transplantation at which time the chambers were implanted. All grafts on these animals were completely rejected (+++) at the time of chamber implantation. As seen in Table II, the mean cell survival ratios were; 21.8% on Day 1, 14.4% on Day 3, and 14.3% on Day 5. Morphologic changes seen in these lymphocytes were similar to those described in group II.

Experimental group V. Chamber implantation was performed 12 weeks after skin grafting. All grafts had been sloughed off at this time. Table II shows the mean cell-survival ratios obtained in this group. On Day 1 of implantation, the mean value of survival ratio was 76.3%, that of Day 3 was 46.3%, and Day 5 was that of 29.1%. Morphologically, these lymphocytes resembled those of the control group. The cell recovery in this group was comparable to those of the control group.

Experimental group VI. In this last experimental group, lymphocytes were obtained from a rat other than the skin-graft donor. Chambers containing these cells were implanted 3 weeks after skin grafting. Cell survival ratios are also shown in Table II; 21.9% on Day 1, 20.7% on Day 3, and 13.5% on Day 5 postimplantation. Morphological changes and the cell recovery in this group were similar to those in experimental group II.

Discussion. Results indicate that more than 50% of lymphocytes enclosed in a diffusion chamber which is implanted in the peritoneal cavity of a nongrafted rat survives for 3 days, and 40% were still alive even after 5 days of *in vivo* incubation (Table II). There seems to be no outstanding morphologic change in these lymphocytes except for minor variations in cell size. On the other hand, nearly 80% of lymphocytes which originated from the skin-graft donor were killed and showed severe morphologic change within 24 hr when implanted into the recipient animals (Table II). In two cases, the number of lymphocytes recovered from a chamber after 1 day of implantation was as low as 14% of the original count, seeming to indicate the disappearance of a large number of enclosed lymphocytes. Since experimental techniques and conditions were the same in both groups except that the control received no skin graft while the other was grafted, such extreme difference in the mean cell-survival ratio (0.001) must result from the transplantation itself. When the animals which had not rejected their skin grafts were used (group III), cell-survival ratios were so much higher that the values resemble those of the control group. The same tendency was noticed in the animals which were implanted with chambers 1 week after transplantation. Except for a few cases, the animals bearing

viable grafts showed higher cell-survival ratios, whereas animals in which the rejection of the graft was advanced or complete, had lower survivals (group I). These results suggest that humoral cytotoxic substances are produced by the stimulation of allografted skin, and that the effect of the substances are not obvious until the graft rejection is completed. There seem to be two ways to explain the latter; first, lymphocytes in a chamber may not be affected when the graft is not completely rejected because of possible immediate fixation of such substances to the grafted tissue, and second, animals which reject their skin graft at a much slower speed may be producing lesser amounts of such substances. It may be noted that Algire's experiments utilizing tumor cells needed repeated immunization of the recipient to evidence humoral cytotoxic substances; in our experiment, a single skin graft was sufficient to demonstrate such substances. Cell-survival ratios determined 6 weeks after grafting (group IV) were still comparable to that of the 3-week group (group II). The results obtained 12 weeks after grafting, however, showed much higher survivals, more nearly comparable to that of the control group, suggesting the virtual disappearance of cytotoxic humoral substances at this time. Lymphocytes collected from unrelated rats (group VI) were also rapidly killed in the animals which had rejected their skin grafts within 3 weeks. Morphologic changes in these cells were as severe as those observed in group II. This is an indication of cross-reactivity within the animals presently used.

Summary. Cytotoxic humoral substances, which are most likely produced by the stimulation of first set skin allograft, were demonstrated by utilizing Millipore diffusion-chamber technique. The cytotoxic effect of the substance was much stronger in the animals which had rejected their grafts, whereas it was least in the animals bearing viable grafts. The presence of such cytotoxic humoral substances was observed as early as 8 days after grafting, and lasted for at least 6 weeks. The results obtained at the twelfth week of grafting suggested the virtual disappearance of the cycotoxic substances. Lymphocytes originating from a rat other than the skin graft donor were also killed rapidly when implanted into recipient rats which had rejected grafts, indicating cross-reactivity within the animals used in this study.

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