Further Studies on Serum Protein Formation by Chimeras V. Effect of Heterologous Antilymphocyte Serum on Formation of Donor Type Serum Proteins *in Vitro*[1] (34853)

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In mice protected from radiation death by allogeneic or xenogeneic bone marrow grafts, there is invariably a high incidence of graft-versus-host reactivity. This reaction has been controlled to some extent in monkeys and mice (1, 2) by the administration of cyclophosphamide (CY) or amethopterin within a few days after grafting and also by the use of CY rather than X-irradiation to induce allogeneic chimerism in mice (3–5). More recently, the relative efficacy of various forms of antilymphocyte serum (ALS) treatment in preventing acute and delayed secondary disease in allogeneic irradiated recipients has been reported (6–9). It was found that treatment of donor mice with rabbit antimouse lymphocyte serum (RAMLS) prevented the acute graft-versus-host reaction elicited by allogeneic donor spleen cells. Also, the incubation of donor allogeneic spleen or bone marrow cells with RAMLS before grafting or the treatment of the hosts with the same suppressed acute secondary disease in allogeneic recipients. However, the latter treatments were found to be less effective than treatment of the donors. The present study was undertaken to investigate the effect of exposing donor bone marrow cells obtained from rats to rabbit anti-rat lymphocyte serum (RARLS) before grafting and also the effect of treating rat donors with RARLS prior to the induction of chimerism on the capacity to reduce graft-versus-host reactivity of the grafted cells in xenogeneic hosts, using the synthesis of donor type serum proteins *in vitro* as a marker.

[1] This investigation was supported by Grant 5 RO1 CA-07863 from the U.S. Public Health Service.

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Materials and Methods. Preparation and absorption of antisera. Adult male New Zealand rabbits weighing 2 kg, were given four inoculations of 6–24 $\times$ 10^8 thymocytes or lymphocytes, obtained from cervical and axillary lymph nodes, in a volume of 2 ml. Male Sprague-Dawley rats, weighing 250 g, were the donors of the cells. The injections, given at weekly intervals, were divided between the iv and ip routes. The rabbits were bled 1 week after the last injection. Assays of the antisera for cytotoxic activity were performed according to a previously described technique (10). The cytotoxic effect, expressed as percentage of lymphocytes or thymocytes lysed at a dilution of 1:160, were found to be 80 and 90%, respectively.

The "activity" of the antisera *in vivo* was measured by the ability of 2 sc injections of 0.5 ml of either ALS or antithymocyte serum (ATS) on Day 2 and Day 5 after grafting to prolong the life of A strain skin grafts on C57Bl/6 normal mice. Full-thickness skin grafts were performed by the method of Billingham and Medawar (11). The average survival time of the graft in this strain combination is 8–10 days. There was no difference in the survival time of grafts from ALS- or ATS-treated animals as compared with grafted animals that did not receive ALS or ATS.

After centrifugation, the antisera were twice absorbed with an equal volume of fresh suspension of packed rat red blood cells. They were then heated to 56° for 30 min, sterilized by Seitz filtration, and stored at −20° until ready for use.

Normal rabbit serum was obtained from rabbits prior to the immunization procedures and from several other normal rabbits. Sera

without any cytotoxic effect were pooled and treated in a manner similar to the RARLS.

For *"in vitro"* chimeras: One ml each of undiluted RARLS or rabbit anti-rat thymus serum (RARTS), 1 ml of sterile fresh rat complement, and the suspension of rat bone marrow cells, collected from tibias and femurs, in Medium 199 (Microbiological Associates, Bethesda, Md.) were incubated at 37° for 1 hr, centrifuged, and resuspended to the desired concentration in Medium 199. Control suspensions of rat bone marrow cells, fresh rat complement, and normal rabbit serum (NRS) were similarly treated.

For donor-treated chimeras: six Sprague-Dawley rats were given 4 ip injections of 0.5 ml RARLS on consecutive days. Bone marrow suspensions from these animals were used to induce chimerism 24 hr later. Control donors received 0.5 ml of NRS at similar intervals.

After either of the above treatments, rat-into-mouse chimeras were produced by the iv injection of $1–6 \times 10^8$ Sprague-Dawley rat bone barrow cells into C57Bl/6 mice with 4 hr after 850 R whole-body X-irradiation. The conditions of the X-irradiation and the preparation of the bone marrow suspension have been previously described (12).

There were 30 animals in each experimental group and in the control groups. All of the animals received neomycin in their water and aureomycin in their food (approximately 6 mg/kg/day, Aurofac, General Biochemicals, Chagrin Falls, Ohio).

Blood samples were obtained from the retroorbital plexus at biweekly intervals after bone marrow transplantation, and the sera were analyzed by immunoelectrophoresis. The presence of transferrin (Tr) in the sera was demonstrated by incubation of the serum for 1 hr at 37° with 2 μCi of $^{59}FeCl_3$ per ml. Autoradiographs of the immunoelectrophoretic patterns of $^{59}FeCl_3$-incubated sera were prepared with Kodak Royal Pan sheet film with an exposure time of 1 week.

The immunoelectrophoretic patterns were prepared with rabbit anti-rat serum specifically absorbed with mouse tissue powders and serum. Rabbit anti-mouse serum, specifically absorbed with rat tissue powders and serum, was also used. The production and absorption of the antisera were described in a previous publication (13).

Chimeric animals were sacrificed at biweekly intervals and their spleens were removed aseptically. Seventy-five to 100 mg of spleen tissue from individual animals and 40–50 mg of mesenteric lymphoid tissue from pooled animals were cultured for 24–48 hr at 37° in roller tubes with 1–2 ml of the appropriate media to which uniformly labeled ^{14}C-lysine and isoleucine had previously been added to a concentration of 1 μCi/ml each.

The culture media, after concentration 10- to 20-fold, were then subjected to immunoelectrophoretic analyses. A nonlabeled rat carrier was added to the antigen well prior to the addition of the culture fluids to provide enough rat proteins to make immunoelectrophoretic patterns. These patterns were developed with absorbed anti-rat sera. Labeled mouse serum proteins were similarly identified by the use of a mouse serum carrier and absorbed anti-mouse serum. Autoradiographs of the dried slides were made and compared with the corresponding immunoelectrophoretic patterns which were stained with amido black.

Results. There were no survivors in either the donor treated or in *"in vitro"* group beyond 4 weeks, with most of the animals dying 10–14 days after bone marrow transplantation. Nor were there any significant differences in the survival rates in either of the two groups of treated aimals or in the treated animals as compared with the controls.

Sera. Donor type serum proteins could be identified in many of the sera obtained from chimeric animals in the *"in vitro"* group during the period of observation. An α line extending into the β region with the typical double-arc appearance of the mouse haptoglobin-rat hemoglobin (HpHb) complex (Fig. 1) was the first to appear. It was observed as early as 6 days after the experimental procedure. Eight sera obtained during the second and third weeks were specifically examined for rat Tr by incubation of the sera with $^{59}FeCl_3$ (Fig. 1). The absorbed anti-rat sera showed a small labeled arc in six of these sera although it was not visible as a

stained arc on the immunoelectrophoretic diagram. Of the six animals that survived 4 weeks, rat IgG_2 was present in four of the sera.

In contrast to the results in the "*in vitro*" group of chimeras, sera from animals in the donor treated group were negative for all rat proteins except Tr which was observed in five of seven sera obtained during the second and third week.

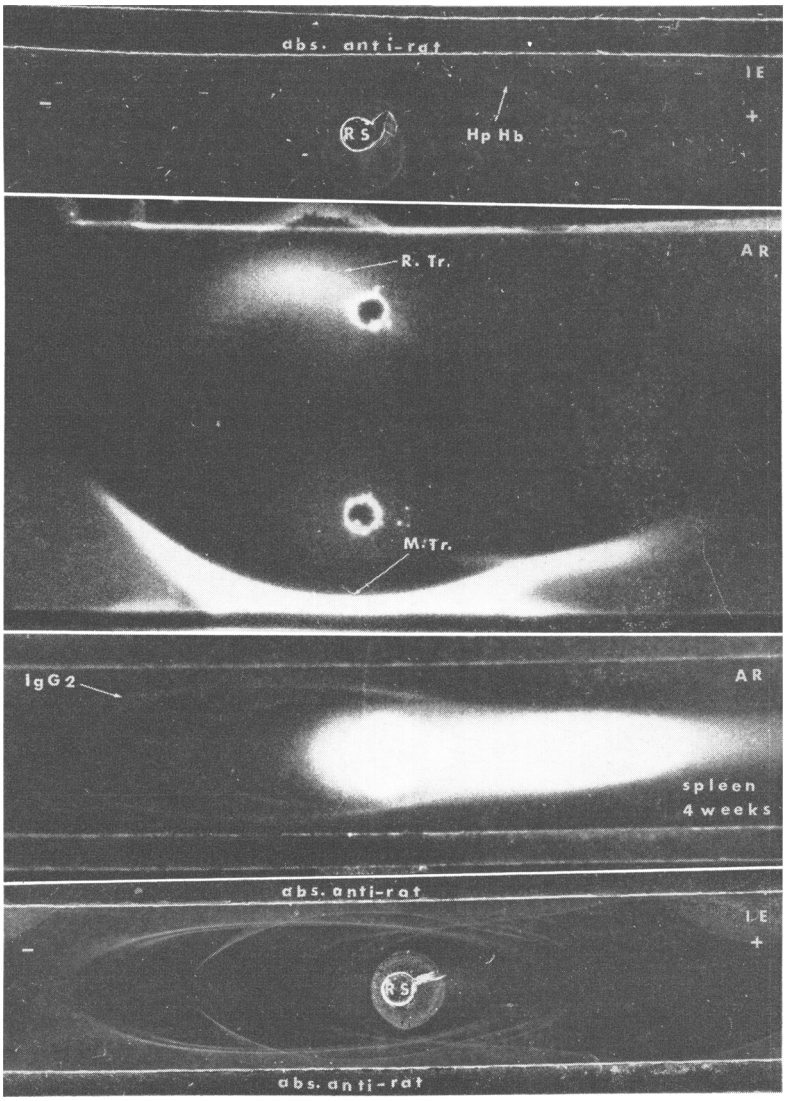

FIG. 1. Immunoelectrophoretic (IE) pattern (top) of serum obtained 6 days after grafting from an *in vitro* chimera. The presence of an α line extending into the β region is characteristic of the HpHb complex. The pattern was developed with absorbed anti-rat serum. The second figure is an autoradiograph (AR) of an IE pattern developed with $^{59}FeCl_3$-incubated serum, obtained 2 weeks after bone marrow transplantation from a chimeric animal in the donor treated group, and adsorbed anti-mouse serum and absorbed anti-rat serum. Rat (R) and mouse (M) transferrin (Tr) are present. The bottom figures show an AR with its IE pattern developed with absorbed anti-rat serum and spleen culture fluid from a chimeric animal in the donor treated group 4 weeks after grafting. Rat serum (RS) was used as a carrier.

TABLE I. Characteristics of Xenogeneic Rat-into-Mouse Chimeras After Various Methods of Induction.

Method of induction	Additional treatment	Longest survival time (weeks)	Donor type serum proteins		Reference number
			In sera of hosts	Labeled *in vitro* by various lymphoid tissues of hosts	
Lethal X-irradiation	Treatment of donors with 0.5 ml RARLS, ip × 4 days	4	Tr	IgG_1, IgG_2, Tr	
Lethal X-irradiation	*In vitro* incubation of donor cells with RARLS or RARTS	4	HpHb, Tr	IgG_2, Tr	
Lethal X-irradiation	—	14	IgG_1, IgG_2, IgM, Tr, HpHb	IgG_1, IgG_2, IgM, B_{1c}, Tr	12
Sublethal X-irradiation	RAMLS treatment of hosts (0.25 mg) twice weekly for 7 weeks	7	Not examined	IgG_2, Tr	13
CY (300 mg/kg ip)	CY (75 mg/kg) ip on days 5, 8, 11, 14 after bone marrow graft	9	HpHb	None	14
Lethal X-irradiation	CY (75 mg/kg) ip on days 5, 8, 11, 14 after bone marrow graft	3	HpHb, Tr	Tr	14
3MCA (5 mg)	—	10	None	IgG_2, Tr	15
3MCA (5 mg)	Sublethal X-irradiation	4	None	IgG_2, Tr	15
Lethal X-irradiation	Thymectomy of hosts, 6–8 weeks of age	4	Tr	HpHb, Tr	16
CY	Thymectomy of hosts, 6–8 weeks of age	7	None	IgG_2, Tr	16
CY (300 mg/kg ip)	—	11	None	IgG_2	16
Lethal X-irradiation	Neonatal thymectomy of donors	16	Tr	IgG_2, HpHb, Tr	16

Culture fluids. There were no differences in the synthesis of serum proteins by the two groups of chimeras. Nor were there any differences in the "*in vitro*" groups between those mice receiving bone marrow incubated with RARLS or with RARTS. Many of the spleen and mesenteric lymph node culture fluids from both groups showed labeling of IgG_2 and Tr. Labeling of Tr appeared during the second week. Three of six chimeras in the "*in vitro*" group and four of seven in the donor treated group that survived 4 weeks showed labeling of IgG_2 (Fig. 1). There were no differences in the intensity of labeling of IgG_2 in the two groups. In addition, several culture fluids obtained from spleens from donor treated chimeras showed labeling of IgG_1. Table I summarizes the findings in the present study as well as the characteristic features of chimeric animals in previous studies from this laboratory.

Discussion. The beneficial effect of RAMLS on secondary disease in allogeneic recipients has been well documented (6–9). The rationale behind pretreatment of donor animals on the development of secondary disease in the recipient was based on the assumption that a lowering of the immunological ractivity of the lymphoid cells in donor material could be expected to ameliorate the graft-versus-host reaction (6). The *in vitro* exposure of donor cells to ALS in the production of allogeneic chimerism stems from the idea that an immunosuppressive agent must selectively inactivate immunonocompetent cells in order to be useful and effective. This

is particularly true when bone marrow cells, which contain mixtures of lymphoid and hematopoietic cells, are transplanted. In such instances, the ability to selectively inactivate the lymphoid cells in order to suppress the acute secondary disease without eliminating hematopoietic precursors which would induce bone marrow aplasia, is of importance (9).

In the present study, the fact that the animals in the *in vitro* and donor treated groups were chimeras was demonstrated by the *in vitro* synthesis of donor-type serum proteins by the lymphoid tissues of the host. However, in contrast to the findings of others with allogeneic chimeras (6–9), the present experiments failed to reveal any beneficial effect of donor treatment or *in vitro* incubation of donor cells on establishment of xenogeneic chimeras. However, treatment of the hosts with RAMLS (14) did produce better survival rates than either of these two treatments. Previous studies in this laboratory on the induction of xenogeneic chimeras by various methods (13–17) have shown that the best survival rates were obtained in those chimeras induced by lethal X-irradiation with or without neonatal thymectomy of the donors (13, 17). Acute and delayed secondary disease were also less severe in those chimeras induced with CY (15) than 3MCA (16).

The work of van Bekkum *et al.* (6) clearly demonstrated that the results with different types of exposure to ALS were dependent on the species of animals. In contrast to the results obtained in mice, donor treatment of monkeys or *in vitro* exposure of bone marrow obtained from monkeys were ineffective in suppressing secondary disease while the treatment of the recipients of allogeneic bone marrow with ALS was equally as effective as CY in its effect on secondary disease.

It is known that xenogeneic chimeras, under any circumstances, are more difficult to establish than allogeneic chimeras and that there are variabilities in the action of ALS obtained from different species. In this study, the failure of donor treatment and *in vitro* exposure of donor cells to ALS or ATS in suppressing secondary disease was most probably due to their lack of "activity" *in vivo*. Although the cytotoxic titers of the antisera used were high, it has been shown that ALS titers for lymphocyte toxicity, lymphoagglutination, and lymphocyte transofrmation do not correspond with the degree of immunosuppressive activity (18, 19).

Summary. Post X-irradiation rat-into-mouse chimeras were produced after treatment of the donors with rabbit antirat lymphocyte serum (RARLS) or the *in vitro* incubation of rat bone marrow cells with RARLS. The survival rate in both groups of animals was poor, with none of the animals surviving beyond 4 weeks. The *in vitro* synthesis of donor-type serum proteins by the lymphoid tissues of the host was demonstrated by the technique of radioimmunoelectrophoresis. There were no significant differences in the *in vitro* synthesis of serum proteins of donor type in the two groups of animals.

There was no correlation between the *in vitro* cytotoxic effect of the antilymphocyte sera and its *in vivo* activity as measured by allogeneic graft survival.

The excellent technical assistance of Mrs. Yvonne Simmons is gratefully acknowledged.

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Received Nov. 21, 1969. P.S.E.B.M., 1970, Vol. 134.