ROLE OF THE THYMUS IN TOLERANCE

I. Tolerance to Bovine Gamma Globulin in Thymectomized, Irradiated Rats Grafted with Thymus from Tolerant Donors*

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The starting point for the present study was provided by Gowans' observation that tolerance is a property of the recirculating pool of small lymphocytes (2, 3). These cells circulate continuously, over long periods of time (4, 5), between the blood and the parenchyma of peripheral lymphoid organs (splenic white pulp, lymph node cortex) (6), and appear to be directly responsible for antibody formation (7), homograft rejection (2), and possibly other types of "delayed" hypersensitivity (8). It is probable that they are produced in the thymus and other central organs, such as the bone marrow, the avian bursa of Fabricius, and possibly the gastrointestinal lymphoid tissue (9–12). Our working hypothesis was that tolerance may result from the interaction of antigen with small lymphocytes or their precursors within these source organs.

In order to evaluate this possibility, thymectomized, irradiated rats were grafted with thymus and marrow, one or both being obtained from donors tolerant to bovine γ-globulin. It was hoped that the recipients, when challenged later, would show tolerance for one or more of the standard immune responses. The data obtained imply that different source organs are concerned in delayed sensitization and certain types of antibody formation and that tolerance, for a least two of these responses, is a property associated with the thymus.

Materials and Methods

The antigen used throughout the present study was bovine γ-globulin (BGG), obtained from Armour Pharmaceutical Company, Kankakee, Illinois. All manipulations of cells or grafts were carried out in Hank's balanced salt solution at 4°C.

Thymectomy.—Inbred Lewis rats were obtained from Microbiological Associates, Bethesda,

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Maryland. They were thymectomized at 5 weeks of age by essentially the same technique employed for neonatal thymectomy (13), ether being used as the anesthetic.

Irradiation.—At 8 weeks, all rats received 800 roentgen whole body x-irradiation from a 250 kv machine with 2 mm aluminum filtration. The beam had a half value layer of 0.8 mm copper. The cone was centered directly over the 3 section turntable assembly containing the animals, with an average target distance of 55 cm. The dose was monitored with a Victoreen 1000 r chamber having a Victoreen 2269 electrode, embedded in "rice" in the rotating container. The corrected dose in a typical run was 134.6 r per minute.

Grafting.—Within 24 hours, each animal received 1 to 2 $\times$ 10^8 nucleated marrow cells intravenously and a single thymus, cut into 4 or 5 large fragments and placed subcutaneously in the left axilla. These were taken from 10-week-old donors of the same sex, either normal or tolerant to BGG following intraperitoneal injection of 20 mg at birth and 50 mg at 4 weeks of age. The marrow cells were prepared as a pool of femoral and humeral marrow from several donors; the dose administered represented the number obtained from a single donor. In certain recipients, BGG was injected intravenously at the time of grafting with normal (non-tolerant) thymus and marrow.

Challenge.—Each rat was challenged, 3 or 6 weeks after irradiation and grafting, by injection of BGG 500 μg in 0.1 ml of adjuvant mixture (10 parts saline containing protein, 1.5 parts arlacel A, 8.5 parts mineral oil, and heat-killed tubercle bacilli at a final concentration of 3 mg per ml) in one hind foot-pad, and boosted by an intravenous injection of 1 mg BGG 25 days later. All rats were bled 10, 20, and 32 days after challenge and skin tested with 30 μg of BGG at 10 and 20 days. Arthus and delayed skin reactions were read at 3 to 4 and 24 hours respectively (13), and the sera stored for later serologic study. Certain animals were challenged and tested with chicken ovalbumin (Ea) (Armour) in parallel with the BGG animals.

Study of Blood and Tissues.—White cell and differential counts were performed at fixed intervals after grafting, to evaluate recovery from the irradiation and grafting procedure. All rats were sacrificed at 32 days. The mediastinum was examined grossly and histologically, and animals with more than 20 mg of residual thymus discarded. The graft, the spleen, and draining and non-draining lymph nodes were studied histologically in hematoxylin-eosin stained sections.

Serologic Procedures.—Passive hemagglutination of tanned, formalinized sheep red cells and double diffusion in agar gel (Ouchterlony) followed the same protocol utilized in our previous study with bovine serum albumin (13). All sera were tested before and after inactivation for 1 hour with 0.125 M 2-mercaptoethanol.

RESULTS

All tolerant donors were challenged at 8 weeks of age, bled, and skin tested 10 to 14 days later (Table I). Only 2 of 62 showed evidence of skin reactivity to BGG; these were not used as donors. Several also formed mercaptoethanol-sensitive antibody. No attempt was made to ascertain whether this antibody was specific for BGG or for a contaminant of the BGG preparation (see reference 14). Skin reactivity and antibody levels in recipients of thymus grafts from donors which formed antibody were indistinguishable from those in animals grafted with thymus from completely non-reactive donors.

The majority of rats in all experimental groups had fully recovered from irradiation and the operative procedure by the time of the 3 week challenge. The few which showed signs of intercurrent infection at either 3 or 6 weeks, and one

rat found to have residual thymus in the mediastinum, are omitted from consideration here. Animals in which no functioning thymus could be identified *postmortem* at the site of grafting showed persistent lymphopenia in the blood and peripheral lymphatic organs (spleen, lymph nodes) and were deficient in several immune responses (see below).

Skin Reactions.—In normal Lewis rats, delayed reactivity was maximal by 10 days and waning by 20 days after a foot-pad injection of BGG and adjuvant (Fig. 1). Arthus reactivity, on the other hand, was still increasing at 20 days. The delayed skin reactions obtained in thymectomized, irradiated rats, 3 or 6 weeks after grafting, are shown in Table II. Average figures are plotted in Fig. 2. Reactivity was essentially normal, at the earlier time, in animals grafted

TABLE I

*Degree of Tolerance Produced in Rats Used as Donors**

Experiment	No. of rats responding			Average positive titer	
	Delayed reactivity	Arthus reactivity	Antibody formation	Total	ME-resistant
1	0/6	0/6	2/10	5.0	1.0
2	0/12	0/12	0/9	—	—
3	0/12	0/12	7/11	4.1	0
4	0/12	0/12	0/9	—	—
5	2/12‡	0/12	4/10	4.8	1.0
6	0/6	0/6	2/9	1.5	0

* Donors received 20 mg BGG intraperitoneally at birth and 50 mg at 4 weeks. After standard challenge at 8 weeks, they were skin tested at 10 days and bled at 12 to 14 days.

‡ Skin-positive rats were not used as donors.

with normal thymus and marrow. Also in the group receiving normal thymus and tolerant marrow, reactivity appeared normal, though here the experiment was marred by several graft failures. Rats given tolerant thymus, whether with normal or tolerant marrow, failed to develop sensitization to BGG, though responding with normal intensity to a heterologous antigen (hen Ea). At 6 weeks, rats grafted with tolerant thymus showed minimal impairment of reactivity to BGG. In those receiving both tolerant thymus and tolerant marrow the impairment was definite, even at the time of the second skin test. Again a comparable group reacted well to Ea.

Arthus reactivity to BGG failed to develop in any of the rats challenged 3 weeks after grafting (Table III). By 6 weeks reactivity had returned to an equal extent in all groups, though remaining at a subnormal level. The intensity of the Arthus response, in individual animals, showed no correlation with antibody titers, measured by hemagglutination or precipitation. A high level of Arthus

reactivity was obtained in animals challenged with Ea, even 3 weeks after grafting; there was no morphologic evidence that these reactions were qualitatively different from those obtained with BGG.

Control rats, grafted with normal thymus and marrow, received various doses of BGG intravenously at the time of grafting and were challenged in the usual manner. Amounts of BGG up to 100 μg had little effect on either delayed or Arthus sensitization (Table IV). With 500 and 1000 μg, there was some reduc-

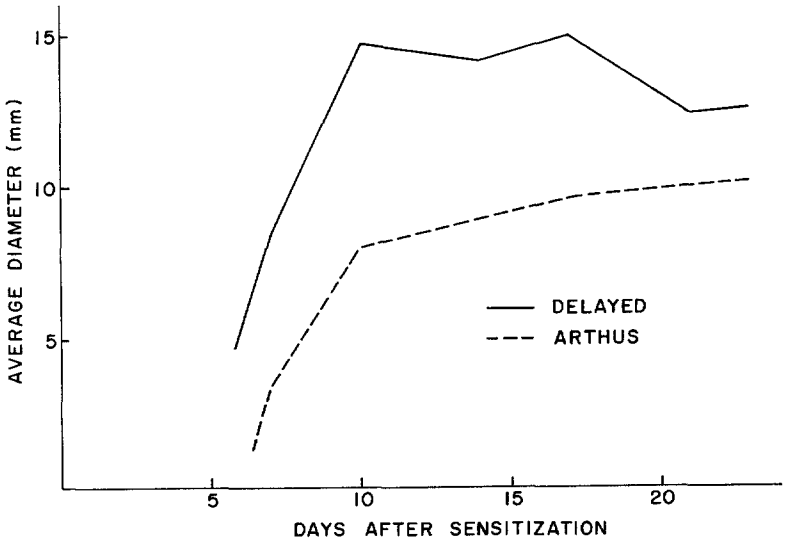

FIG. 1. Course of delayed and Arthus sensitization in groups of 6 normal rats sensitized in the usual manner and skin tested at various intervals with 30 μg BGG. Average figures are given for the diameter (in mm) of skin reactions 24 and 3 to 4 hours after testing. (Reprinted from *Science*, 1965, **148**, 1333, by permission of the Editors.)

tion in the intensity of delayed sensitization 3 weeks after grafting, but little or no effect on Arthus reactivity.

Failure of thymus grafting was associated with failure of delayed sensitization. Of 12 rats which did not show sensitization (in groups other than those grafted with tolerant thymus), only 3 were found *postmortem* to have functional thymus grafts, whereas active grafts were present in 19 of 21 animals which reacted. Conversely, of rats which did not receive a graft or in which the graft failed to survive, most did not develop delayed sensitization or developed it poorly (Table V). Arthus sensitization occurred to the same extent in animals without a graft and in those successfully grafted.

Serologic Data.—In normal rats, following challenge with BGG in adjuvant, hemagglutinating antibody appears at 1 week, rises to a maximum at 20 to 25

TABLE II

Delayed Skin Reactions at Various Times after Grafting with Thymus and Marrow*

Thymus graft	Normal	Normal	BGG	BGG	BGG
Marrow graft	Normal	BGG	Normal	BGG	BGG
Challenge antigen	BGG	BGG	BGG	BGG	Ea
Challenge, 3 weeks after grafting	18+++, 23+++ 18+++, 16++ 17+++, 22+++ 15+++, 19+++ 14++, 22+++ 13++, 18+++ 13+, 18+++ 11++, 19+++ 11+, 11+‡	19+++, 17+++ 15+++, 15+++ 13++, — 12++, 0‡ 9+, 17+++ 0, 17++ 0, 0‡ 0, 0‡	9+, 9± 6±, 0 0, 0 0, 0 0, 0 0, 0 0, 0 0, 0‡	10±, 12+ 0, 0 0, 0 0, 0 0, 0 0, 0 0, 0 0, 0 0, 0‡ 0, 0‡	22+++, 20+++ 18+++, 20+++ 18+++, 19++ 17+++, 19+++ 15++, 15+++ 15+, 24+++ 7±, 19+++ 0, 0‡
Challenge, 6 weeks after grafting	20+++, 15++ 17+++, 19+++ 17+, 0 16++, 15++‡ 15++, 18+++ 14++, 22+++ 10±, 7± 8±, 14+ 0, 15++‡	20+++, 15+++ 15++, 13++ 12+, 11++ 11++, 19+++ 9+, 6± 9+, — 8±, 14+ 0, 11+ 0, 8±	14+++, 13++ 14+, 11+ 9+, 17++ 7±, 15++ 6±, 8+ 6±, 7+ 0, 20+++‡ 0, 0‡	11++, 18+++ 11+, 9± 10+, 13+ 9±, 6± 9±, 0‡ 8±, 6± 7±, 10+ 0, 0‡ 0, 0‡	23+++, 18+++ 22+++, 21+++ 22+++, 20+++ 19+++, 16+++ 16++, 17+++ 12++, 18+++ 0, 14++‡

* First and second figures of each pair represent reactions to skin test 10 and 20 days after challenge respectively. Diameter in mm and degree of induration are given for each reaction.

‡ Thymus graft not found postmortem.

days, then falls gradually (Fig. 3). The first antibody formed is mercaptoethanol-sensitive (MES) but, by 30 days, this has been almost entirely replaced by a mercaptoethanol-resistant (MER) type of antibody. At intermediate times both are present, in proportions which vary in different series of rats. About

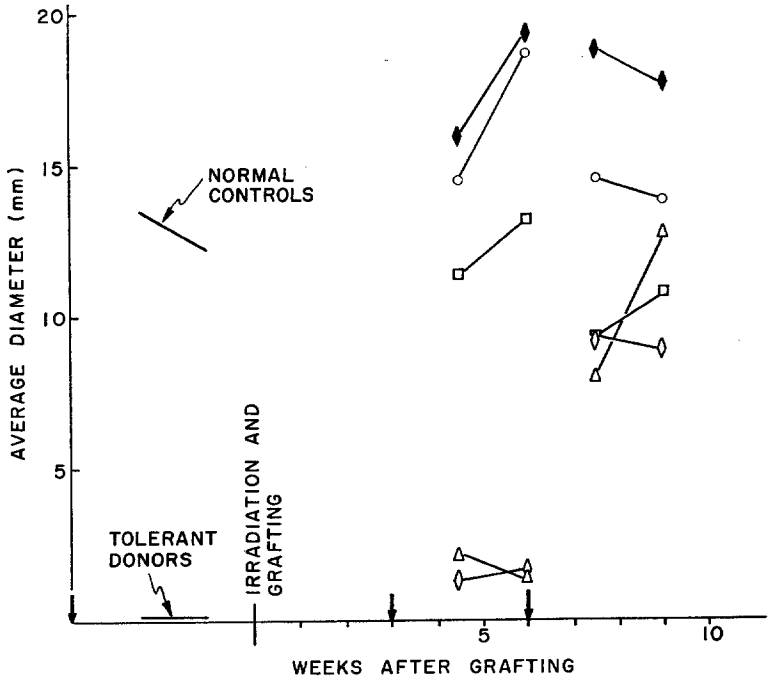

FIG. 2. Average diameter of delayed skin reactions in rats of the different experimental groups. Values obtained 10 and 20 days after challenge in any one group are connected by a line.

Thymus	*Marrow*
Normal	Normal ○——○
Normal	Tolerant □——□
Tolerant	Normal △——△
Tolerant	Tolerant ◇——◇
Tolerant	Tolerant ◆——◆
	(Challenge with Ea)

half the sera are positive by gel diffusion from 14 days on. Following a booster dose of antigen (1 mg intravenously), MES antibody appears again, but now high titers of MER antibody are formed rapidly, reaching a peak at 7 to 10 days. Virtually all sera are positive by gel diffusion at 5 and 10 days. MES and MER hemagglutinating antibodies always showed a reciprocal relation in our experiment, high titers of one being accompanied by low titers of the other. Neither was well correlated with the presence of precipitating antibody.

TABLE III

Arthus Skin Reactions at Various Times after Grafting with Thymus and Marrow

Thymus graft	Normal	Normal	BGG	BGG	BGG
Marrow graft	Normal	BGG	Normal	BGG	BGG
Challenge antigen	BGG	BGG	BGG	BGG	Ea
Challenge, 3 weeks after grafting	8±, 0 6±, 9± 6±, 6± 6±, 0 5±, 8+ 0, 7±‡ 0, 0 0, 0 0, 0	6±, — 5±, 0‡ 0, 9+ 0, 0 0, 0 0, 0 0, 0‡ 0, 0‡	0, 8± 0, 7± 0, 7± 0, 0 0, 0 0, 0 0, 0 0, 0‡	7±, 11± 0, 7± 0, 0 0, 0 0, 0 0, 0 0, 0 0, 0‡ 0, 0‡	0, 21+++ 0, 20+++ 5±, 14++ 0, 13+++ 0, 13+++ 0, 11± 6±, 10+ 0, 6±‡
Challenge, 6 weeks after grafting	11++, 14++ 11+, 11+ 9+, 14++ 9+, 14+‡ 5±, 0 5±, 0 0, 13++ 0, 8±‡ 0, 0	0, 14++ 6±, 14+ 8+, 10± 8±, 7± 0, 7± 0, 6± 0, 0 0, 0 0, —	12++, 13++ 9+, 14++ 7±, 11+ 7±, 7± 6±, 0 0, 7± 0, 0‡ 0, 0‡	6±, 14++ 8+, 7± 6±, 10± 6±, 6± 6±, 6±‡ 6±, 0‡ 0, 8+‡ 0, 8+ 0, 8±	11++, 16+++ 10+, 18+++ 10+, 10++ 8±, 12++ 6±, 10+ 0, 11+++ 0, 0‡

*, ‡, Footnotes as in Table II.

In animals undergoing thymectomy, irradiation, and grafting of normal thymus and marrow, there was partial recovery of both antibody responses at 3 weeks (Fig. 4). MES titers were low 10 days after challenge. MER antibody formation was minimal at 20 days; and even 7 days after a secondary stimulus, MER titers were lower than in controls. The gel diffusion test was negative throughout. At 6 weeks, the responses approached those of normal animals. However, only 32 day sera with high hemagglutination titers were positive by gel diffusion. Rats which received intravenous doses of BGG at the time of grafting showed no sign of either tolerance or immunization to BGG.

TABLE IV

Number of Delayed and Arthus Reactions of Different Intensities in Rats Given Free BGG Intravenously at Time of Grafting

BGG dose at time of grafting	3 weeks challenge						6 weeks chall ge					
	10 days			20 days			10 days			20 days		
Delayed reactions, *mm.*.	0	7–12	>12	0	7–12	>12	0	7–12	>12	0	7–12	>12
μ_g												
500, 1000	4	3	2	0	6	3	1	5	3	0	4	4
1, 10, 100	1	5	5	0	3	8	0	4	7	0	5	6
0	0	2	7	0	1	8	1	2	6	0	1	7
Arthus reactions, *mm.*.	0	6–10	>10	0	6–10	>10	0	6–10	>10	0	6–10	>10
μ_g												
500, 1000	9	0	0	5	4	0	7	1	0	2	3	3
1, 10, 100	10	3	0	11	2	0	9	2	0	2	5	4
0	5	4	0	5	4	0	5	2	2	3	1	5

Rats grafted with thymus from tolerant donors, when challenged at 3 weeks, showed a substantial diminution in formation of MER antibody (Fig. 4). In the majority of animals which received both tolerant thymus and marrow, this response was entirely lacking. Formation of MES antibody was diminished as well; but, even in the group getting both grafts from tolerant donors there were appreciable titers by 32 days. The antibody response to Ea in a comparable group of rats was normal. Six weeks after irradiation and grafting all rats responded more or less normally. However the hemagglutination titers were uniformly higher in animals grafted with tolerant thymus, whether with tolerant or normal marrow; and over half the sera from these animals were Ouchterlony positive at 32 days (Table VI). Animals receiving marrow from tolerant donors reacted, at both 3 and 6 weeks, like those grafted with normal tissues.

Two additional findings should be noted. Where MER antibody was not formed, as in the 3 week group given tolerant thymus, the titer of MES antibody

was unusually high. Secondly, in almost every instance (6 out of 7) of failure to form MER antibody following a secondary stimulus, aside from the 3 week groups receiving tolerant thymus, no functional remnant of thymus could be identified at the graft site. Of 26 rats which formed MER antibody, 21 had active grafts. This relationship is also shown by the marked diminution in for-

TABLE V

*Comparison of Immunologic Reactivity in Normal and Thymectomized, Irradiated Rats**

Experimental group	No. of rats responding	Average response at		
		10 days	20 days	32 days
Delayed sensitivity				
Normal	6/6	13.5	12.2	—
Thymectomy + irradiation				
3 weeks	2/7	3.3	1.6	—
≥6 weeks	8/12	4.4	8.2	—
Arthus response				
Normal	6/6	8.2	11.3	—
Thymectomy + irradiation				
3 weeks	1/7	0.7	1.0	—
≥6 weeks	6/12	2.2	4.1	—
Hemagglutinating antibody				
Normal	12/12	6.9	7.7	9.5
Thymectomy + irradiation				
3 weeks	6/7	0.6	1.9	4.4
≥6 weeks	7/7	3.3	2.6	6.4
ME-resistant antibody				
Normal	12/12	2.2	4.0	8.0
Thymectomy + irradiation				
3 weeks	2/7	0	0	2.0
≥6 weeks	3/7	0.3	0.3	3.3

* Includes rats of experimental groups found postmortem to lack functional thymus grafts, plus 5 animals not grafted with thymus.

mation of MER antibody in rats not grafted or lacking a satisfactory thymus graft (Table V).

Histologic Findings.—Thymus grafts, 32 days after challenge at both 3 and 6 weeks, showed a normal or slightly distorted structure with, however, a full complement of lymphocytes. One graft contained oil vacuoles and epithelioid cells; and a large sector of this specimen resembled normal lymph node, containing germinal centers and medullary cords filled with plasma cells.

The spleen, 32 days after the 3 week challenge, showed moderate to marked

depletion of lymphocytes in the white pulp, both in rats which received tolerant thymus and/or marrow grafts and those which received normal tissue. (The single rat found to have residual normal thymus had a normal spleen, and those in whom no graft was found had few lymphocytes or none at all). Germinal

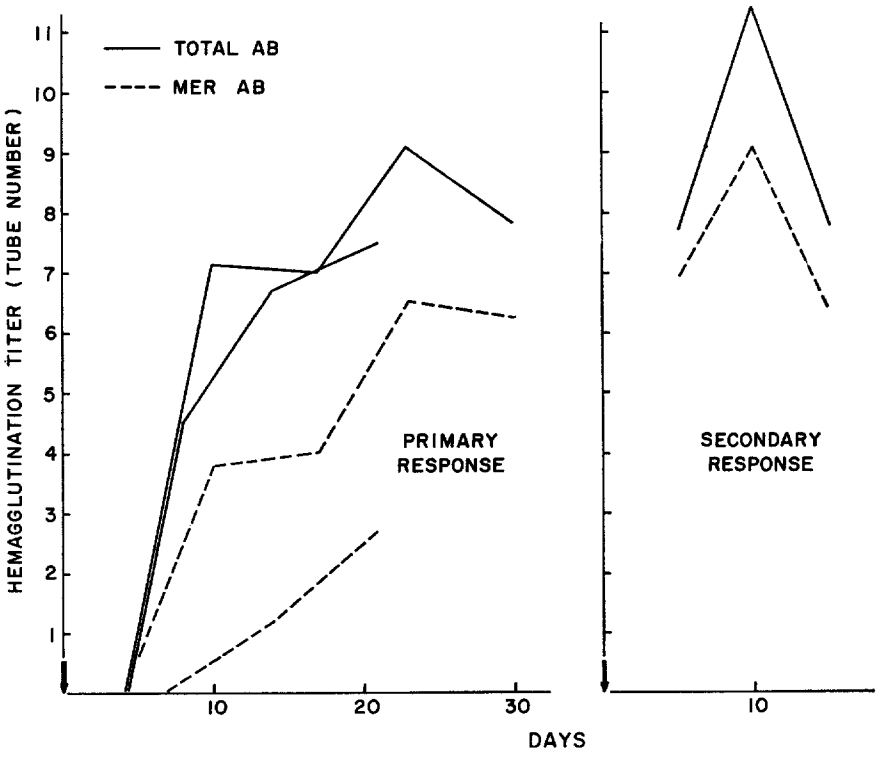

FIG. 3. Hemaglutination titers (log_2) in two groups of normal rats challenged with BGG and adjuvant in the usual manner and bled at various intervals. Secondary response followed intravenous booster dose of 1 mg BGG at 40 days. Average values, obtained before and after treatment of sera with mercaptoethanol, are plotted.

centers were normal or decreased in number; all were small and consisted of small dark cells. The marginal zone of phagocytic cells was uniformly and strikingly increased. Animals challenged at 6 weeks showed the same changes in lesser degree. Lymphocytes, however, were not at the normal level.

In lymph nodes, the diffuse cortex was easily distinguished from the follicles, with their germinal centers, and from the medullary cords containing immature and mature cells of the plasma cell series. In nodes draining the inoculation site, prominent oil vacuoles, masses of epithelioid cells, giant cells, and occasional

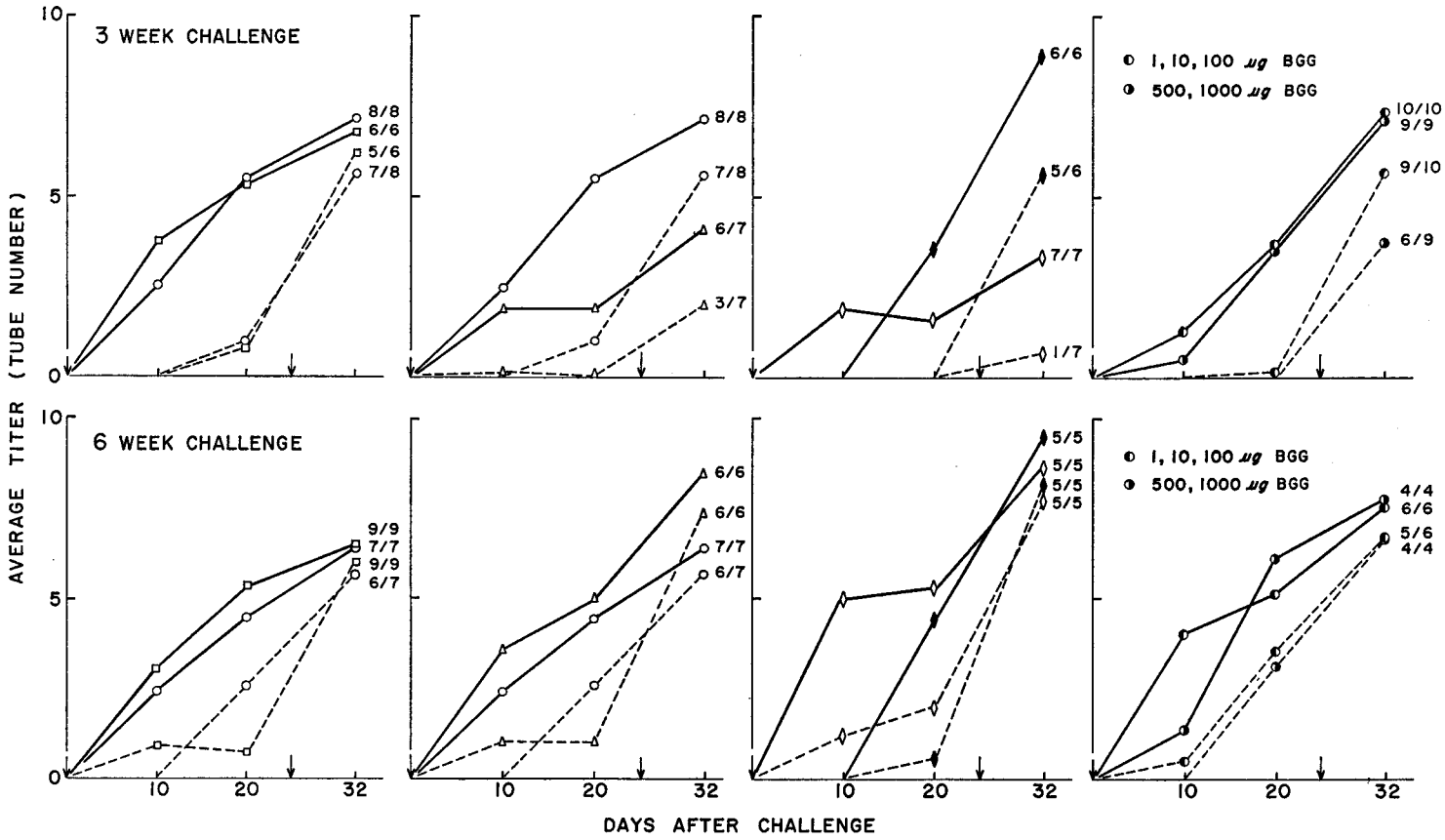

FIG. 4. Average hemagglutination titers (log_2) before (——) and after (------) mercaptoethanol in sera from rats of different experimental groups. Arrows indicate challenge with BGG in adjuvant and intravenous booster dose at 25 days. Only values obtained in healthy rats possessing a functional thymus graft are included in averages. Figures show number of rats developing significant titers over number included in group. Symbols as in Fig. 2.

foci of necrosis were also present. After challenge at 3 weeks, there was a limited complement of small lymphocytes, apparently repopulating the depleted cortex. In some nodes, these were prominent principally about postcapillary venules, in others they were numerous in the most superficial zone of the cortex, while in still others they were distributed diffusely throughout the cortex. In draining nodes, they were densest about zones of epithelioid cells and, in more than half, extended into the medullary sinuses as tongues of rather uniform, small cells. In non-draining nodes, they tended to be less numerous and did not enter the sinuses. In many nodes the cortex was filled with a disorganized mass of epithelioid cells, lymphocytes, medullary elements, and sharply circumscribed aggregates of lymphocytes (in sinuses ?). Plasma cells were frequently increased.

TABLE VI

Relationship between Hemagglutination Titer and Precipitin in Rats of Different Experimental Groups

Type of graft		Antibody response*			
		3 week challenge		6 week challenge	
Thymus	Marrow	Hemagglutinin	Precipitin	Hemagglutinin	Precipitin
Normal	Normal	8/8 (7.3)	1/7	7/7 (6.4)	0/6
Normal	BGG	6/6 (6.7)	0/5	9/9 (6.4)	0/9
BGG	Normal	6/7 (4.1)	0/7	6/6 (8.4)	3/5
BGG	BGG	7/7 (3.2)	0/8	5/5 (8.7)	4/6

* Number of sera containing antibody over total number tested. Average hemagglutinin titer in parentheses. Only values obtained at 32 days, 7 days after secondary stimulus, are shown.

After challenge at 6 weeks, almost all lymph nodes showed an essentially normal complement of lymphocytes; in a few, some architectural disorganization persisted. No difference was noted between lymph nodes of rats in the different treatment groups. In animals in whom satisfactory grafts were not identified, there was little lymphocyte repopulation of the nodes. There was a definite difference at both 3 and 6 weeks between the lymph nodes and spleen, the latter showing far less lymphocyte repopulation, in accord with Harris and Ford's observation (15) that thymus cells populate lymph nodes but not spleen.

Blood lymphocyte counts rose from 3 to 6 thousand per mm^3 10 days after grafting to 5 to 12 thousand 6 weeks after. There was no difference between treatment groups. The counts in individual animals were poorly correlated with the status of the thymus grafts.

An unexpected finding was the presence of thyroiditis of moderate or in some instances severe degree in almost half the animals examined (18 out of 43). This

change was equally frequent and severe 3 weeks and 6 weeks after challenge and in animals possessing and lacking effective thymus grafts. It may have been induced as a consequence of the cervical and mediastinal trauma accompanying thymectomy.

DISCUSSION

The present experiments demonstrate that rats deprived of immunologically competent cells by the combination of thymectomy and irradiation (16, 17) are promptly restored to normal reactivity by implantation of normal adult thymus and infusion of normal marrow cells. Yet implantation of a tolerant thymus was found to result in specific tolerance for two different types of immune response lasting several weeks. The thymus does not itself contain immunologically competent cells (18–20, 8); yet it seeds the peripheral lymphoid tissues, lymph nodes in particular, with lymphocytes (21–23, 15). It follows that tolerant cells are actually produced in the thymus or, if produced in another source organ, pass through the thymus and perhaps mature there. Control experiments appear to rule out the possibility that free BGG transferred at the time of grafting with tolerant thymus could be responsible for tolerance in the recipient. Of the 70 mg of protein injected in the donor rats probably less than 200 μg penetrates the thymus.[1] If this entire amount remained in the animal, it would be insuficient to induce tolerance when transferred to the recipient at the time of grafting. In the adult rat, the half-life of BGG is approximately 3 days (24, 25). The amount remaining at 10 weeks in the animal as a whole would be of the order of 5 μg. Antigen in the thymus may not diminish at a comparable rate but is, in any event, too little to account for the result observed.

In intact animals, the waning of tolerance to protein antigens is frequently followed by a period of increased reactivity to antigen resembling that which follows primary immunization (26, 27). Recipients of tolerant thymus, in the present experiments, while they showed a specific loss of immune reactivity at 3 weeks, formed antibody (hemagglutinin and precipitin) in higher titer after challenge at 6 weeks than animals grafted with normal tissues, *i.e.* they behaved like animals exposed previously to antigen.

These observations suggest the hypothesis that antigen must penetrate the thymus and persist there to induce tolerance in cells which, after maturation, enter the competent small lymphocyte pool (Fig. 5). A blood-thymus barrier interferes with penetration into the thymus of antigens present in the circulation (28–31). Proteins such as ferritin and human serum albumin enter the thymus in low concentration, while aggregated materials such as colloidal iron and heavily iodinated bovine serum albumin fail to do so (29). On the other

[1] After injection of I^{125}-labelled BGG intraperitoneally into newborn and 4-week-old rats, 0.1 to 0.3 per cent of the injected dose was found in the thymus 24 hours later (unpublished data of Dr. I. Gery and Dr. M. Mueller).

hand, aggregates penetrate the thymus relatively freely in the neonatal period (32). No study has been carried out of the persistence of soluble or aggregated antigens in the thymus. However mice with neonatally induced tolerance to homografts may have large numbers of donor cells (up to 86 per cent) in the thymus over a period of many weeks, and this thymic chimerism shows some correlation with persistence of the tolerance state (33, Trenton and Session in

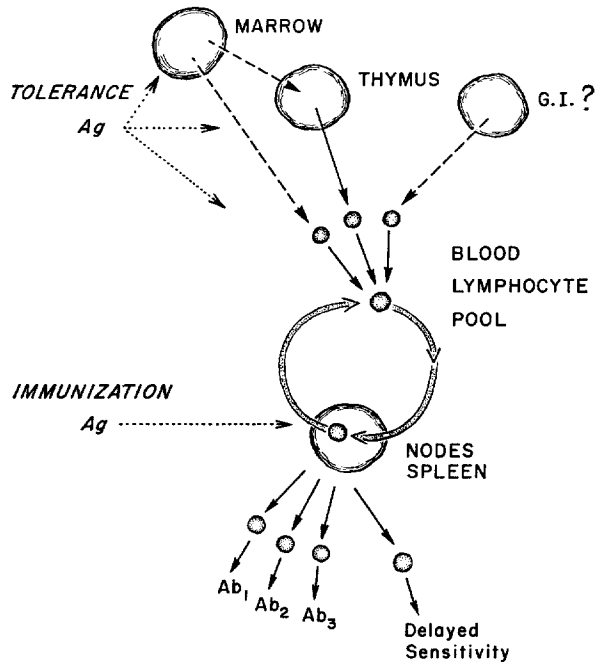

FIG. 5. Hypothetical scheme relating the site of action of antigen (Ag) to the induction of tolerance or of immunization, *i.e.* formation of antibody (Ab) or delayed sensitization.

reference 34). Several authors have shown that thymus grafts become tolerant of host antigens, whether in thymectomized (22, 35) or normal (36) hosts. The implication is that tolerance is induced in the thymus itself by continued exposure to antigen.

This hypothesis provides a simple explanation for several well established features of the tolerance phenomenon. The requirement that large or repeated doses of antigen be used in the induction of tolerance (26, 27, 34), even in the adult (37, 38), is accounted for by the barrier which inhibits the penetration of antigen. The greater ease of inducing tolerance in the neonate may result from the absence of a pool of competent cells and the relative inefficiency of the blood-thymus barrier at this period of life. The requirement that antigen be systemically disseminated to induce tolerance may

follow from the fact that it must reach the thymus to do so. The production of tolerance in adult mice by administration of antigen (BGG) in a non-aggregated form (39, 40) may be regarded as a consequence of the more ready penetration of the thymus by non-aggregated materials of this type. The requirement that antigen persist, either in the form of living cells (in homograft systems), of polysaccharides which are very slowly catabolized (in immunological paralysis), or of proteins which must be injected repeatedly (27, 37, 38), follows from the requirement that new populations of small lymphocytes during their maturation process in the thymus, interact with antigen. It is equally possible that tolerance is due to elimination of clones of specifically reactive cells, normally present in the small lymphocyte pool, or to presence of antigen or an antigenic fragment within all reactive cells in the pool. A number of studies have shown that antigen does not persist in free form at an extracellular site but must be in some critical location (intracellular? intrathymic?) (see references 27, 28). The hypothesis does not explain the failure of adult animals to react when injected with an overwhelming dose of *e.g.* pneumococcal polysaccharide ("immunologic paralysis"). Eisen and Karush (41) have proposed an explanation of tolerance, based on the proposition that complexes of antigen with preformed ("natural") antibody must be ingested by competent cells to induce an immune response and that complexes formed in the presence of excess antigen may not be phagocytizable. Such a hypothesis may account for the behavior of normal adult animals treated with large doses of antigen.

If tolerance requires persistence of antigen within the thymus and its interaction there with newly formed lymphocytes, eventual depletion of thymic antigen will lead to formation of new, non-tolerant lymphocytes. This may explain the waning of tolerance by 6 weeks in rats grafted with "tolerant" thymus. Claman and Talmage (42) and Taylor (40) have shown that continued presence of the thymus is essential to the waning of tolerance. Mice made tolerant to BSA or BGG by repeated injections from birth or by a dose of centrifuged BGG at the age of 3 months failed to regain immune reactivity if thymectomized after the establishment of tolerance. Adults thymectomized and then given a massive dose of BGG show a persistence of tolerance unlike that produced by antigen in control animals. The waning of tolerance can be accelerated by irradiation in animals possessing a thymus, but this procedure is without effect in thymectomized mice (43). On the other hand, termination of tolerance by immunization with cross-reacting antigens may not depend on loss of tolerance to major antigenic determinants of the molecule, *i.e.* production of a new population of non-tolerant cells, but on immunization against non-tolerated minor determinants. Not unexpectedly, the abrogation of tolerance to BSA in 3- to 4-month-old rabbits by immunization with arsanil-sulfanil BSA is not prevented by thymectomy (44).

Several authors have recently published data showing that restoration of irradiated animals with spleen or lymph node cells derived from specifically tolerant donors results in tolerance of the recipients for the specific antigen. Here one is concerned with transfer of competent cells which, as in the Gowans experiment cited earlier, are specifically non-reactive when derived from tolerant donors. These experiments have a different significance from ours: the transferred thymus does not contain competent cells but is engaged in producing them or perhaps controlling their production in other organs. The reported experiments involve both mice and rats and homograft tolerance (45) as well as tolerance to erythrocyte antigens (46, 47), purified protein (48), and

bacterial lipopolysaccharide (49). Dietrich and Weigle, for example, have found that mice lethally irradiated (825 r) and given 75 or 100 $\times$ 10^6 normal spleen cells intravenously, show normal antibody formation after challenge with antigen (human γ-globulin) and adjuvant 7 days later (48). If restored with spleen cells from tolerant donors, these animals fail to form antibody immediately but may show immune elimination of antigen by 17 to 18 days. Apparently the irradiated animals, if provided with a pool of tolerant lymphocytes, remain tolerant until the central lymphoid organs have recovered from irradiation injury and initiate the formation of new non-tolerant cells. Similarly 10-week-old rats receiving 1000 r, followed by isologous spleen or lymph node cells from donors tolerant to sheep erythrocytes, remained specifically unreactive to sheep cells for as long as 6 weeks (46).

The two antibody responses measured by hemagglutination (MES and MER) presumably correspond to the early macroglobulin (19S) and late 7S antibodies described by a number of authors in rats given protein antigens plus Freund's adjuvant (50, 51). The latter may be of the γG type or possibly γA in electrophoretic mobility (52, 53). The present experiments demonstrate a clear relationship of the thymus to the production of MER antibody: such antibody was not formed in animals lacking a satisfactory thymus graft nor in those grafted successfully with thymus from donors tolerant to BGG. The tolerance effect was apparently specific. MES antibody was formed both in rats lacking a graft and in those receiving tolerant thymus. This finding agrees with the observation that after neonatal thymectomy, in both the rat and mouse, the formation of γM antibodies is relatively unimpaired (53–55) and plasma cells and germinal centers are formed normally (13, 16). Perhaps the thymus plays no role as a source organ in relation to this type of immune response or to the corresponding form of tolerance. The striking dissociation of the two antibody responses is reminiscent of the dissociation in their susceptibility to whole body irradiation (56) or antimetabolites such as 6 MP and methotrexate (57, 58), the 19S response being the more resistant in each case. Delayed sensitization is relatively resistant to irradiation and antimetabolites (59, 60); yet in the present experiment, the delayed response appeared to parallel formation of MER antibody.

Arthus reactivity was dissociated from each of the other immune responses studied. By 3 weeks after grafting of normal thymus and marrow, delayed sensitization was normal or nearly so; yet skin reactivity of the Arthus type was entirely absent. Even at 6 weeks this function had not returned to normal (reactivity to Ea recovered more rapidly than reactivity to BGG). If an organ other than thymus or marrow produces the responsible precursor cells, recovery from irradiation in the recipient would necessarily be slow since, even in intact animals given large doses of irradiation, the return of immune function and of blood and tissue lymphocytes to normal levels takes 6 weeks or more (61–64). We may be concerned with a gastrointestinal source organ, such as the appen-

dix, acting as a mammalian homologue of the bursa of Fabricius (12), or perhaps the spleen. In agreement with this possibility, specific inhibition of Arthus sensitization was not observed in recipients of thymus and marrow grafts from tolerant donors.

The principal histological finding in the present study was the progressive repopulation of lymph nodes and the much slower repopulation of the spleen with small lymphocytes in animals with successful thymus grafts. The lymphoid tissues of tolerant animals have been reported to show little or no histologic response following challenge with the tolerated antigen (38, 65). A meaningful analysis of lymph node changes following immunization in our animals could not be undertaken, since the architecture of the nodes was greatly affected by irradiation and since the use of adjuvant presumably results in active immune responses to non-tolerated antigens of the tubercle bacilli.

SUMMARY

Rats thymectomized and irradiated as adults were restored to immunologic reactivity by grafts of normal adult rat thymus and bone marrow. Reactivity of the delayed (cellular) type and formation of mercaptoethanol-sensitive (MES) and mercaptoethanol-resistant (MER) antibody returned within 3 weeks, while Arthus reactivity remained subnormal till 9 weeks after irradiation and grafting. When the thymus donor was tolerant to BGG, the recipient showed specific non-reactivity to this antigen 3 weeks and, to a much lesser extent, 6 weeks after grafting. This non-reactivity affected delayed responses and MER antibody. No effect was noted on Arthus reactivity and a slight effect on MES antibody. Controls showed that the non-reactivity was not due to transfer of free antigen at the time of grafting. It was concluded that different source organs are responsible for different immune functions and that specific immunologic tolerance may be induced within such an organ as the thymus.

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