these deviations from expectation are statistically significant. It should be noted that $MnCl_2$ dialysis did not appreciably activate *in vitro* the AHF of hemophilic plasma being prepared for transfusion, and that this mixture containing $MnCl_2$ did not "release" or "activate" AHF within the hemophiliac after transfusion.

Discussion and summary. These experiments show that the AHF activity of plasma is enhanced by dialysis, especially against $MnCl_2$, and that thrombic destruction of AHF can be prevented by prior dialysis of plasma against $MnCl_2$. Manganese chloride is clearly the most effective cation tested with respect to protection of AHF against thrombic destruction and 0.02 M is the optimal concentration. Manganese does not protect AHF, however, by destroying thrombin or by altering the thrombin destroying properties of antithrombin. This specific effect of Mn++ in protecting AHF is not surprising because others have observed that Mn++ both activates and stabilizes AHF in plasma(11,12). Our experiments suggest that the protective action of $MnCl_2$ may be related in some manner to changes induced in the AHF molecule. The nature of any such change remains to be clarified.

Identification of the AHF activity in the treated plasmas was established by injection into both normal and hemophilic dogs. These experiments showed that the AHF activity observed *in vitro* could also be observed *in vivo*. Normal dogs circulated somewhat more and hemophilic dogs somewhat less AHF than expected. It is suggested by these results and a similar result using $MnCl_2$ alone that AHF may become "activated" by Mn++ *in vivo* in normal but not hemophilic animals. This apparent "activation" may, however, be nothing more than an indirect method of producing the well known "adrenaline effect."

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Role of the Thymus in Tolerance. II. Transfer of Specific Unresponsiveness to BSA with Thymus Grafting.* (30949)

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In a previous study(1), we have established that thymectomized, irradiated rats, which receive grafts of thymus from donors made tolerant by neonatal exposure to protein antigen (bovine γ-globulin, BGG), themselves show specific inhibition of certain immune responses, notably delayed sensitization

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TABLE I. Number of Rats Developing Different Degrees of Delayed Skin Reactivity After Thymus and Marrow Grafting and Challenge.

Experimental group	3 week challenge						6 week challenge					
	10 days			20 days			10 days			20 day		
	0	7-12	>12mm	0	7-12	>12mm	0	7-12	>12mm	0	7-12	>12mm
Normal thymus, normal marrow	1	2	3	2	2	2	1	0	3	0	2	2
Normal thymus, normal marrow, iv BSA*	9	5	1	11	2	1	6	5	6	6	6	5
Normal thymus, pretreated marrow	2	1	1	3	1	0	2	1	2	3	1	1
Pretreated thymus, normal or pretreated marrow	10	2	1	13	0	0	7	2	3	7	4	1
Thymus and marrow from Ea treated donors	–	–	–	–	–	–	2	1	3	2	1	3
Unsuccessful thymus graft	7	0	1	6	1	1	6	3	0	8	1	0

* Recipients given up to 1000 μg BSA intravenously at time of grafting.

and formation of mercaptoethanol-resistant (MER) antibody. This inhibition, which lasts several weeks, resembles tolerance in a number of respects and is presumed to result from interaction of antigen with precursors of immunologically active lymphocytes in the thymus. In the present study an attempt was made to extend these observations to a second antigen (bovine serum albumin, BSA) and to determine whether similar transfer of an adult thymus, exposed to antigen over a period of only a few days, would also result in a specific unresponsive state in the recipient.

Materials and methods. Crystalline BSA was obtained from Pentex, Inc., Kankakee, Ill. Inbred Lewis rats (Microbiological Associates), 8-10 weeks old, were used as donors and recipients. Donors either were normal or received 50 mg BSA intraperitoneally each day for 3 days prior to transfer. Recipients were thymectomized at 5 weeks of age and received 800r of whole body irradiation immediately before transfer. Each recipient was grafted subcutaneously with a single donor thymus and injected intravenously with approximately 10^8 marrow cells. Different groups of animals received different combinations of thymus and marrow from normal donors and donors pretreated with BSA. Control groups received thymus and marrow from donors pretreated in the same manner with chicken ovalbumin (Armour, Ea) or normal grafts accompanied by an intravenous injection of BSA (doses between 1 and 1000 μg). All animals were challenged, 3 or 6 weeks after grafting, by footpad injection of 500 μg BSA in complete adjuvant, bled and skin tested with 30 μg BSA at 10 and 20 days, injected with an intraperitoneal booster dose of 1 mg BSA at 25 days, and subjected to a final bleeding at 32 days. Skin reaction diameters were recorded at 3-4 hours (Arthus) and 24 hours (delayed). Hemagglutination titers ($\log_2$) of all sera were determined before and after treatment with 0.125 M mercaptoethanol. At the time of sacrifice, the possible presence of residual thymus in the mediastinum and the status of the thymus graft were evaluated in each animal, both grossly and by examination of multiple histologic sections. A detailed description of the methodology is given in our earlier paper (1).

Results. The development of delayed sensitization and antibody formation (Tables I and II) were markedly deficient in animals with unsuccessful thymus grafts, *i.e.*, in ani-

TABLE II. Average Hemagglutination Titers Against BSA Developed by Rats Grafted with Normal or "Tolerant" Lymphoid Tissues.

Experimental group	3 week challenge		6 week challenge	
	20 days	32 days	20 days	32 days
	Total MER	Total MER	Total MER	Total MER
Normal thymus, normal marrow	1.5, .5 (4/6)*	3.8, 1.8 (5/6)	3.3, 1.5 (4/4)	5.0, 5.0 (3/4)
Normal thymus, normal marrow, iv BSA†	.8, 0 (5/13)	2.1, .7 (9/14)	2.0, 0 (14/16)	3.3, 2.2 (16/16)
Normal thymus, pretreated marrow	.8, 0 (2/4)	2.3, .5 (4/4)	2.2, .4 (4/5)	4.4, 3.2 (5/5)
Pretreated thymus, normal or pretreated marrow	.1, 0 (/13)	.3, 0 (3/11)	3.0, 1.3 (9/9)	6.8, 3.8 (5/5)
Thymus and marrow from Ea-treated donors	—	—	1.6, .8 (5/5)	7.0, 5.8 (5/5)
Unsuccessful thymus graft	.2, 0 (2/9)	1.0, .6 (4/9)	1.0, .4 (4/8)	1.5, .1 (5/8)

* No. of positive sera given in ( ).
† Recipients given up to 1000 μg BSA intravenously at time of grafting.

mals thymectomized, irradiated and given 10^8 marrow cells. Both functions were restored in animals grafted with normal thymus and bone marrow though not to the levels observed in normal animals.

Table I shows that thymectomized, irradiated rats grafted with thymus from donors pretreated with BSA were almost completely unable to develop delayed responses 3 weeks after grafting and still showed reduced reactivity at 6 weeks. However, the groups receiving bone marrow from pretreated donors or given BSA intravenously at the time of grafting with normal thymus and marrow also showed some reduction in reactivity.

Little hemagglutinating antibody was found in the 10 day bleedings, whether 3 or 6 weeks after grafting. However, the bleedings at 20 and 32 days (following a booster antigen dose at 25 days) showed striking differences among the experimental groups (Table II). Rats receiving thymus grafts from pretreated donors showed almost no antibody formation 3 weeks after grafting, both mercaptoethanol-sensitive and resistant, (MES and MER) antibodies being absent. This effect had disappeared at 6 weeks. Again, animals given marrow from pretreated donors and normal thymus or normal thymus and marrow plus an injection of BSA showed some reduction in reactivity.

No significant Arthus reactions could be obtained 3 weeks after grafting. Following the 6 week challenge, tests at 10 days were positive in only 3 rats, 2 of which did not show significant titers of circulating antibody. At 20 days, 3 rats gave minimal reactions (7-8 mm), and these had antibody titers of borderline significance. Eleven others gave larger reactions (9-14 mm), and all but one of these had high 20 and 32 day titers in comparison with other rats in the associated groups. There was no clearcut effect of donor treatment on the expression of Arthus reactivity, less than half the animals in each group reacting after the 6 week challenge.

Histologic studies confirmed the findings of our earlier investigation. Successful thymus grafts showed an essentially normal architecture and were fully populated with lymphocytes. The peripheral lymphoid tissues, in thymectomized animals possessing a functional thymus graft, showed some depletion, even 6 weeks after grafting. The nodes had returned to normal to a much greater extent than the spleen in this length of time. The nodes in animals lacking a satisfactory graft, however, remained entirely depleted of lymphocytes at 6 weeks. Blood lymphocyte counts were normal in the thymectomized, successfully grafted group, averaging 10,900 at 20 days (16 rats) and 8,200 42 days after grafting (11 rats). Rats lacking a graft had very low counts at 20 days. The value in 7 animals averaged 5,200 and 4 of these had counts below 3,000. However, by 42 days the

average value was 12,000.

Discussion. The distinctions observed here between experimental groups and controls were less sharp than in the study in which BGG was used as antigen(1), perhaps because of the inferior immunogenicity of BSA. There was nevertheless a clearcut depression of immune reactivity of the delayed type and the formation of antibody in rats receiving thymus grafts from donors pretreated over a 3-day period with BSA, and this effect exceeded the depression observed in various control groups. As in the BGG study, delayed sensitization appeared to be inhibited even 6 weeks after thymus grafting while antibody-forming capacity had returned to the control level. Arthus reactivity was slow in reappearing in all experimental groups and was uninfluenced by pretreatment of the donor with BSA.

These findings provide support for the thesis advanced earlier that interaction of antigen with lymphocyte precursors in the thymus may be the basis of tolerance for certain immune responses, the delayed response in particular, though not necessarily for others such as the Arthus. The possibility cannot be ruled out, however, that antigen carried into the recipient with the pretreated thymus graft produced unresponsiveness by its action at another site. The graft may have contained several hundred μg of BSA (unpublished data), and the controls demonstrate that amounts of BSA below 1000 μg given intravenously to the recipients of normal grafts inhibited later reactivity. Conversely, BSA given at the time of grafting may have exerted its inhibitory effect within the grafted thymus.

The present observations imply that tolerance is induced in the adult thymus by the penetration of antigen. Such penetration occurs readily with soluble, non-aggregated proteins such as BSA(2). In the normal adult receiving a sufficient dose of antigen this tolerance induction must occur, though it may be masked by the immune response of lymphocytes in the peripheral lymphoid tissues. If the immune response is avoided by use of a monomeric antigen, which is not taken up by phagocytic cells and therefore induces no response, tolerance is readily observed(3,4,5). It is similarly brought out in animals whose peripheral response is temporarily suppressed by irradiation(6,7) or the use of antimetabolites(8). Perhaps immunologic paralysis(9) or states in which long continued immunization leads to specific unresponsiveness(10, 11) also involve tolerance and some unidentified type of exhaustion of peripheral lymphocytes, *i.e.*, two distinct phenomena occurring simultaneously.

As in our earlier study, Arthus reactivity did not reappear in the various recipients until 2 months after irradiation and grafting. This immune response depends perhaps on a source organ other than the thymus.

Summary. In rats thymectomized and irradiated as adults and grafted with thymus from adult donors given 150 mg of BSA over the 3-day period immediately preceding grafting, sensitization of the delayed type and antibody formation against BSA were both inhibited. An inhibition of lesser degree was observed in rats grafted with normal thymus and given BSA intravenously at the time of grafting or given marrow from pretreated donors. It is concluded that tolerance for certain immune responses is induced in the adult thymus, even in normal animals given large systemic doses of antigen which can penetrate this organ.

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