ROLE OF THE THYMUS IN TOLERANCE

III. Tolerance to Bovine Gamma Globulin after Direct Injection of Antigen into the Shielded Thymus of Irradiated Rats*

By PARKER J. STAPLES,‡ M.D., IGAL GERY,§ Ph.D., and BYRON H. WAKSMAN, M.D.

(From the Department of Microbiology, Yale University, New Haven, Connecticut)

(Received for publication 11 April 1966)

Several recent studies have directed attention to the possibility that specific immunologic tolerance, induced by antigen in newborn or irradiated adult animals, may result from interaction of antigen with immature lymphoid cells in central lymphatic organs such as the thymus (1–3). Nonaggregated proteins introduced into the circulation enter the thymus readily, even in the adult (4–5), and the thymus is generally recognized as a source of small lymphocytes which participate in homograft immunity, delayed sensitivity, and some forms of antibody synthesis.

Three times as many lymphocytes are found leaving the thymus as entering (6). These seed peripheral lymphoid organs (7–9) and appear to act as precursors of the large, pyroninophilic cells characteristic of the homograft response (10). Neonatally thymectomized animals show a striking immunologic deficiency (7, 11, 12), which is promptly corrected by supplying competent lymphocytes in sufficient number (13). However thymus lymphocytes themselves appear to be incompetent, and some maturation of these cells in the thymus must be postuated (13–15).

The present report describes an attempt to produce tolerance to bovine gamma globulin by injecting this antigen directly into the thymus of adult rats, deprived of a competent pool of peripheral small lymphocytes by irradiation. The results obtained confirm our earlier findings (1, 2) and provide support for the initial hypothesis.

Material and Methods

Lewis rats of both sexes (Microbiological Associates, Bethesda, Maryland), 6 wk of age at the start of the experiment, were used throughout. They were maintained in groups of 5 in plastic cages and fed ad lib. with standard laboratory chow. After irradiation, each group

* Supported by Grants AI-06112, AI-06455, and NB-05187 from the National Institutes of Health, Bethesda, Maryland. A preliminary report of this work was presented before the 50th annual meeting of the American Association of Immunologists, April, 1966.

‡ Senior medical student, Yale University School of Medicine, 1965 to 1966.

§ Helen Hay Whitney Research Fellow, on leave of absence from the Hebrew University-Hadassah Medical School, Department of Medical Ecology, Jerusalem, Israel.

received daily fresh water containing oxytetracycline in a concentration of 0.5 g/liter until the termination of the experiments.

Treatment Groups.—The treatment groups included normal rats, rats given 800 R whole-body irradiation with lead shielding of the thymus (T) or the spleen (S), and similar rats injected immediately after irradiation with either bovine γ-globulin (BγG) (Armour Pharmaceutical Laboratory Kankakee, Illinois) or chicken ovalbumin (Ea) (Nutritional Biochemicals Corporations, Cleveland) directly into the thymus or spleen (iT, iS) or intraperitoneally (i.p.) BγG was injected in doses of 40, 20, 2.0, 0.2, or 0.02 mg iT in T-shielded, iS in S-shielded, and i.p. in T-shielded animals; and 40 mg Ea was injected iT in some T-shielded rats as a specificity control. For each replication of the experiment, closely matched animals were assigned at random to each of the treatment groups, all groups being represented. These animals were carried together through the irradiation and injection procedure and the later challenge, skin testing, etc. Ten successive replications were initiated over a 7 day period.

All animals were challenged 3 wk after the irradiation and injection procedure by foot-pad injection of 500 μg BγG in complete adjuvant, followed by an intravenous booster dose of 1.0 mg BγG at 25 days. The details of irradiation and of the challenge procedure are given in our earlier publication (1). Skin tests with 30 μg BγG were carried out 10 and 20 days after challenge, and Arthus and delayed reactions read at 3 and 24 hr. Sera obtained at 0, 10, 20, and 32 days were assayed for hemagglutinating antibody and by gel diffusion by the techniques used earlier (1, 12). For microimmunoelectrophoresis, the method of Scheidegger (16) was employed.

Shielding and Injection of Thymus and Spleen.—The thymus was protected during irradiation with a 2 × 2.5 cm lead shield, 0.16 cm in thickness, taped over the upper midsternal thorax. For the spleen, a 21 × 10 × 5 cm box was fashioned of 0.16 cm lead sheeting. The spleen was brought out through a left subcostal skin incision and hung through a 1.2 mm hole in the box; and the animal was positioned so that radiation was perpendicular to the box. All irradiation was carried out on a large backdrop of 0.16 cm lead sheeting, to minimize scatter. The approximate radiation dose to the shielded thymus was 75 R and to the shielded spleen was 7 R, as measured by prior Victoreen electrode determinations. Thymus shielded rats received 59.3 R per minute whole-body irradiation for 13.5 min and spleen-shielded rats 64.3 R per minute for 12.5 min.

Within 10 min after irradiation, each rat received the iT, iS, or i.p. injection of antigen. The thymus was approached by a midline sternal incision and the gland exposed by either a mastoid retractor or two 4.0 silk sutures applied to either edge of the wound. Thymus injections were made slowly with a 1.0 ml syringe and a 30 gauge needle; 0.1 ml of protein solution was injected into each lobe of the thymus without leakage. Prior injection studies with Evans blue dye demonstrated that at least 80% of the thymus could be completely injected in this manner. The sternum was closed with 4.0 silk sutures and the skin apposed with metal wound clips. Splenic injection was carried out in a similar manner. All operations were performed under clean conditions.

RESULTS

The experimental animals withstood irradiation and the operative procedure well. At the time of challenge, 8 rats out of 200 had died in the various groups. No animals were used that did not appear to be in good health.

At autopsy, approximately 8 wk after the irradiation and injection procedure, thymus and spleen weights had not returned to normal levels (Table I). In the spleen-shielded group the thymus weighed approximately three quarters of the normal value. Histologically no significant difference was found in the

thymus of thymus-shielded and spleen-shielded groups; and there was no clear-cut difference between injected and non injected thymus in the former or between injected and noninjected spleen in the latter.

Skin Reactions.—The development of delayed skin reactivity in rats challenged 3 wk after the treatment procedure was essentially normal in animals whose spleen or thymus was shielded during irradiation (Table II). It was inhibited in the group which had received specific antigen directly in the shielded thymus. Rats given 20 or 40 mg BγG showed no significant delayed response, those given 2 mg gave a minimal response, and even animals receiving 200 μg of antigen showed a substantial reduction of reactivity. Controls in which similar quantities of BγG had been injected intraperitoneally, with thymus shielding, or intrasplenically, with spleen shielding, showed a reduction in reactivity only at the highest doses used; injection of large doses of heterologous antigen (Ea) di-

TABLE I

Weight of Lymphoid Organs in Irradiated and Shielded Rats

Treatment group			No. of rats	Average weight	
800 R	Shielded organ	Injected organ		Thymus	Spleen
				mg	*mg*
−	—	—	5	364	472
+	T	—	4	326	375
+	T	T	5	344	420
+	S	—	5	264	408
+	S	S	6	288	407

Weights recorded 8 wk after irradiation, 5 wk after challenge.

rectly into the thymus had little inhibitory effect. The differences in reactivity were particularly striking at the time of the second skin test, 20 days after challenge (Fig. 1).

Arthus reactivity failed to return to normal in any of the irradiated animals during the interval before challenge (Table III). At the time of the second skin test, i.e. 6 wk after irradiation, reactivity was still slightly subnormal in the spleen-shielded group, and in the thymus-shielded group it was very low. Reactivity was inhibited in spleen-shielded rats given higher doses of intrasplenic antigen. The effect of antigen pretreatment could not be evaluated in the thymus-shielded groups.

Serologic Data.—Hemagglutinin measurements against crude BγG showed that antibody formation at 10 days was significantly inhibited in rats pretreated with intrathymic antigen (Figs. 2 and 3). Some inhibitory effect was still evident 20 days after challenge. Low titers were seen in animals injected even with

the lowest pretreatment dose of antigen (20 μg). There was no evidence that antibody formation was inhibited in any of the control groups.

It could be shown, however, by gel diffusion and by immunoelectrophoresis that rats, like mice (17), when hyperimmunized with the crude BγG preparation used in these studies, formed 3 or 4 antibodies directed respectively at the BγG itself, a major component with β-mobility, and one or two minor compo-

TABLE II

Specific Depression of Immunologic Reactivity of the Delayed Type after Intrathymic Injection of Antigen

Treatment group					No. of rats	Average diameter of delayed reactions to skin test at	
800 R	Shielded organ	Injection route	Antigen	Injected dose		10 days	days 20
				mg		*mm*	*mm*
−	—	—	—	—	18	17.3	15.2
+	T	—	—	—	10	15.8	15.3
+	S	—	—	—	10	15.2	16.1
+	T	iT	Ea	40	9	12.8	14.7
+	T	iT	BγG	20, 40	18	3.9	0
+	"	"	"	2.0	15	8.3	3.7
+	"	"	"	0.2	13	10.5	9.8
+	"	"	"	0.02	14	12.3	12.6
+	T	i.p.	BγG	20, 40	13	10.1	9.2
+	"	"	"	2.0	8	13.7	12.4
+	"	"	"	0.2	9	11.8	13.9
+	"	"	"	0.02	8	11.9	14.2
+	S	iS	BγG	20, 40	14	11.2	9.0
+	"	"	"	2.0	10	12.7	12.4
+	"	"	"	0.2	10	13.6	13.1
+	"	"	"	0.02	10	15.2	14.9

Challenge 3 wk after irradiation and injection.

nents of γ_1-mobility (Figs. 4 and 5). When sera from animals of the present experiment were studied by gel diffusion against whole bovine serum or crude BγG, it became clear (Table IV) that intrathymic antigen exerted a highly specific inhibitory effect on formation of antibody to γ-globulin. Irradiation alone resulted in slowed antibody formation against this antigen, i.e. fewer responders at 20 days, and diminished response to the minor γ-contaminants even at 32 days. Almost no antibody to γ-globulin (major or minor) was formed in animals given as little as 200 μg of antigen intrathymically at the time of irradia-

tion and shielding; and less than half of those given 20 μg formed measurable antibody after challenge (Table IV). On the other hand, rats given antigen intrasplenically or intraperitoneally showed a diminished response only at the 20 to 40 mg level. Antibody to the β-component was formed by all animals, though there was a delayed response in those pretreated with the higher doses of intrathymic antigen. It is presumed that hemagglutination observed with many of the sera from the group injected intrathymically depended on antibody to this β-contaminant.

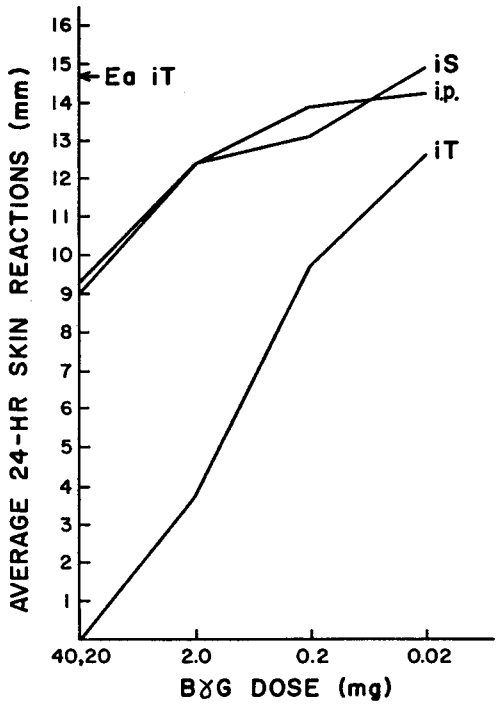

FIG. 1. Relation of delayed skin reactions, elicited 20 days after challenge, to dose and route of antigen injection at time of irradiation 3 wk earlier.

DISCUSSION

Any investigation of specific immunologic unresponsiveness must concern itself with the possible effect of antigen on several different types of cell, which are recognized as playing a role in immune responses. These include precursor cells in the bone marrow; immature lymphoid cells derived from these precursors and sojourning in the thymus or other "central" lymphoid organs; the mature, competent cells of the peripheral small lymphocyte pool, which recirculate between the blood and the parenchyma of peripheral lymphoid organs; committed

cells such as the plasma cell and the large, pyroninophilic cell of "cellular" hypersensitivity; and the phagocytic cells which process antigen and may provide the effective antigenic stimulus to competent cells. Studies of classic tolerance, induced with large doses of antigen in fetal or newborn animals or in older animals treated with X-ray or radiomimetic agents, are concerned only with the

TABLE III

Effect of Intrathymic Injection of Antigen on Immunologic Reactivity of the Arthus Type

Treatment group					No. of rats	Average diameter of Arthus reactions to skin test at	
800 R	Shielded organ	Injection route	Antigen	Injected dose		10 days	20 days
				mg		*mm*	*mm*
−	—	—	—	—	18	13.6	14.0
+	T	—	—	—	10	0	9.4
+	S	—	—	—	10	3.9	12.4
+	T	iT	Ea	40	9	0	5.8
+	T	iT	BγG	20, 40	18	0	2.7
+	"	"	"	2.0	15	0	2.1
+	"	"	"	0.2	13	0	2.6
+	"	"	"	0.02	14	0	5.2
+	T	i.p.	BγG	20, 40	13	2.4	4.2
+	"	"	"	2.0	8	0	6.0
+	"	"	"	0.2	9	0	6.0
+	"	"	"	0.02	8	0	6.2
+	S	iS	BγG	20, 40	14	2.6	5.0
+	"	"	"	2.0	10	2.0	7.8
+	"	"	"	0.2	10	0	10.3
+	"	"	"	0.02	10	0	8.8

Challenge 3 wk after irradiation and injection.

first two of these and with the phagocytic cells, since competent and committed cells either have not yet appeared or have been ablated by the agents used.

Gowans' observations that the property of tolerance is conferred on irradiated rats by transfer to them of small lymphocytes from specifically tolerant donors and, conversely, that tolerant rats are rendered normal by a transfusion of normal lymphocytes (18, 19) speak against the possibility that phagocytic cells play a role in tolerance and suggest that tolerance is a property of the peripheral pool of competent small lymphocytes. The failure to demonstrate any peculiarity in phagocytic uptake of tolerated antigens (20) is consistent with this suggestion.

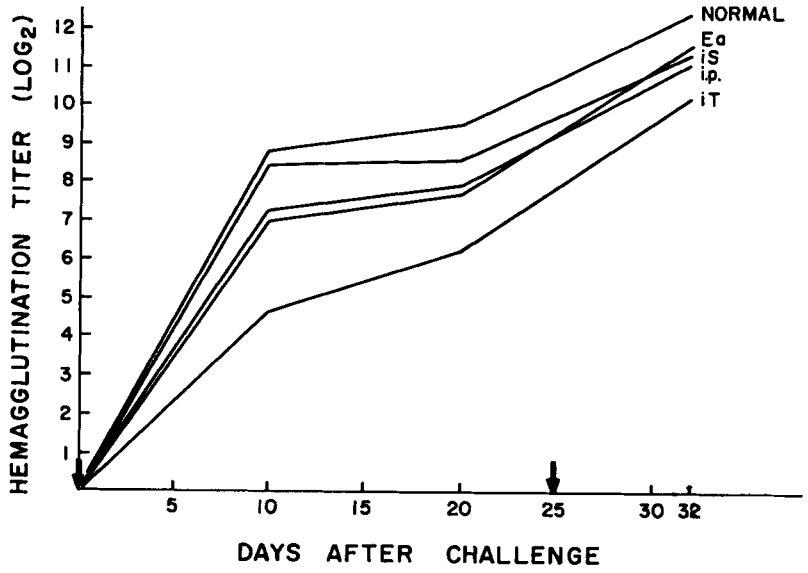

FIG. 2. Average hemagglutinin titers in animals pretreated with irradiation and injection of 20 or 40 mg of antigen by various routes. Arrows indicate challenge and booster doses of antigen.

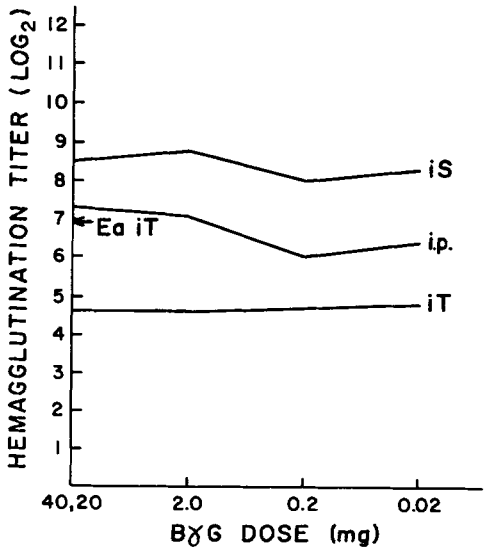

FIG. 3. Average hemagglutinin titers at 10 days and relation to dose and route of antigen injection at time of irradiation 3 wk before challenge.

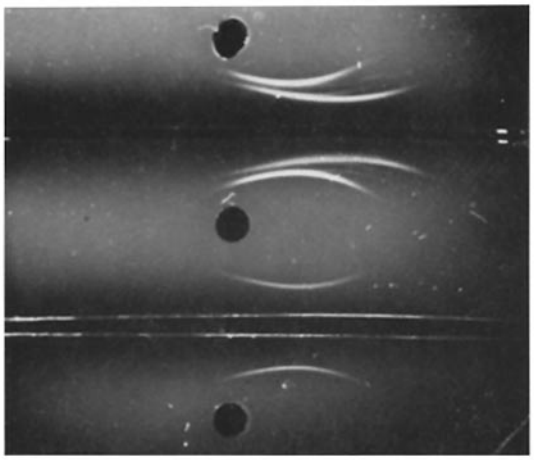

FIG. 4. Immunoelectrophoretic pattern of crude BγG used in present experiments. Upper and lower troughs contain 32-day sera, respectively, of rats from groups given 40 mg Ea and 40 mg BγG intrathymically. Note absence in latter of antibodies against major or minor γ-components.

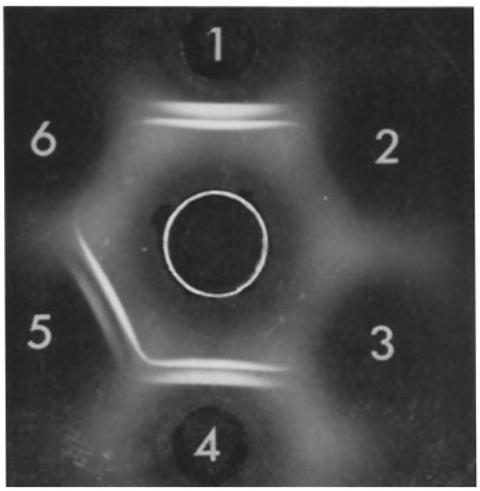

FIG. 5. Ouchterlony pattern given by rat sera with whole, normal bovine serum (center well). Wells 1 and 4 contain hyperimmune rat serum and show 2 lines. Wells 2, 3, and 6 contain 32-day sera from rats injected intrathymically with 40, 2.0, and 20 mg BγG and show a very faint line with the β-contaminant. Well 5 contains 32-day serum from an unirradiated rat and shows 3 lines.

We have therefore sought evidence that interaction of antigen with precursors of the competent lymphocytes, either in the bone marrow or the thymus, might be responsible for tolerance. It was established that thymectomized, irradiated adult rats grafted with thymus from isogenic donors tolerant for BγG, show a

specific inability to respond immunologically to BγG for a period of several weeks (1). The transfer of marrow from tolerant donors failed to convey such nonreactivity. Grafting of thymus from normal adult donors, treated with large doses of BSA over the preceding 36 hr, also conferred specific nonreactivity on the recipient (2). The apparent unresponsiveness in each case concerned "de-

TABLE IV

Depressed Antibody Formation after Intrathymic Injection of Antigen

Treatment group					Antibody by gel diffusion							
					20 days				32 days			
						Antibody against				Antibody against		
800 R	Shielded organ	Injection route	Antigen	Injected dose	No. of rats	BγG	Contaminants		No. of rats	BγG	Contaminants	
							β	γ1			β	γ1
				mg								
−	—	—	—	—	18	18	18	0	11	11	11	6
+	T	—	—	—	10	1	8	0	10	10	10	1
+	S	—	—	—	10	4	9	0	10	10	10	1
+	T	iT	Ea	40	9	0	5	0	9	8	9	1
+	T	iT	BγG	20, 40	17	0	0	0	15	0	13	0
+	"	"	"	2.0	13	0	4	0	12	2	12	0
+	"	"	"	0.2	13	0	5	0	12	1	12	0
+	"	"	"	0.02	12	0	8	0	12	5	12	0
+	T	i.p.	BγG	20, 40	12	0	10	0	12	11	12	1
+	"	"	"	2.0	8	0	5	0	7	6	7	1
+	"	"	"	0.2	8	0	7	0	8	8	8	1
+	"	"	"	0.02	8	1	6	0	8	7	8	3
+	S	iS	BγG	20, 40	14	0	9	0	13	8	13	0
+	"	"	"	2.0	9	0	7	0	9	9	9	0
+	"	"	"	0.2	10	3	9	0	9	9	9	2
+	"	"	"	0.02	9	2	8	0	7	7	7	1

No precipitating antibody was found at 10 days.

layed" hypersensitivity and the formation of mercaptoethanol-resistant antibody.

The present study confirms these earlier observations in that BγG introduced directly into the shielded thymus, in rats deprived of a competent peripheral pool by irradiation, gave a striking degree of tolerance for both types of immune response 3 wk later, even when microgram amounts of antigen were employed. The effect was shown by suitable controls not to involve action of antigen at a site outside the thymus nor to be duplicated by interaction of comparable amounts of antigen with mature cells in the shielded spleen. The inference seems

inescapable, from this series of three studies, that in classic tolerance antigen acts on immature cells within the thymus and perhaps in other central lymphoid organs as well. It is difficult to see how antigen injected directly into the thymus could produce a *specific* suppressive effect on distant immune responses by way of a humoral agent produced in the thymus. Another alternative, that antigen is processed in the thymus and released in a form which inhibits specific responses elsewhere, also seems far-fetched.

There is no implication in these findings that the effect of antigen on thymus lymphocytes differs qualitatively from its effect on mature, competent lymphocytes in the peripheral pool of intact adult animals. Several recent studies (17, 21–23) show a specific suppressive effect of protein antigen injected into adults on immune responses; and Mitchison has advanced evidence that this effect is mediated by its interaction with peripheral lymphocytes (24). Indeed an exactly comparable effect can be demonstrated in animals lacking a thymus (24, 25). In the present study, when antigen was allowed to interact with competent cells in the shielded spleen, it produced inhibition only in amounts 100 to 1000 times greater than those effective in the thymus. The final concentrations of antigen in the two organs may have been slightly influenced by difference in their size (volume) but it seems clear that a much higher concentration of antigen was required to produce a suppressive effect in the spleen. This result implies a quantitative difference in reactivity to antigen of immature and mature lymphocytes. No evidence was found for inhibition of mature cells by very low antigen concentrations, such as has been observed in normal adult mice and guinea pigs (21, 26), but such an effect may have been masked by introduction of antigen directly into an organ in which the immune response occurs. The suppression by antigen of already established immunologic responses, demonstrated in a number of systems (27–29), may require still larger amounts of antigen or involve a qualitatively different mechanism, as implied in the term "desensitization". Finally with very large amounts of material, nonspecific blockade of phagocytic cells may occur (30).

The relationships uncovered in the present studies concern certain protein antigens and the limited class of thymus-mediated immune responses, which includes delayed hypersensitivity and formation of mercaptoethanol-resistant antibody. Vojtíšková and Lengerová have reported that injection of allogeneic lymphoid cells into the thymus of irradiated mice results in specific homotransplantation tolerance (3), an experiment formally comparable to that reported here; and we have found (31) that grafts of thymus from tolerant donors to neonatally thymectomized recipient mice confer specific homograft tolerance on the recipients (see also reference 32). The relationships, then, may be the same in the case of homograft immunity as in protein sensitization.

We have confirmed here the finding (1) that ability to develop Arthus reactivity recovers more slowly from irradiation injury than delayed sensitization

or formation of hemagglutinating and precipitating antibody. Arthus reactivity was also found to be readily suppressed by intrasplenic antigen; it differs in this respect as well from these immunologic responses. With other types of response, still other, qualitatively distinct patterns of antigen action may be discovered.

SUMMARY

Rats subjected to high doses of whole-body irradiation, with simultaneous shielding of the thymus or spleen, recovered at 3 wk the ability to develop delayed sensitization and to form hemagglutinating and precipitating antibody following foot-pad injection of BγG in complete adjuvant. Injection of BγG into the shielded thymus immediately after irradiation, in amounts between 20 μg and 40 mg, inhibited these response to later challenge partially or completely. A comparable effect on immune responses to BγG was not seen after injection of heterologous antigen (Ea) intrathymically, BγG intraperitoneally, or BγG into the shielded spleen. However high doses (20 or 40 mg) of antigen given by the latter routes resulted in some diminution of later response. Arthus reactivity recovered partially in the spleen-shielded group and was readily suppressed by intrasplenic administration of antigen.

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