DUAL REGULATORY ROLE OF THE THYMUS IN THE MATURATION OF IMMUNE RESPONSE IN THE RABBIT

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The role of the thymus in the immune response of the rabbit is still uncertain. This is probably because neonatal as well as adult thymectomy has much less effect on the subsequent immune response of rabbits than rodents (1–3). Furthermore, Richter and his co-workers have reported that antigen-reactive cells, comparable to thymus-derived lymphocytes (T cells) of rodents, were found in the bone marrow but not in other lymphoid tissues of rabbits (4–6). Nevertheless, the multicellular model for humoral antibody response is applicable to that of rabbits, as shown by three different lines of evidence: (*a*) cooperation between different antigenic determinants on the same molecule occurs in the secondary antibody response of the rabbit, as revealed by both in vivo and in vitro studies (7–10); (*b*) preimmunization with carrier antigen enhances the primary antibody response to a hapten-carrier conjugate (10); (*c*) at least two different cell types provided by the thymus, bone marrow, and appendix are required to reconstitute the early antibody response of lethally X-irradiated rabbits to sheep erythrocytes (11, 12). Although there is no clear indication that the thymus-derived lymphocytes actually act as helper cells in the rabbit, it may be predicted from these studies that the thymus is endowed with an important role in the immune response of the rabbit.

Another important indicator of maturation of the immune response, demonstrated mainly in the rabbit, is the time-dependent increase of antibody affinity that reflects the differentiation of antibody-forming cells (13–17). The increase in antibody affinity appears to result from selection of antibody-forming cell precursors by antigen and is thus primarily determined by the effective concentration of antigen available to such precursor cells (18–20). However, Gershon and Paul (21) have recently shown that the affinity of antibody produced by mice is also influenced by the quantity of T cells present in the immunized animals. They found that mice relatively deficient in T cells could produce only low affinity antibodies upon immunization with small amounts of a hapten-carrier conjugate.

The present studies were undertaken to learn the regulatory role of the thymus in the immune response of the rabbit with respect to the amount and

affinity of antibody produced. The basic motive of the experiments derives from our previous studies on the regulatory activity of T cells on antihapten-antibody formation in the rat (22–25) in which we showed that T cells primed with a carrier protein first assist in the production of antihapten homocytotropic antibody, but later negatively regulate preestablished antibody formation. We have tried to find evidence for a similar regulatory influence of the thymus on antibody-forming cells in rabbits, since the rabbit has a highly differentiated immune system. The data presented below clearly show that the rabbit thymus is indeed endowed with an important regulatory role over the formation and maturation of antihapten antibody.

Materials and Methods

Proteins and Chemical Reagents.—Bovine serum albumin (BSA)[1] and bovine fibrinogen (BF) were obtained from Armour Pharmaceutical Co., Kankakee, Ill. Human serum albumin (HSA) and bovine gammaglobulin (BGG) were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. 2,4-dinitrobenzenesulfonic acid (DNBS) was obtained from K & K Laboratories, Inc., Plainview, N. Y. and twice recrystallized from 90% ethanol. Amberlite IRA 400, disodium ethylenediamine tetraacetate (EDTA), and epsilon-aminocaproic acid (EACA) were purchased from Nakarai Chemicals LTD., Kyoto, Japan. 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate was obtained from Aldrich Chemical Company Inc., Milwaukee, Wis.

Preparation of Dinitrophenylated (DNP) Proteins.—Dinitrophenylated bovine gammaglobulin (DNP-BGG), dinitrophenylated bovine fibrinogen (DNP-BF), and dinitrophenylated bovine serum albumin (DNP-BSA) were prepared by the reaction of DNBS with proteins at room temperature under alkaline conditions as described by Eisen et al. (26). DNP-BGG containing 43 groups/mol, DNP-BF containing 107 groups/mol, and DNP-BSA containing 7 groups/mol were used throughout.

Preparation of Radioactive Compounds.— 1-fluoro-[2,4-^{3}H]dinitrobenzene ([^{3}H]DNFB) (sp act 19.0 Ci/mmol) was obtained from Radiochemical Centre, Amersham, England. [^{3}H]DNP-EACA was prepared by mixing [^{3}H]DNFB with a 100-fold molar excess of EACA dissolved in 0.1 ml of 5% Na_2CO_3. After stirring at room temperature for 5 h, the reaction mixture was applied to analytical thin layer chromatographic plates composed of silica gel. The thin layer chromatograph was developed with toluene-pyridine-ethylenchlorohydrin-0.8 N ammonia solution (100:30:60:60). The product was eluted from the silica gel with phosphate-buffered saline (PBS, 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.6). [^{131}I]DNP-BSA was prepared by the reaction of DNP-BSA with Na^{131}I in the cold by the chloramine T method (27).

Preparation of DNP Carboxymethyl Cellulose Immunosorbent.—Carboxymethyl (CM) cellulose (Whatman CM-32) was obtained from W. & R. Balston, LTD., England. DNP-CM cellulose was prepared by the reaction of aminoethylated-CM cellulose with DNBS under alkaline conditions for 24 h at room temperature. Aminoethylated-CM cellulose was made by the reaction of CM cellulose with a 100-fold molar excess of anhydrous ethylenediamine and

[1] *Abbreviations used in this paper*: ATS, antirabbit thymocyte serum; BF, bovine fibrinogen, BGG, bovine gamma globulin; BSA, bovine serum albumin; CFA, Freund's complete adjuvant; CM, carboxymethyl; DNBS, 2,4-dinitrobenzenesulfonic acid; [^{3}H]DNFB, 1-fluoro-[2,4-^{3}H]dinitrobenzene; DNP, dinitrophenylated; EACA, epsilon-aminocaproic acid; EDTA, disodium ethylendiamine tetra acetate; HSA, human serum albumin; SRBC, sheep red blood cell; Tx, adult thymectomized.

1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide toluenesulfonate under acid conditions for 20 h.

Preparation of Antirabbit Thymocyte Serum.—Antirabbit thymocyte serum (ATS) was prepared in goats injected with 5×10^9 rabbit thymocytes in Freund's complete adjuvant (CFA). Goats were boosted with five additional injections at 2-wk intervals. They were exsanguinated by heart puncture 2 wk after the last injection, and the antisera were absorbed with normal rabbit serum, erythrocytes, and a small amount of bone marrow cells. The pooled antiserum killed 50% of rabbit thymocytes in the presence of guinea pig complement (by dye exclution), at a 1:250 dilution, and agglutinated thymocytes at 1:640 dilution.

Quantitation of Antibodies in the Serum.—The amount of anti-DNP antibody was measured by quantitative precipitation with DNP-BF (28) and by Farr's ammonium sulfate precipitation technique (ABC-33) using [^{131}I]DNP-BSA (29). The values obtained by both methods were so close that the ABC-33 method was used in most of the experiments. The amount of anticarrier antibodies was determined by quantitative precipitation.

Purification of Anti-DNP Antibodies.—Anti-DNP antibody was specifically purified from individual serums by adsorption to and elution from a DNP-CM cellulose immunoadsorbent column in the presence of 0.01 M EDTA pH 7.6. Antibody was eluted with 0.1 M DNP-OH. Hapten was removed by extensive dialysis against PBS in the presence of Amberlite IRA 400 in PBS. The eluate was concentrated by vacuum dialysis. Antibody concentration was estimated by nesslerization.

Measurement of Antibody Affinity.—The association constants (K_0) for the reaction of purified anti-DNP antibodies were measured by equilibrium dialysis using tritiated DNP-EACA ([^{3}H]DNP-EACA) as ligand, according to the method described by Eisen (30). 0.2-ml samples of specifically purified antibody in PBS pH 7.6 (about 100 μg/ml) were placed inside the dialysis chamber which was made of 15 mm $\times$ 3 mm Tygon tube (Norton Chemical Process Prods Div., Akron, Ohio) covered with cellophane membranes on both sides. Dialysis was carried out at 37°C for 48 h (constant shaking) in a large volume (about 100 ml) with various concentrations of the ligand. 0.1 ml of the samples from inside and outside of the dialysis chamber were collected, dissolved in 1 ml of Soluene (Packard Instrument Co., Downers Grove, Ill.), and diluted with 10 ml of scintillation liquid. The radioactivity in the samples was counted in a Beckman LS-100 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.). Average intrinsic association constants (K_0) were calculated from equilibrium dialysis data (31, 32). The standard free energy change ($-\Delta F^\circ$) for the reaction between antibody and hapten was calculated from the equilibrium constant (K_0) by the use of the usual thermodynamic relationship

$$-\Delta F^\circ = RT \ln K_0$$

where R is gas constant, T is absolute temperature, and $\ln K_0$ is the natural log of the average intrinsic association for the reaction.

Thymectomy.—Litters of young adult rabbits at 1 kg body weight were divided into two groups. One group was thymectomized under anesthesia with pentobarbital. The thymus was removed through a median sternotomy, taking care to remove the last piece and not to damage the pericardium or pleura. The other group was sham thymectomized by cutting open the thorax and immediately suturing the wound closed. The animals were kept for about 3 wk, and when body weights reached more than 1.5 kg each group was immunized as described below.

Experimental Design.—Four separate experiments were designed (Fig. 1) to learn the regulatory influence of the thymus on antihapten antibody formation. In experiment 1, nine thymectomized and nine sham-thymectomized rabbits were immunized with 500 μg of DNP-BGG in CFA divided among the four footpads. They were boosted monthly with 500 μg of DNP-BGG in CFA in the back muscles. Sera were taken at 15, 30, 90, and 150 days after the start of immunization for measurement of antibody concentration and affinity.

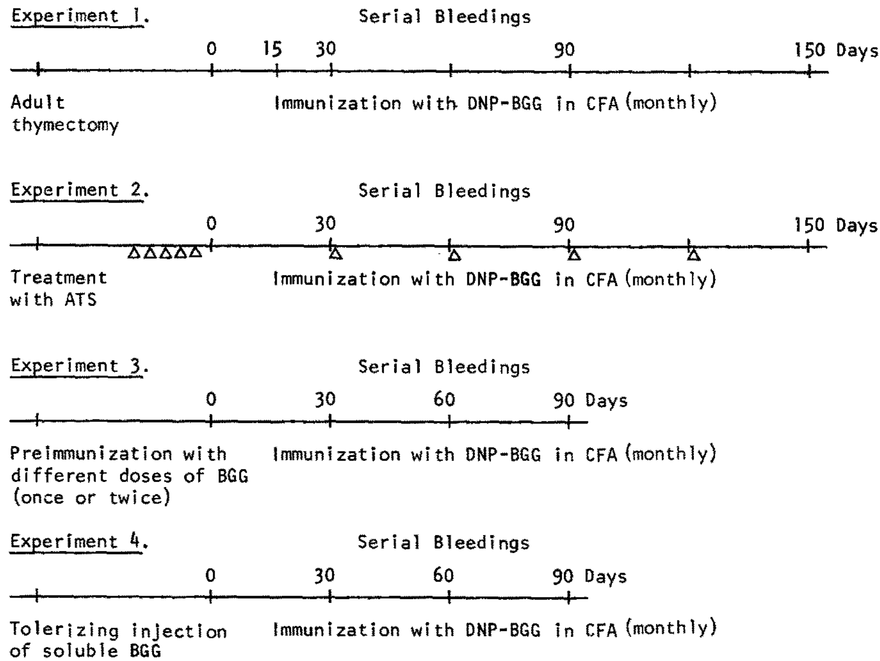

FIG. 1. Experimental protocols.

In experiment 2, a group of five adult rabbits weighing about 2 kg was treated with five consecutive daily injections of 2 ml of ATS intramusculary before the immunization was started. Animals were immunized according to the same schedule as in experiment 1, consisting of monthly injections of 500 μg of DNP-BGG in CFA. Additional monthly injections of 2 ml of ATS were given to each experimental rabbit 1 day before the boosting injections of DNP-BGG. Anti-DNP antibody concentration and affinity were measured at 30, 90, and 150 days after immunization was started.

Experiment 3 was designed to study the effect of preimmunization with different doses of the carrier molecule. Groups of four rabbits were preimmunized with different doses of unconjugated BGG in CFA 2 wk before the immunization with DNP-BGG was started. The priming dose of unconjugated carrier ranged from 5 μg to 500 μg. Four control rabbits were given no BGG. All groups were then immunized with monthly injections of 500 μg of DNP-BGG in CFA.

To see the effect of more profound carrier immunization, a separate group of rabbits was preimmunized 2 wk apart with two injections of 500 μg of BGG in CFA, and then was given DNP-BGG 2 wk after the second BGG injection. As a control for this experiment, a group of rabbits was preimmunized with an unrelated antigen, HSA (500 μg in CFA given twice 2 wk apart), and then immunized with DNP-BGG by the same schedule. The immunization with DNP-BGG in CFA was performed monthly as in experiment 1, and the amount of anti-DNP antibody and its affinity were measured at 30, 60, and 90 days after the start of immunization with DNP-BGG (see Fig. 1).

In experiment 4, adult animals were partially tolerized with unconjugated carrier by the intravenous injection of 0.5–500 mg of soluble BGG 1 mo before the immunization with DNP-BGG. These injections resulted in the production of only small quantities of antibody after subsequent immunization with DNP-BGG 1 mo later (see below). They were thus con-

sidered to be "tolerizing" rather than priming. Such rabbits, partially tolerized with the carrier, were immunized with monthly injections of 500 μg of DNP-BGG in CFA. Sera taken at 30, 60, and 90 days after the first immunization with DNP-BGG were examined for the amount and affinity of anti-DNP antibody (see Fig. 1).

RESULTS

Enhancement of Hapten-Specific Antibody Responses by Adult Thymectomy (Experiment 1).—A group of nine rabbits thymectomized in young adulthood was immunized with monthly injections of DNP-BGG in CFA, and the amount and affinity of anti-DNP antibody produced were compared with those of the sham-thymectomized control. Fig. 2 shows the kinetics of anti-DNP antibody

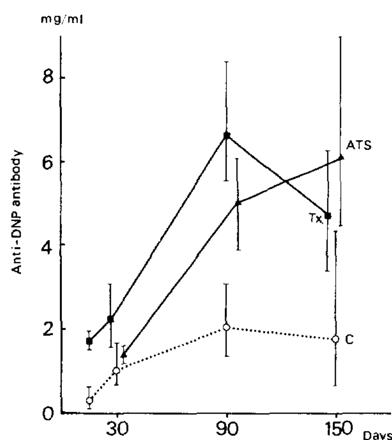

FIG. 2. Anti-DNP antibody response in adult thymectomized (Tx, ■—■), ATS-treated (▲—▲), and sham-thymectomized control (C, ○····○) rabbits immunized with monthly injections of 500 μg of DNP-BGG in CFA. Each point and bracket are the geometric mean and standard deviation of five to nine similarly treated animals.

formation in adult thymectomized rabbits, as measured by ABC-33, in comparison with those in the sham-thymectomized control. The amount of anti-DNP antibody produced in all nine thymectomized rabbits was much higher than that of controls on day 15. The amount further increased between 30 and 90 days (Fig. 2) and, though falling by day 150, remained well above control values. The mean amount of antibody produced in the thymectomized group on day 90 was about four times higher than that of the controls (Table I).

Changes in affinity of the anti-DNP antibody produced in the thymectomized and control groups are shown in Fig. 3. Up until day 15, the affinity values of thymectomized rabbits were lower than those of the controls, whereas after day 15 the affinity of antibodies from thymectomized animals consistently increased to values significantly higher than that in sham-thymectomized animals. After day 30, values in the control group, by contrast, actually decreased. As shown in Table I, the difference in the association constants (K_0)

TABLE I

Anti-DNP Antibody and its Hapten-Binding Affinity in Sham-Thymectomized, Thymectomized, and ATS-Treated Rabbits 90 Days after the Start of Immunization with DNP-BGG

Treatment	No. of animals	Anti-DNP antibody*		Affinity* (K_0)
		ABC-33	Quant. P‡	
		mg/ml	*mg/ml*	$\times 10^{-6}$ *liters/mol*
Sham thymectomized	9	1.88 (1.13–3.13)	1.65 (1.09–2.50)	6.5 (3.1–13.6)
Thymectomized	9	6.81 (4.92–9.44)	6.55 (5.48–8.39)	44.7 (18.6–107.6)
ATS treated	5	4.99 (3.77–6.63)	4.81 (3.49–6.05)	13.5 (5.8–31.3)

* Geometric means, lower and upper limits of standard deviations.
‡ P, precipitation.

between those two groups was about one log scale on day 90, which amounted to a difference of about 1.2 kcal/mol in free energy change ($-\Delta F°$), a margin that was even more striking on day 150 (2.2 kcal/mol).

Enhancement of Hapten-Specific Antibody Responses by Treatment with ATS (Experiment 2).—In this experiment, animals were treated with ATS instead of by adult thymectomy. Animals that were extensively treated with the lymphopenic dose of ATS and then immunized with monthly injections of DNP-BGG in CFA produced greater amounts of anti-DNP antibody than did controls, especially in the later days of immunization (Fig. 2). Table I includes the results obtained in ATS-treated animals on day 90, and shows that the average amount of anti-DNP antibody in ATS-treated animals was about 2.5 times higher than those of the controls, both by quantitative precipitation and ABC-33. Also in accord with the results of experiment 1, antibody affinity of the group was lower than that of the controls until 30 days, after which it kept increasing (Fig. 3). The difference in $-\Delta F°$ between the control and ATS-treated group on day 90 amounted to about 0.5 kcal/mol, but on day 150 was 1.9 kcal/mol, being comparable to that observed between thymectomized and sham-thymectomized groups (Fig. 3, Table I).

Enhancement and Suppression of Hapten-Specific Antibody Responses by Carrier Preimmunization (Experiment 3).—The above findings indicated that the relative diminution of T cells resulting from adult thymectomy or ATS treatment caused enhanced production of high affinity hapten-specific antibody. These results suggested that T cells suppressed the antibody response, and that the relative depletion of such suppressor T cells resulted in an overproliferation of antibody-forming cells. In order to test whether such postulated regulation by T cells is effected by antigen-specific stimulation, experiment 3 was performed, testing the effect of preimmunization by the carrier protein, BGG, on the subsequent antibody response to DNP-BGG. Groups of four rabbits each were primed with various doses of BGG in CFA given 2 wk before the start of immunization with monthly injections of DNP-BGG in CFA. A control

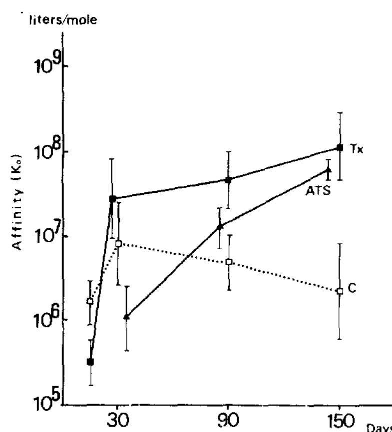

FIG. 3. Antibody affinity of anti-DNP antibodies produced by adult thymectomized (Tx, ■—■), ATS-treated (▲—▲), and sham-thymectomized control (C, ○····○) rabbits immunized with monthly injections of 500 μg of DNP-BGG during the 150-day period. Each point and bracket are the geometric mean and standard deviation of five to nine similarly treated rabbits.

group of four rabbits was not preimmunized with BGG, but received the same monthly injections of DNP-BGG.

As shown in Fig. 4, rabbits that had been preimmunized with unconjugated carrier showed either enhanced or suppressed production of antihapten antibodies, depending on the priming doses of BGG. Animals preimmunized with 5 μg of BGG produced markedly larger amounts of anti-DNP antibody than the control unprimed animals, although the antibody affinities were in the same range. Animals preimmunized with 50 μg of BGG, however, showed no significant difference from controls in the amount of antibody produced on day 90, although its affinity was a half log lower than that of the controls (Table II). Such a difference in free energy change ($-\Delta F°$) was calculated to be 0.53 kcal/mol. By contrast, animals preimmunized with 500 μg of BGG produced slightly less amounts of anti-DNP antibody after subsequent immunization with DNP-BGG in CFA, and the affinity of antibody produced was significantly lower than that of the control. The difference in free energy change ($-\Delta F°$) between this and the control group amounted to 0.82 kcal/mol. Thus it appeared that animals primed with a small dose of BGG produced a markedly enhanced and more mature antihapten antibody response, whereas priming with larger doses of BGG suppressed both amounts and affinities of antibodies produced (Table II).

In order to confirm the carrier effect on the regulation of antihapten antibody response and its maturation, a group of four rabbits was treated with two successive injections of 500 μg of BGG in CFA, given 2 wk apart. 2 wk later they were immunized with DNP-BGG. A control group was pretreated with two

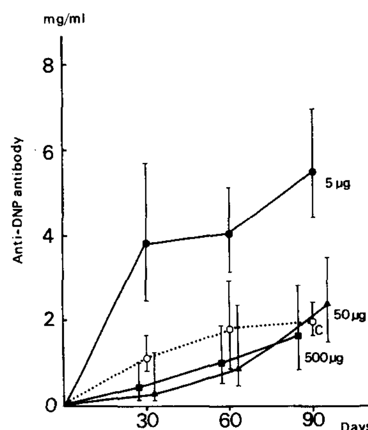

FIG. 4. Anti-DNP antibody responses in rabbits preimmunized with different doses of unconjugated carrier (BGG) 2 wk before the start of monthly injections of DNP-BGG in CFA. The doses of BGG for priming are shown on the right side of the graph. Control animals (C, ○···· ○) were not primed with BGG. Each point and bracket are the geometric mean and standard deviation.

TABLE II

Enhancement and Suppression of Anti-DNP Antibody Formation by Preimmunization with Different Doses of BGG 90 Days after the Start of Immunization with DNP-BGG

Preimmunization*	No. of animals	Anti-DNP antibody‡		Anti-BGG antibody‡	
		ABC-33	Affinity (K_0)	Day 0	Day 90
		mg/ml	× 10^{-6} *liters/mol*	*mg/ml*	*mg/ml*
None	4	1.91 (1.71–2.33)	7.7 (6.2–9.6)	0	0.64 (0.33–1.31)
BGG in CFA					
50 μg	4	5.56 (4.45–6.95)	6.8 (6.3–7.4)	0.31 (0.20–0.47)	3.2 (2.31–4.66)
50 μg	4	2.43 (1.54–3.81)	3.1 (2.0–4.9)	0.78 (0.58–1.04)	1.84 (0.65–5.21)
500 μg	4	1.49 (0.78–2.87)	1.9 (1.0–3.5)	1.49 (1.30–2.78)	6.15 (4.20–8.94)

* Immunized with indicated doses of BGG 2 wk before the start of immunization with DNP-BGG.

‡ Geometric means, lower and upper limits of standard deviations.

injections of an unrelated antigen, HSA, in CFA. At this time members of both groups were producing considerable amounts of anticarrier antibodies.

The results of the above experiment (Fig. 5) confirm the findings of the first part of experiment 3. Pretreatment with BGG in CFA produced markedly smaller amounts of lower affinity anti-DNP antibody than were found in control rabbits pretreated with unrelated antigen. As shown in Table III, on day 30 the amount of anti-DNP antibody in the BGG-preimmunized group was only $\frac{1}{10}$th that in the HSA-preimmunized group and its affinity was more than

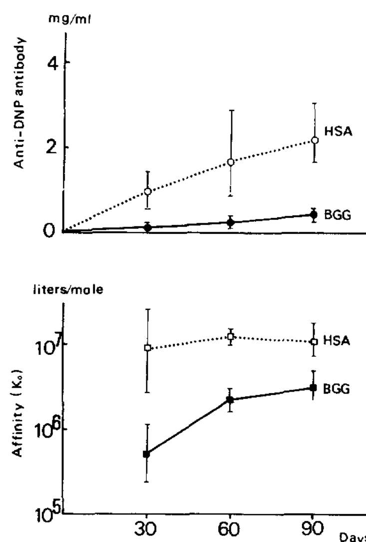

FIG. 5. The amount (upper panel) and affinity (lower panel) of anti-DNP antibodies produced in rabbits preimmunized with two injections of homologous carrier (BGG, ●—● ■—■) or heterologous carrier (HSA, ○····○ □····□) in CFA. Animals were given monthly injections of DNP-BGG in CFA 2 wk after the second injection of BGG or HSA. Each point and bracket are geometric mean and standard deviation of four similarly treated animals.

TABLE III

Effect of Hyperimmunization with Homologous and Heterologous Carriers on Anti-DNP Antibody Formation by Subsequent Immunization 30 Days after the Immunization with DNP-BGG in CFA

Pre-immunization*	No. of animals	Anti-DNP antibody‡		Anti-BGG antibody‡	
		ABC-33	Affinity (K_0)	Day 0	Day 30
		mg/ml	$\times$ *10^{-6} liters/mol*	*mg/ml*	*mg/ml*
HSA	4	0.94 (0.60–1.48)	9.0 (2.8–23.1)	0	0.18 (0.13–0.21)
BGG	4	0.09 (0.01–0.17)	0.5 (0.2–1.2)	3.01 (1.27–7.27)	6.50 (2.74–10.24)

* Immunized with two injections of 0.5 mg of proteins 2 wk apart and then immunized with 0.5 mg of DNP-BGG in CFA.

‡ Geometric means, lower and upper limits of standard deviations.

one log scale lower. This difference in free energy change ($-\Delta F°$) was about 1.7 kcal/mol.

All of the BGG-preimmunized rabbits in experiment 3 showed a substantial anti-BGG secondary response upon immunization with DNP-BGG (Tables II and III). However, no significant correlation was observed between anti-BGG secondary response and DNP-specific antibody response.

Enhancement and Suppression of Hapten-Specific Antibody Response by Partial Tolerance to the Carrier (Experiment 4).—Since carrier preimmunization produced dose-dependent effects on subsequent antihapten antibody responses, the effect of tolerizing injections of the carrier on the antihapten antibody was tested. Two groups of rabbits were given a single intravenous injection of 0.5 mg and 500 mg of soluble BGG. Although a few rabbits produced minute amounts of anti-BGG antibody, barely detectable only by interfacial ring precipitation test 30 days after the intravenous injection, in no case did they produce secondary anti-BGG antibody responses after subsequent immunization with DNP-BGG (Table IV). Thus, it was considered that these animals were partially tolerant to the carrier molecule. In response to a single injection of DNP-BGG in CFA (given 30 days after the tolerizing injection of soluble BGG), these animals produced only small amounts of anti-DNP antibody. However, after second and third injections of DNP-BGG, the group given 0.5 mg of soluble BGG produced almost as much anti-DNP antibody as the untreated controls. On the other hand, three of the five animals pretreated with 500 mg of soluble BGG produced only minute amounts of anti-DNP antibody throughout the 90-day course (Fig. 6, 500 mg [*b*]). By contrast, the other two rabbits in this group showed a rapid increase in anti-DNP antibody after the third injections of DNP-BGG, which rose to about 8 mg/ml (Fig. 6 and Table IV, 500 mg [*a*]).

The affinities of anti-DNP antibodies produced in these groups are shown in Table IV. The association constants of the group pretreated with 0.5 mg of soluble BGG and those of two enhanced rabbits (500 mg [*a*]) were similar to each other and only slightly lower than those of the controls, but the amount of anti-DNP antibody in the suppressed group was about $\frac{1}{100}$th that of the

TABLE IV

Enhancement and Suppression of Anti-DNP Antibody Formation by Tolerizing Injection of Soluble BGG 90 Days after the Start of Immunization with DNP-BGG

Tolerizing injection*	No. of animals	Anti-DNP antibody‡		Anti-BGG antibody‡	
		ABC-33	Affinity (K_0)	Day 0	Day 90
		mg/ml	$\times 10^{-6}$ *liters/mol*	*mg/ml*	*mg/ml*
None	4	1.91 (1.72–2.33)	7.7 (6.2–9.6)	0	0.64 (0.33–1.31)
Soluble BGG (i.v.)					
0.5 mg	4	1.59 (1.18–2.14)	4.5 (3.9–5.3)	<0.1	<0.1
500 mg(*a*)§	2	8.34 (7.46–9.12)	4.5 (4.4–4.7)	<0.1	<0.1
500 mg(*b*)‖	3	0.024 (0.02–0.03)	0.7 (0.5–0.9)	<0.1	<0.1

* Given 30 days before the start of immunization with DNP-BGG in CFA.
‡ Geometric means, lower and upper limits of standard deviations.
§ Enhanced rabbits.
‖ Suppressed rabbits.

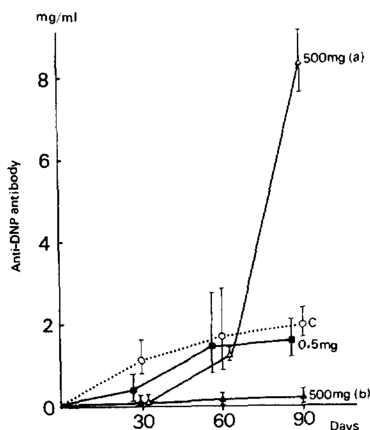

FIG. 6. Anti-DNP antibody responses in rabbits given a tolerizing injection of different doses of soluble BGG 1 mo before the start of monthly immunization with DNP-BGG in CFA. The dose of soluble BGG given to each group is shown on the right side of the graph. Control animals (C, ○····○) were given no tolerizing BGG. Animals given 500 mg of BGG responded in two different ways: two of five animals produced a large amount of anti-DNP antibody (500 mg [*a*]), but the other three rabbits produced a minute amount (500 mg [*b*]).

controls, and its affinity was about $\frac{1}{10}$th of the control value. The difference in $-\Delta F^{\circ}$ between these groups was 1.5 kcal/mol.

DISCUSSION

Data reported here suggest a dual role for thymus-derived lymphocytes in the antibody response and maturation in the rabbit. The first two experiments clearly demonstrated that the relative diminution of T cells, caused either by surgical thymectomy in early adulthood or by treatment with ATS, significantly enhanced the antibody response to a hapten-carrier conjugate, and that the enhancement was associated with a marked increase in affinity of the antibody produced. It appears therefore that both the amount and affinity of antibody are, at least in part, influenced by the number of T cells present in immunized animals, and that a large number of T cells can suppress both these attributes.

Furthermore, experiments 3 and 4 suggested that such a regulatory effect of T cells may be antigen specific. This was shown by two different lines of evidence: (*a*) Preimmunization with carrier molecules either enhanced or suppressed the subsequent antibody response to a hapten on the same carrier, depending on the preimmunization dose of carrier. It similarly altered the average affinities of the antibodies produced. (*b*) A tolerizing injection of a large dose of carrier depressed that antihapten antibody response in some animals, while the same dose markedly enhanced the response of other animals. Such alterations in the quantity of the antibody response were also associated with changes, similar in direction, in antibody affinity. These results suggest

that the number of primed carrier-specific cells, possibly of thymic origin, influences the emergence and maturation of hapten-specific antibody-forming cell precursors.

These results are only partly in agreement with reports by other investigators. Adult thymectomy in the rabbit has been reported to have variable effects on humoral antibody responses in a short-term study (1–3), but thymectomy of rats markedly enhanced and prolonged their production of IgE homocytotropic antibody against a hapten-carrier conjugate (22). The usual effect of ATS treatment in vivo is to depress the humoral antibody response to a variety of antigens (33), but in certain circumstances it enhances antibody formation against some antigens (25, 34, 35). Resolution of these conflicting findings may be obtained from the concept that T cells exert two opposite functions, depending on the experimental conditions: a well-established positive effect as helper cells in some humoral antibody responses, and a suppressive influence on similar humoral antibody responses (23, 24, 36–40). The relative depression of T-cell numbers by surgical, chemical, or immunochemical means might have permitted synthesis of more antibody if a critical supply of helper cells was preserved, while the number of suppressor cells was effectively depressed.

Of more interest in our present studies is the increase in binding affinities of antibodies produced in relatively T-cell-deprived animals. Recently, Gershon and Paul (21) clearly demonstrated an influence of T cells on antibody affinity. They reported that adult-thymectomized, lethally irradiated, bone marrow-reconstituted mice produced only small amounts of antibody of low affinity when immunized with DNP-BSA in the presence of a small number (0.33×10^8) of syngeneic thymocytes. The passive transfer of a somewhat larger number (1×10^8) of thymocytes into recipient mice resulted in an increase of both amount and affinity of antibody produced. On the other hand, when such animals were immunized with DNP-keyhole limpet hemocyanin and were given 0.33×10^8 thymocytes, they produced normal amounts of anti-DNP antibody with affinity comparable to that of control mice. The authors suggested that T cells are important in increasing the rate of change in the precursors of antibody-forming cells, upon which antigen-driven selection operates. The present results support their idea that T cells regulate the stimulation of precursors of antibody-forming cells by antigen, although our results indicate a depression rather than enhancement of antibody affinities. We believe that our results do not necessarily contradict those of Gershon and Paul (21), if we assume that T cells may either facilitate or suppress the proliferation and differentiation of antigen-stimulated B cells. The lack of such suppression by T cells may have allowed the increases of both amount and affinity of the antibody seen in thymectomized and ATS-treated animals, who perhaps retained enough T cells to serve as helper cells.

The second part of our results concerns the effects of priming and tolerizing with carrier molecules on the antihapten antibody response. Katz et al. (10)

reported that the primary antihapten antibody response of rabbits and guinea pigs to a hapten coupled to a carrier was enhanced if the animals had been preimmunized with small doses of free carrier. Carrier preimmunization had no effect on the affinity of the antihapten antibody, however. A similar enhancing effect of carrier preimmunization on an in vitro antihapten antibody response in mice was reported by Falkoff and Kettman (41) using trinitrophenyl as a hapten and sheep red blood cell (SRBC) as a carrier. In these experiments the enhancing effect was dependent on the dose of carrier used for priming. Only small doses of carrier, suboptimal for the induction of a primary anticarrier antibody response, were effective in enhancing subsequent hapten-specific antibody responses to hapten-carrier conjugate. Grantham (42) also reported that the secondary response of mice to SRBC was significantly enhanced by a low dose priming rather than by high dose priming with SRBC. More recently, Ishizaka and Okudaira (43) studied the relationship between the dose of carrier used for preimmunization of mice and the magnitude of hapten-specific IgG and IgE antibody responses produced by immunization with a hapten-carrier conjugate. They also found that only small doses of carrier could effectively enhance the anti-DNP antibody responses of both classes; doses that were optimal or supraoptimal for inducing anticarrier antibody suppressed the antibody responses. Our previous study on the rat antihapten IgE antibody response (24) also demonstrated a definite suppressive effect of carrier preimmunization. Since in the present studies the secondary anticarrier response occurred in all cases, suppression by carrier preimmunization is not considered to be due to the lack of carrier-specific helper cells. Therefore, it seems reasonable to conclude that only in suitable numbers can carrier-specific cells effectively cooperate with hapten-specific antibody-forming cell precursors to induce antihapten antibody, while larger numbers may suppress stimulation of the latter cell type.

Although Katz et al. (10) found no change in affinity after preimmunization of rabbits with 1–50 μg of BGG, our results clearly demonstrated that 500 μg of BGG decreased the average association constant of rabbit antihapten antibody produced by subsequent immunizations with DNP-BGG. This effect was more striking when animals were preimmunized with two injections of 500 μg BGG. On the other hand, preimmunization with 5 μg of BGG had no effect on the affinity of anti-DNP antibody, although it significantly elevated the quantities produced. These results suggest that high affinity cells are more sensitive than low affinity cells to the suppressive effects of carrier-specific cells.

Since the preimmunized animals had moderate to large amounts of anticarrier antibodies in their circulation, one might reasonably argue that anticarrier antibody was responsible for the observed suppression. At the present time this possibility cannot be denied, but we prefer to think that carrier-specific cells are responsible for this suppression, for the following reasons: (*a*) There was no strict correlation between the amount of circulating anticarrier antibody and the degree of suppression (Tables II and III), and in some cases antihapten

antibody was even enhanced in the presence of moderate amounts of anticarrier antibody. (*b*) In the presence of moderate to large amounts of circulating anticarrier antibody, secondary anticarrier antibody responses were never suppressed. Thus, the helper function of carrier-specific helper cells was not blocked by anticarrier antibody. (*c*) In the suppressed groups, affinity for DNP of the antibody produced was lowered; if the circulating anticarrier antibody had reacted with antigen to diminish the effective antigen concentration available to antibody-forming precursor cells, the affinity of antibody produced should have increased rather than decreased (18). Moreover, antibody-mediated suppression is, in most cases, determinant specific (44, 45) although some investigators reported that anticarrier antibody can suppress the production of antihapten antibody as well (46). Thus, at the present time it is reasonable to assume that carrier-specific cells in large numbers may have inhibited the immune response to hapten coupled to the homologous carrier, and that the same carrier-primed cells in optimal numbers may have facilitated the B-cell response as suggested by Katz et al. (47).

The last experiment demonstrated that partial tolerance induced in carrier-specific cells significantly diminished the antibody response to the hapten-homologous carrier complex. Such suppression was also accompanied by a marked decrease in the antibody affinity. Some of the tolerized animals suddenly began producing large amounts of antihapten antibody late in the course of immunization. This burst of activity was accompanied by a quick recovery of antibody affinity. The observed suppression by the tolerizing injection of the carrier may have resulted from a lack of helper activity in carrier-specific cells (24, 48). Since the tolerance was induced by carrier and not by hapten, the observed suppression cannot be explained by lack of hapten-specific antibody-forming cell precursors, and therefore the decrease in antibody affinity was not due to hapten-specific tolerance (49). Termination of carrier-specific tolerance would, at least transiently, result in the appearance of a suitable number of carrier-specific helper cells to allow prompt production of hapten-specific antibody. The somewhat lower affinity of such antibody, despite its higher amount, may be due to a slow start of B-cell response or to inactivation of high affinity cells by the hapten under a circumstance in which helper cells had been absent.

Although it is still not determined whether the carrier-specific cells heretofore mentioned are actually thymus derived, the above results give reason to assume that these cells may be at least functionally identical to carrier-specific T cells found in other species. Our simplified conclusions are as follows: (*a*) Relative diminution of T cells resulted in an increase in both amount and affinity of antibody produced by immunization with a hapten-carrier conjugate; (*b*) Overstimulation of T cells by the carrier caused a suppression of antihapten antibody formation and a decrease in antibody affinity; (*c*) The presence of an optimal number of carrier-stimulated T cells is necessary for production of antihapten antibody response and for the increase in antibody affinity. These

conclusions imply that T cells have a dual regulatory role on the maturation of the immune response, although the possibility that helper and suppressor functions are conducted by different cell types cannot be denied, especially in view of the recognized heterogeneity among T cells (50) and the fact that carrier-specific B cells as well as T cells coexist.

It has been well documented by Siskind and Benacerraf (20) that maturation of the immune response is explained in terms of the thermodynamically driven selection of B cells by antigen and is hence primarily determined by the concentration of the antigen in the microenvironment of B cells. B cells with higher affinity antigen receptors can more easily capture the antigen, allowing them to proliferate and differentiate into antibody-forming cells. Recent studies by Davie and Paul (51, 52) presented direct evidence that antibody maturation results from the continued proliferation of higher affinity cells after an immunization, while the number of cells with lower affinity falls rapidly. However, recent advances in cellular immunology argue that selection by antigen is not by itself enough to stimulate antibody-forming cell presursors, and that "helper" T cells are necessary for maximal stimulation of B cells (53–55). Mediators driving this cellular interaction have recently been reported by several authors (56–61). A most important point, considered critical to the regulatory influence of T cells on antibody maturation, is how the T cells influence selection of B cells by an antigen. Gershon and Paul (10) as well as Katz and Benacerraf (62) hypothesized that T cells indirectly influence B cell selection, independently of an antigen-driven mechanism, by increasing the rate of proliferation and accelerating the change of the B-cell population leading to more rapid appearance of B cells bearing higher affinity receptors. We would propose a somewhat different hypothesis in which a more direct action of T cells is considered. By the simple selection theory, cells with higher affinity receptors would be expected to be more easily selected by antigen. However, such selected cells would not proliferate and differentiate into antibody-forming cells without the help of antigen-stimulated T cells. If an optimal number of T cells is present, they would specifically cooperate with B cells which had already been selected by the antigen, and the latter would now proliferate and synthesize antibody with the destined affinity. Therefore, the T cells' help would preferentially be directed to B cells which easily capture the antigen, leading to production of high affinity antibody by their progeny. In this manner optimal numbers of T cells would facilitate the maturation of immune response.

On the other hand, supraoptimal numbers of antigen-stimulated T cells would "inactivate" the B cells that had already been selected by antigen. Thus B cells with higher affinity receptors would be more easily influenced by T cells' suppressive activity, leaving low affinity cells relatively unaffected and leading ultimately to synthesis of low affinity antibody. This interpretation is in accord with the selection theory of Siskind and Benacerraf (20) and further permits

an explanation for the decrease in affinity after long-term immunization. A proof at the cellular level for the preferential suppression of high affinity cells by carrier-specific T cells is in progress in our laboratory.

SUMMARY

Rabbits thymectomized in early adulthood produced more antihapten antibody than sham-thymectomized controls after hyperimmunization with 2,4-dinitrophenyl bovine gamma globulin (DNP-BGG). The average associated constant of anti-DNP antibody produced by thymectomized animals was more than 10 times higher than that of the controls. Similar effects were obtained by extensive treatment of rabbits with antithymocyte serum (ATS) before and during the immunization with DNP-BGG. The results indicated that relative diminution of thymus-derived lymphocytes (T cells) resulted in a stimulation of antibody-forming cells with a higher affinity.

On the other hand, preimmunization of rabbits with different doses of BGG caused either enhancement or suppression of the hapten-specific antibody response, depending on the priming dose of BGG. The suppressed antibody response was always associated with a marked decrease in the antibody affinity. If rabbits were partially tolerized with a large dose of soluble BGG, some of the animals produced little antibody against hapten (DNP) coupled to this carrier, and the affinity of produced antibody was low. However, other rabbits tolerized with BGG produced large amounts of anti-DNP antibody upon hyperimmunization with DNP-BGG, whose affinity was only slightly lower than that of the control.

These results can be harmonized if it is assumed that the thymus plays an important role in the maturation of the immune response. It is postulated that T cells, in numbers ordinarily available, would first assist in the proliferation of antihapten antibody-forming cell precursors already selected by antigen, thus accounting for the rapid increase of antibody affinity in the early stage of immunization. However, after a larger number of carrier-specific T cells are made in response to continued immunization, these would suppress antibody-forming cells. The suppression would be greater for cells with higher affinity for antigen, resulting in a decrease in antibody affinity. This postulate explains preferential stimulation and suppression of cells having higher affinity receptors under circumstances in which T cell are relatively depleted or overstimulated, and further permits an explanation for the decrease of antibody affinity after long-term immunization.

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