DIFFERENCES IN THE MECHANISM OF TOLERANCE TO DINITROPHENYLATED BOVINE GAMMA GLOBULIN WHEN INDUCED IN NORMAL ADULT MICE OR IN RECONSTITUTED IRRADIATED MICE: DEPENDENCE OF THE MECHANISM OF TOLERANCE ON THE STRUCTURAL ORGANIZATION OF THE LYMPHOID SYSTEM*

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In recent years it has become apparent that a variety of distinct mechanisms can lead to a state of specific immunologic tolerance. Clonal deletion of T and/or B lymphocytes (1), activation of specific suppressor T lymphocytes (2), and the production of small amounts of high affinity antibody (3) are among the well-defined mechanisms of immunologic unresponsiveness. At least two types of B-lymphocyte tolerance (reversible and irreversible) plus peripheral neutralization of antibody ("treadmill hypothesis") have been definitively shown to operate in tolerance to T-independent antigens (4). In addition, recent studies have suggested that the interaction of antigen with antibody-producing B lymphocytes can lead to a decrease in the rate of antibody secretion (effector cell blockade) (5) and that anti-idiotype antibody might be capable of mediating tolerance (6). Other than the observations of Weigle et al. (1) that higher doses of antigen are required for tolerance induction in B lymphocytes than in T lymphocytes, relatively little is known as to what factors determine which mechanism will be operative in any given unresponsive state.

In the present study it was found that the same procedure for tolerance induction, when applied to normal intact adult mice and to reconstituted lethally irradiated mice, led to unresponsive states having distinctly different properties and clearly mediated by different mechanisms. The intravenous injection of a modest dose of dinitrophenylated bovine gamma globulin (DNP-BGG)[1] induces an antibody-mediated tolerance state in intact mice. The same procedure applied to irradiated mice reconstituted with B cells alone or with T and B cells leads to a B-cell clonal deletion-type tolerance. The findings emphasize that the outcome of antigen exposure is determined by complex, highly

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[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; DNFB, 1-fluoro-2,4-dinitrobenzene; DNP-BGG, dinitrophenylated bovine gamma globulin; EACA, ϵ-amino-*N*-caproic acid; HBSS, Hanks' basic salt solution containing 0.02 mg/ml sodium heparin sulfate; PBS, phosphate-buffered saline; PFC, plaque-forming cells.

structured interrelationships within the normal lymphoid system. The application of observations on cell transfer or tissue culture systems to the intact animal must consequently be carried out with considerable caution.

Materials and Methods

Mice. 6- to 8-wk-old LAF_1 male mice (The Jackson Laboratory, Bar Harbor, Maine) were used.

Antigens and Haptens. The 2,4-dinitrophenyl (DNP) derivative of BGG (Miles-Yeda, Kankakee, Ill.) was prepared by the reaction of 1-fluoro-2,4-dinitrobenzene (DNFB; Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) with the protein under alkaline conditions essentially as described by Eisen et al. (7). The conjugated protein was purified by extensive dialysis against 0.001 M phosphate buffer, pH 7.4. The concentration of the product was determined from its "dry weight" and its degree of hapten substitution was estimated from its absorbancy at 360 nm (ϵ for DNP-lysine was taken at 17,400). A single preparation of DNP-BGG was used which had an estimated 44 DNP groups per molecule of protein. DNP-ϵ-amino-*N*-caproic acid (DNP-EACA) was prepared by the reaction of DNFB with EACA (Sigma Chemical Co., St. Louis, Mo.) under alkaline conditions and was purified by repeated crystallization from hot water as described previously (8).

Tolerance Induction. Tolerance was induced by a single intravenous injection of 0.5 mg DNP-BGG dissolved in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M K phosphate buffer, pH 7.4). Although the antigen preparation employed for tolerance induction was soluble, it should be noted that it was highly coupled with hapten, and was therefore undoubtedly significantly denatured. It was not cleared of aggregated material before use. Animals were challenged with DNP-BGG in complete Freund's adjuvant (CFA) 1 wk after tolerance induction.

Immunization. Mice were immunized by the intraperitoneal injection of 400 μg DNP-BGG-emulsified CFA (containing 1.5 mg/ml *Mycobacterium butyricum*) so as to be in a final vol of 0.2 ml. Animals were sacrificed by cervical dislocation and their spleens assayed for plaque-forming cells (PFC) 13 or 20 days after antigen injection.

Cell Transfers. Transfer studies were carried out in thymectomized, lethally irradiated mice (800 R from a gamma cell 40; Atomic Energy of Canada, Ltd.). The mice were reconstituted with syngeneic adult bone marrow or spleen cells which had been treated with anti-brain θ-antiserum plus complement (C). In addition some of the animals received 1×10^8 syngeneic adult thymus cells. The anti-brain θ-antiserum was prepared by immunizing rabbits with CBA/J mouse brain in CFA as described by Golub (9). The antiserum was found to be specific for T cells in that at a dilution of 1:80, in the presence of a 1:3 dilution of fresh guinea pig serum which had been absorbed with 8% agarose, it was cytotoxic for 95% of mouse thymocytes and less than 5% of mouse bone marrow cells. It behaved similar to an authentic anti-θ-antiserum in cytotoxic assays on spleen and lymph nodes. In each experiment "B cells" (anti-brain θ-antiserum plus C-treated bone marrow or spleen) and thymus cells from several donors were pooled so that a reasonably constant population of cells was given to experimental and control animals. Cells were obtained by teasing the tissue in Hanks' basic salt solution, pH 7.2, containing 0.02 mg/ml sodium heparin sulfate (HBSS; Grand Island Biological Co., Grand Island, N. Y.). The cells were filtered through a thin layer of cotton gauze to remove clumps, were washed once with HBSS, and were resuspended in HBSS for injection. The cells were injected intravenously 2–4 h after lethal irradiation. Recipients received either $5–7 \times 10^7$ bone marrow cells or the equivalent of the cells from one spleen (approximately 10^8 nucleated cells). In those experiments where the recipients were to receive thymus cells, they were added to the bone marrow or spleen cell suspension before injection. Each recipient received either none or 1×10^8 thymus cells. The tolerance-inducing injection of antigen was given 1 day after cell transfer and challenge immunization was carried out 7 days later. In those studies in which tolerance was induced in the absence of T lymphocytes, all animals received 1×10^8 thymus cells intravenously 1 day before challenge immunization. If thymus cells were not given, essentially no anti-DNP PFC were seen after immunization with DNP-BGG in CFA.

Assay and Number and Avidity of Anti-DNP PFC. Splenic anti-DNP PFC were determined by the method of Jerne et al. (10) as modified for slide assay by Dresser and Greaves (11). Washed sheep red blood cells (SRBC) were conjugated by the Rittenberg and Pratt method (12) with 2,4,6-trinitrobenzene sulfonic acid (Sigma Chemical Co.). The slides were incubated for 1 h at 37°C. Freshly frozen guinea pig serum, absorbed with SRBC, was added as a source of C at a predeter-

TABLE I
*T-Cell Dependence of the Anti-DNP PFC Response to DNP-BGG**

Experiment	Reconstitution (no. of Mice)	Indirect Anti-DNP PFC/spleen	
1	BMθ (6)	650 ± 44	($P < 0.02$)
	BMθ + thymus (4)	3,863 ± 2,363	
2	BMθ (6)	33 ± 52	($P < 0.004$)
	BMθ + thymus (5)	20,560 ± 58	

* Thymectomized lethally irradiated mice were reconstituted with 5–7 × 10^7 syngeneic adult bone marrow cells which had been treated with anti-brain-θ and C (BMθ). Where indicated, the animals also received 1 × 10^8 syngeneic thymus cells. 1 day after cell transfer, all mice were immunized with 400 μg DNP-BGG in CFA, intraperitoneally. The anti-DNP PFC in the spleen were assay 13 days (exp. 1) or 20 days (exp. 2) after immunization. The results are expressed as arithmetic mean ± standard deviation of four to six animals in each group. The Mann-Whitney "U" test was employed to evaluate the significance of the difference in the number of PFC in the two groups. Two representative experiments are presented.

mined optimal dilution (1/30) and the slides were incubated for an additional 45 min. Rabbit anti-mouse gamma globulin was used at the predetermined optimal dilution (1/200) to develop indirect plaques.

The avidity distribution of the anti-DNP PFC was assayed by the inhibition of plaque formation using various concentrations of DNP-EACA, essentially according to the method of Andersson (13) as validated by previous work (14, 15). Concentrations of DNP-EACA ranging from 1 × 10^{-9} to 1 × 10^{-5} M, in half-log increments, were used. Details of the PFC assay, as performed in our laboratory, were presented in an earlier publication (16).

Results

T-Cell Dependence of the Anti-DNP PFC Response to DNP-BGG. In Table I is illustrated the anti-DNP PFC response 13 and 20 days after DNP-BGG immunization of lethally irradiated, thymectomized mice reconstituted with syngeneic bone marrow which had been treated with anti-brain θ-antiserum and C. Half of the animals also received 1 × 10^8 syngeneic thymus cells. Clearly, DNP-BGG is a thymic-dependent antigen. It is also clear that 10^8 adult thymus cells provide sufficient T-lymphocyte helper activity to support a marked immune response.

Induction of Tolerance in the Absence of T Lymphocytes. We have previously shown (3) that tolerance to DNP-BGG could be induced in adult mice by a single intravenous injection of 0.5 mg DNP-BGG. Evidence was offered which was consistent with the hypothesis that this tolerant state was mediated by the production of small amounts of high affinity antibody in response to the tolerance-inducing injection of antigen. Since DNP-BGG is a highly thymic-dependent antigen, it might be expected that induction of tolerance by this mechanism would be dependent upon the presence of helper T lymphocytes. The T-cell dependence of tolerance induction to DNP-BGG in adult mice was, therefore, studied in a cell transfer system. Lethally irradiated, thymectomized mice were reconstituted with anti-brain θ-antiserum-treated bone marrow with or without 1 × 10^8 thymus cells. 1 day after cell transfer, half of each group received 0.5 mg DNP-BGG in PBS intravenously. 1 wk thereafter all animals received 1 × 10^8 thymus cells intravenously and were immunized 24 h later with DNP-BGG in CFA. The anti-DNP PFC response was assayed 13 days after immunization.

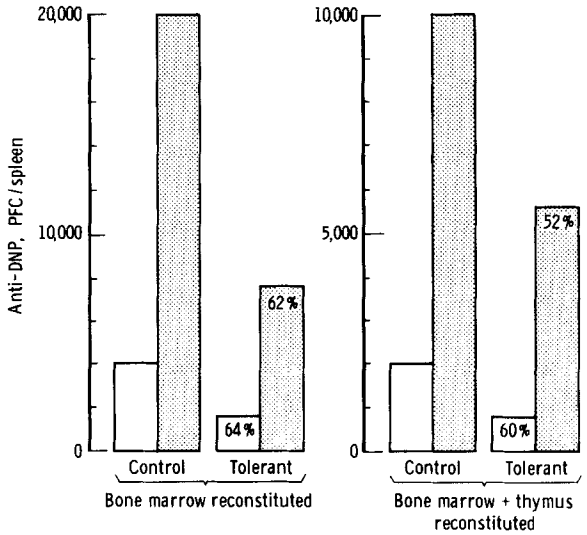

FIG. 1. Induction of tolerance to a thymic-dependent antigen in the presence and absence of thymus cells. Irradiated, thymectomized mice were reconstituted with anti-brain θ-antiserum-treated syngeneic bone marrow cells together with or without 1×10^8 syngeneic thymus cells. 1 day after cell transfer half of the animals received a tolerance-inducing injection of 0.5 mg DNP-BGG in PBS intravenously. 5 days later all animals received 1×10^8 syngeneic adult thymus cells and were immunized with 400 μg DNP-BGG in CFA, intraperitoneally. Splenic anti-DNP PFC were assayed 13 days after immunization. Direct anti-DNP PFC are indicated by the open columns and indirect anti-DNP PFC by the solid columns. The number in each column indicates the percent depression relative to the appropriate control. The results are expressed as the arithmetic mean of the data from five independent experiments each of which contained four to six animals per group.

As was the case with the intact animals previously studied (3), a single intravenous injection of 0.5 mg DNP-BGG resulted in a marked reduction in the number of both direct and indirect anti-DNP PFC (Fig. 1). It is apparent from the data that the same degree of tolerance was induced in the animals which received thymus cells before tolerance induction as in those animals in which tolerance was induced in the absence of T lymphocytes. Thus, T lymphocytes were neither required for tolerance induction nor did they prevent tolerance induction in this system. It should be noted that the control animals which had received two injections of 1×10^8 syngeneic adult thymus cells showed a marked reduction in the number of anti-DNP PFC as compared to controls which received only a single injection of thymus cells. This suggests the presence of nonspecific suppressor cell activity in the thymus cell population used for reconstitution. Since the lack of T-cell dependence would cast doubt upon an antibody-mediated mechanism for tolerance to DNP-BGG, it was viewed as important to characterize the cellular basis of the tolerant state induced in the reconstituted irradiated animals and to compare it with the unresponsive state induced, by the identical procedure, in intact mice.

Effect of Tolerance on the Avidity of Anti-DNP PFC. The effect of partial tolerance on the distribution of avidities of residual PFC was studied when tolerance was induced in intact normal mice and when tolerance was induced in reconstituted irradiated animals. Reconstituted, irradiated mice made tolerant

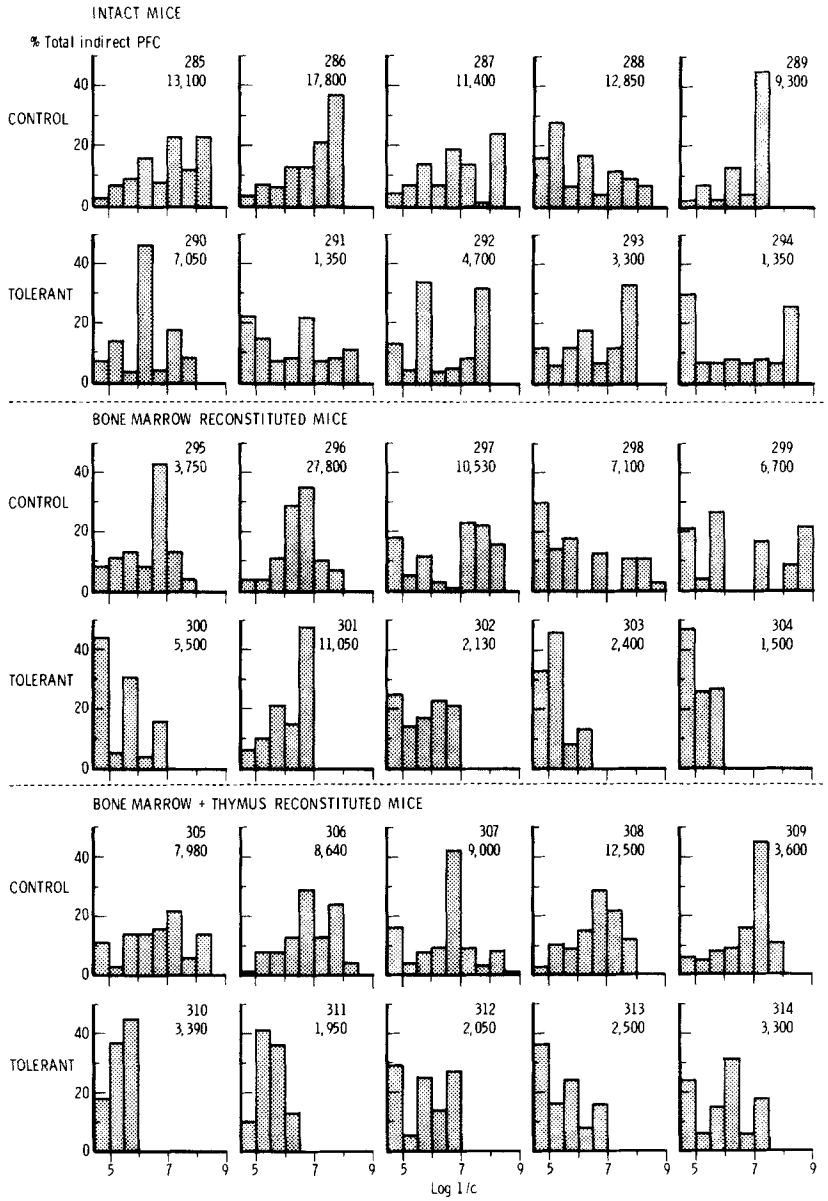

FIG. 2. Effect of partial tolerance on antibody avidity. Each histogram illustrates the distribution of indirect anti-DNP PFC with respect to avidity in the spleen of an individual mouse. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. The animal identification number (top) and total indirect PFC per spleen are given in the upper right corner of each histogram. Avidity increases to the right. "Tolerant" mice had received a tolerance-inducing injection of 0.5 mg DNP-BGG intravenously. "Controls" were injected with saline. 5 days later all animals were immunized with 400 μg DNP-BGG in CFA intraperitoneally. The anti-DNP PFC in the spleen were assayed 20 days after immunization. The top two rows illustrate data on tolerance induction in intact adult mice. The middle two rows illustrate data on tolerance induction in irradiated, thymectomized mice reconstituted with anti-brain θ-antiserum-treated bone marrow cells. (Animals received 1×10^8 syngeneic thymus cells 1 day before immunization with DNP-BGG in CFA.) The bottom two rows illustrate data on tolerance induction in irradiated, thymectomized mice reconstituted with anti-brain θ-antiserum-treated bone marrow cells plus 1×10^8 syngeneic thymus cells. The tolerance-inducing injection of antigen was given 1 day after reconstitution.

TABLE II

*Effect of Partial Tolerance on Antibody Avidity and Heterogeneity**

Experimental animals	Time between reconstitution and tolerance induction	Treatment (no. of mice)	% Depression of PFC in tolerant mice	Avidity‡ $K_{50\%}$ ($\times 10^6$)	Heterogeneity index§	
	days					
Intact	–	Control (4)	72 ($P < 0.008$)	9.1	2.40 ± 0.57	($0.6 < P < 0.7$)
	–	Tolerant (5)		2.2	2.54 ± 0.25	
Irradiated, thymectomized, reconstituted with BMθ	1	Control (4)	60 ($P < 0.028$)	5.7	2.48 ± 0.13	($P < 0.01$)
	1	Tolerant (5)		0.4	1.88 ± 0.29	
Irradiated, thymectomized, reconstituted with BMθ and thymus	1	Control (5)	68 ($P < 0.008$)	7.8	2.59 ± 0.17	($P < 0.01$)
	1	Tolerant (5)		0.5	1.99 ± 0.35	
Irradiated, thymectomized, reconstituted with spleenθ	14	Control (5)	50 ($P < 0.008$)	7.2	2.96 ± 0.22	($P < 0.01$)
	14	Tolerant (4)		0.9	2.13 ± 0.35	
Irradiated, thymectomized, reconstituted with spleenθ and thymus	14	Control (6)	62 ($P < 0.032$)	5.0	2.54 ± 0.29	($0.2 < P < 0.3$)
	14	Tolerant (5)		1.7	2.76 ± 0.26	

* Intact mice or lethally irradiated thymectomized mice reconstituted as indicated in the first column were used. 1 or 14 days later, as indicated in the second column, half of the animals received a tolerance-inducing injection of 0.5 mg DNP-BGG intravenously while the remaining mice were used as controls. 1 day before immunization, tolerant or control mice which had been reconstituted only with B cells were injected intravenously with 1×10^6 syngeneic thymus cells. 5 days after tolerance induction all animals were immunized with 400 μg DNP-BGG in CFA intraperitoneally and the anti-DNP PFC in the spleens were assayed 20 days after immunization. The animals are the same as those whose PFC avidity distributions are illustrated in Figs. 2 and 4. The Mann-Whitney "U" test was employed to evaluate statistical significance of the depression in PFC and a "T" test was used to evaluate 'significance of differences in the heterogeneity indices.

‡ Geometric mean of the reciprocal of the concentration of hapten required for 50% inhibition of the number of PFC.

§ The Shannon heterogeneity index (31) was used to describe the degree of heterogeneity of avidity of the PFC population of individual animals. The average value, ± standard deviation, of this index for each of the experimental groups is presented. The larger the index, the greater the heterogeneity.

by the intravenous injection of 0.5 mg DNP-BGG showed a marked reduction in the avidity of their residual anti-DNP PFC as compared with control animals which did not receive tolerogen (Fig. 2 and Table II). A similar decrease in avidity was obtained whether tolerance induction took place in the presence or absence of thymus cells. In marked contrast, intact animals made tolerant by the same procedure had PFC of approximately the same high avidity and heterogeneity of avidity as did the controls (Fig. 2 and Table II). These findings suggested that the state of tolerance induced in an adoptive cell transfer system is mediated by a different mechanism from that operating in the intact animal. A decrease in affinity as a result of partial tolerance is most typical of unresponsiveness due to B-cell clonal deletion or specific suppressor T-cell activity (17–21). High affinity residual antibody production by tolerant animals would suggest an antibody-mediated tolerance (3).

Stability of Tolerance upon Cell Transfer. The stability upon cell transfer of the tolerance induced in intact mice, and in reconstituted irradiated mice, was compared. Spleen cells obtained 5 days after tolerance induction were transferred into irradiated recipients which were immunized with DNP-BGG in CFA 1 day later. Controls received spleen cells from similarly reconstituted or intact mice which had not been rendered tolerant. It is clear from Table III that

TABLE III

*Stability of Unresponsiveness upon Transfer of Spleen Cells from Tolerant Mice into Irradiated Recipients**

Primary recipients		Secondary recipients				
			Anti-DNP PFC/spleen			
Preparation	Treatment	No.	Direct PFC		Indirect PFC	
Irradiated, thymectomized, reconstituted with BMθ and thymus	Control	17	9,520	($P < 0.002$)	20,120	($P < 0.002$)
	Tolerant	19	490		2,880	
Irradiated, reconstituted with spleenθ and thymus	Control	9	6,520	($P < 0.008$)	11,740	($P < 0.008$)
	Tolerant	9	1,200		2,050	
Intact	Control	13	5,170	($0.50 < P < 0.60$)	15,480	($0.50 < P < 0.60$)
	Tolerant	15	4,660		17,080	

* Lethally irradiated, thymectomized or just irradiated mice were reconstituted as indicated in the first column. Animals received 5–7 × 10^7 anti-brain θ-treated bone marrow cells or 7 × 10^7 anti-brain θ-treated spleen cells as indicated. 1 day after cell transfer half of the mice were injected intravenously with a tolerance-inducing dose of 0.5 mg DNP-BGG. Intact adult mice were treated in a similar manner. Controls did not receive the tolerance-inducing injection of antigen. 5 days after tolerance induction, spleen cells from these tolerant and control mice were transferred into lethally irradiated, syngeneic recipients. All secondary recipients were immunized with 400 μg DNP-BGG in CFA, intraperitoneally, 1 day after cell transfer. The anti-DNP PFC in the spleens were assayed 13 days after immunization. The results are expressed as the geometric means of the data from two to four independent experiments each of which contained four to five animals per group. The Mann-Whitney "U" test was employed to evaluate statistical significance.

tolerance induced in reconstituted irradiated animals was stable upon transfer of spleen cells into a second irradiated recipient. In contrast, spleen cells from intact mice which had been rendered tolerant to DNP-BGG gave a PFC response identical in magnitude to that of recipients of normal spleen cells. Thus, the tolerant state induced in intact animals was not stable on cell transfer.

Lack of Evidence for Increased Specific Suppressor Activity in Tolerant Reconstituted Irradiated Mice. The possibility that the stable tolerant state induced in reconstituted irradiated mice was mediated by suppressor T cells was evaluated by use of the usual mixed cell transfer system in which irradiated mice are reconstituted with spleen cells from either tolerant mice or normal mice, or with a mixture of cells from tolerant and normal animals. The results are illustrated in Fig. 3. It is clear that the mixed cell transfer did not demonstrate any increased suppressor activity in the spleen of reconstituted irradiated mice rendered tolerant in the presence of T lymphocytes. The tolerant state was stable on transfer of spleen cells from tolerant donors into irradiated recipients.

Evidence Supporting a B-Cell Clonal Deletion Mechanism for Tolerance Induced in Reconstituted Irradiated Mice. Mixed cell transfer experiments were employed to define the cellular basis for the tolerant state induced in reconstituted irradiated mice. The results are summarized in Table IV. Tolerance induced in irradiated mice which were reconstituted with anti-brain θ-treated spleen cells was stable on cell transfer. Normal thymus cells could not restore normal immune reactivity. Tolerant spleen cells did not suppress the reactivity of normal spleen cells on mixed cell transfer. In addition, it was found that tolerance induced in irradiated mice which were reconstituted with anti-brain θ-treated spleen cells plus normal thymus cells was also stable on cell transfer. Normal thymus cells failed to restore immune reactivity on mixed cell transfer. In contrast, anti-brain θ-treated normal spleen did restore normal reactivity to recipients of the tolerant spleen cell population. The results are

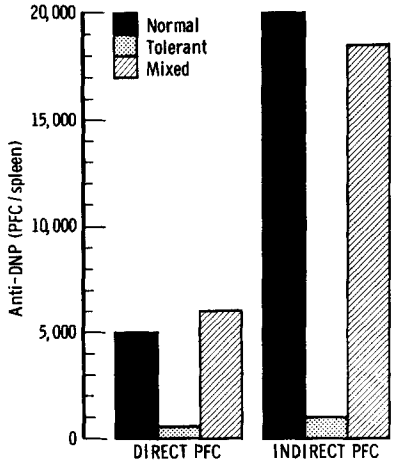

FIG. 3. Failure to demonstrate increased suppressor cell activity in spleens from tolerant reconstituted mice. Irradiated, thymectomized mice reconstituted with anti-brain θ-antiserum-treated syngeneic bone marrow plus syngeneic thymus cells were made tolerant by the intravenous injection of 0.5 mg DNP-BGG in PBS. 5 days later spleen cells from either these tolerant animals or normal mice or an equal mixture of spleen cells from tolerant and normal animals were injected intravenously into irradiated syngeneic recipients. Each recipient received the equivalent of one half a spleen from a tolerant or a normal donor or one half of a spleen from a tolerant donor plus one half of a spleen from a normal donor. Recipients were immunized with 400 μg DNP-BGG in CFA 1 day after cell transfer and splenic anti-DNP PFC were determined 13 days later. The data illustrated are from one representative experiment out of four independent experiments. The data are expressed as the arithmetic mean of PFC assays on groups of four to five mice.

thus consistent with the interpretation that the tolerant state induced in reconstituted irradiated mice is a B-cell clonal deletion. This appears to be true both in animals reconstituted with B and T cells and in animals reconstituted with only B cells.

Restoration of a "Normal" Response to Tolerance Induction in Reconstituted Irradiated Mice. It is clear that the mechanism of tolerance induced by the intravenous injection of 0.5 mg DNP-BGG is different in intact and in reconstituted irradiated mice. Previous studies (3) suggest that the unresponsive state induced by this procedure in intact adult mice is mediated by serum antibody operating through a suppression type mechanism (22). Based upon the results presented here it would seem that the tolerance induced in reconstituted, irradiated mice is the result of a B-cell clonal deletion. This appears to be the mechanism of tolerance in reconstituted mice whether T lymphocytes are present or absent when the tolerance-inducing injection of antigen is given. It was hypothesized that different tolerance mechanisms were activated in the different experimental models as a result of differences in the "internal microenvironment" of these animals. One would expect that given sufficient time, the transferred cells in a reconstituted, irradiated recipient would restore a normal "internal microenvironment" and the reconstituted animal would then behave like a normal intact mouse with respect to tolerance induction. Lethally irradiated, thymectomized mice were reconstituted with anti-brain θ-antiserum-treated syngeneic spleen cells. Half of the animals also received syngeneic

TABLE IV

*Characterization of the Cellular Basis of Tolerance Induced in Reconstituted Irradiated, Thymectomized Mice**

Tolerant spleen donors	Cells used for reconstitution (no. of mice assayed)	Anti-DNP PFC/spleen	
		Direct PFC	Indirect PFC
None	Normal spleen (9)	13,350	25,230
	Anti-brain θ-treated normal spleen (5)	544	903
Irradiated, thymectomized, mice reconstituted with anti-brain θ-treated spleen cells	Tolerant spleen (5)	1,697	3,794
	Tolerant spleen + normal thymus (5)	988	2,345
	Tolerant spleen + normal spleen (5)	15,210	23,680
Irradiated, thymectomized, mice reconstituted with normal thymus cells and anti-brain θ-treated spleen cells	Tolerant spleen (5)	1,139	3,233
	Tolerant spleen + normal thymus (5)	1,843	3,803
	Tolerant spleen + anti-brain θ-treated normal spleen (5)	11,100	22,130

* Lethally irradiated, thymectomized mice were reconstituted with 7×10^7 anti-brain θ-treated syngeneic spleen cells together with or without 1×10^8 syngeneic thymus cells. 1 day after cell transfer all animals were tolerized with a single intravenous injection of 0.5 mg DNP-BGG. 5 days later animals were sacrificed and the tolerant spleens were either injected into irradiated mice or were mixed with normal spleen cells or anti-brain θ-treated spleen cells or 1×10^8 normal thymus cells and were injected into irradiated mice. The type of tolerant spleen cell donor used is indicated in the first column (left) and the cell mixture used for reconstitution of the secondary recipients is indicated in the second column. Secondary recipients received the equivalent of one tolerant spleen and when indicated $7\text{–}10 \times 10^7$ normal spleen cells. Groups of irradiated mice were also reconstituted with 1×10^8 normal spleen cells or with 7×10^7 anti-brain θ-treated normal spleen cells. All of the secondary recipients were immunized with 400 μg DNP-BGG in CFA and the anti-DNP PFC in their spleens were assayed 13 days later. The data presented are from one of two experiments which yielded similar results. The data are expressed as geometric means of five to nine animals in each group, as indicated in parentheses.

thymus cells. 2 wk after cell transfer those mice which were to be rendered tolerant received 0.5 mg DNP-BGG intravenously. Control animals did not receive the tolerizing injection of antigen. 5 days after tolerance induction half of each group of mice was immunized with DNP-BGG in CFA. These animals were assayed 20 days later for the number and avidity distribution of their splenic anti-DNP PFC. The other half of each group was sacrificed at day 5 after tolerance induction and their spleens transferred into lethally irradiated mice. Recipients were immunized with DNP-BGG in CFA 1 day after cell transfer and assayed for anti-DNP PFC 13 days later. In all cases where T cells were not given at the time of reconstitution the animals received 1×10^8 thymus cells 1 day before challenge with antigen in CFA. The results are presented in Fig. 4 and Tables II and V. It is clear that when 2 wk are allowed to elapse before tolerance induction, irradiated mice reconstituted with spleen and thymus cells behave like normal intact animals. The avidities of the anti-DNP PFC of control and tolerant animals are roughly equivalent. In addition, the tolerance induced

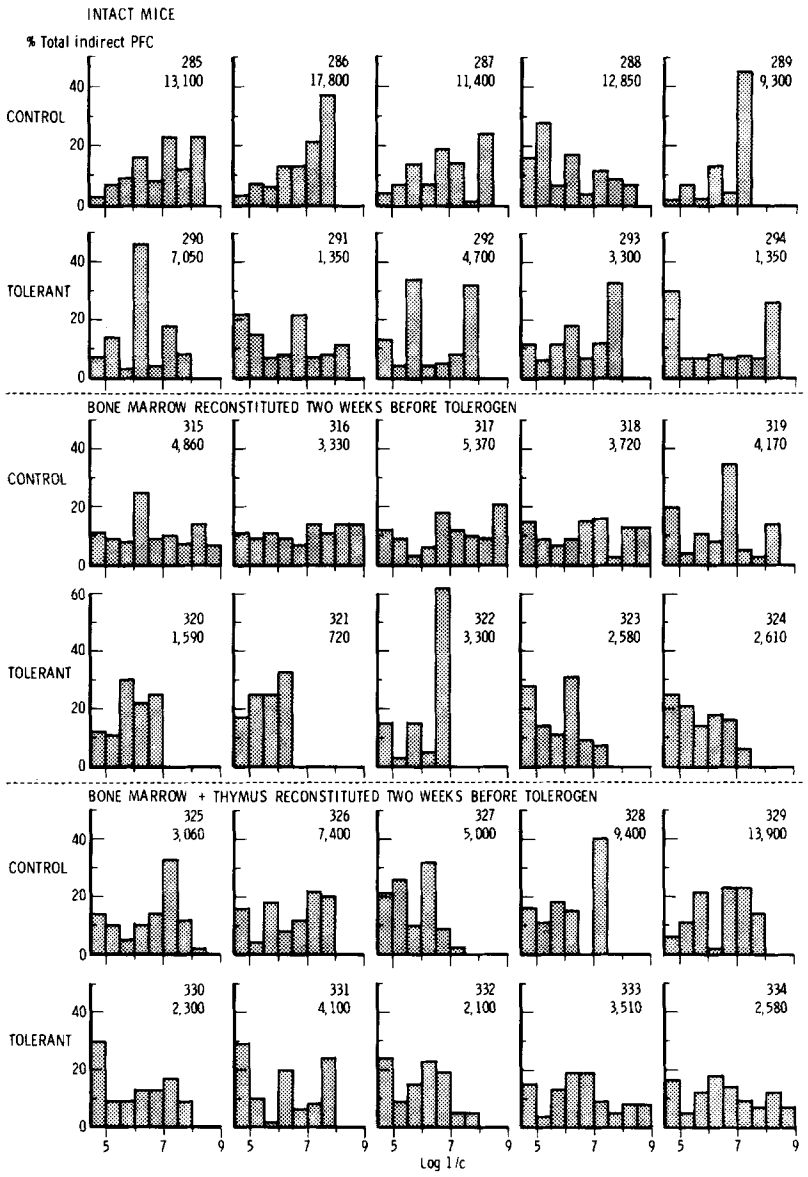

FIG. 4. Restoration of a "normal" response to tolerance induction 2 wk after reconstitution of irradiated, thymectomized mice with syngeneic anti-brain θ-antiserum-treated spleen and normal thymus cells. Each histogram illustrates the distribution of indirect anti-DNP PFC with respect to avidity in the spleen of an individual mouse. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. The animal identification number (top) and total indirect PFC per spleen are given in the right upper corner of each histogram. Avidity increases to the right. "Tolerant" mice received a tolerance-inducing injection of 0.5 mg DNP-BGG in PBS intravenously 2 wk after reconstitution. "Controls" received saline. 5 days later all animals were immunized with 400 μg DNP-BGG in CFA intraperitoneally. The anti-DNP PFC in the spleens were assayed 20 days after immunization. The top two rows illustrate data on tolerance induction in intact adult mice. These data are the same as were presented in Fig. 2 and are repeated here only to facilitate comparisons. The middle two rows illustrate data on tolerance induction in irradiated, thymectomized mice reconstituted with anti-brain θ-antiserum-treated spleen cells 2 wk before tolerance induction. (Animals received 1×10^8 thymus cells 1 day before immunization with DNP-BGG in CFA.) The bottom two rows illustrate data on tolerance induction in irradiated, thymectomized mice reconstituted with anti-brain θ-antiserum-treated spleen cells plus 1×10^8 thymus cells.

TABLE V

*Recovery of Normal Response to the Intravenous Injection of DNP-BGG 2 wk after Reconstitution of Irradiated Mice**

Experiment no.	Primary Recipients		Secondary Recipients				
				Anti-DNP PFC/spleen			
	Reconstituted with:	Treatment	No.	Direct PFC	(% Depression)	Indirect PFC	(% Depression)
1	Spleenθ	Control	4	4,125		7,225	
		Tolerant	2	2,700	(35)	4,850	(33)
	Spleenθ + thymus	Control	2	2,750		4,350	
		Tolerant	3	3,320	(0)	8,495	(0)
2	Spleenθ	Control	2	4,140		11,660	
		Tolerant	2	2,330	(44)	6,800	(42)
	Spleenθ + thymus	Control	5	12,100		21,920	
		Tolerant	5	12,670	(0)	27,140	(0)
3	Spleenθ	Control	4	16,850		48,360	
		Tolerant	5	11,660	(31)	17,350	(64)
	Spleenθ + thymus	Control	5	16,550		25,290	
		Tolerant	5	17,330	(0)	27,710	(0)

* Lethally irradiated, thymectomized mice (primary recipients) were reconstituted with 7×10^7 anti-brain θ-treated syngeneic spleen cells (spleenθ) with or without 1×10^8 syngeneic thymus cells. 2 wk were allowed to elapse before a tolerance-inducing injection of 0.5 mg DNP-BGG was given intravenously to half of the animals. Primary recipients which were not injected with DNP/BGG served as controls. 5 days after tolerance induction, spleen cells from the tolerant mice or from the control mice were injected intravenously into irradiated syngeneic recipients (secondary recipients). Spleen cells from tolerant or control mice which had been reconstituted only with spleenθ were mixed with 1×10^8 syngeneic thymus cells before injection into secondary recipients. All secondary recipients were immunized with 400 μg DNP-BGG in CFA, intraperitoneally, 1 day after cell transfer. The anti-DNP PFC in the spleen were assayed 13 days after immunization. The results are expressed as the geometric mean of the number of splenic anti-DNP PFC. Three independent experiments are presented.

under these conditions is unstable upon tranfser of spleen cells from unresponsive mice into irradiated recipients. In contrast, irradiated, thymectomized mice reconstituted only with anti-brain θ-treated spleen cells still behave like "reconstituted" mice with regard to tolerance induction 2 wk after reconstitution. That is, the unresponsive state is stable upon transfer of spleen cells from tolerant into irradiated mice and residual PFC in partially tolerant animals are of relatively low avidity. Thus, the recovery of a normal "internal microenvironment" appears to require the presence of thymus cells.

Discussion

Data have been presented in this paper which show that different mechanisms for tolerance operate in intact mice and in reconstituted, irradiated mice even though the same tolerance-inducing procedure was employed. The tolerant

state induced in intact mice was shown to be unstable on cell transfer and was characterized by a high avidity of residual PFC in partially tolerant animals. In addition, it was previously shown (3) that a low magnitude, but relatively high avidity PFC response occurs after the tolerance-inducing injection of antigen and that the carrier specificity of the tolerant state was identical to that of antibody-mediated immune suppression. We have previously suggested (3) that the properties of this tolerant state are consistent with the hypothesis that it is mediated by the suppressive effect of a small amount of high affinity antibody which is produced in response to the tolerance-inducing injection of antigen. On the other hand, the tolerant state induced in reconstituted, irradiated mice was shown here to be stable on cell transfer and to be characterized by a marked depression in the avidity of residual PFC in partially tolerant mice. Tolerance could be induced in reconstituted, irradiated animals in the presence or absence of thymic cells. That is, T lymphocytes neither prevented tolerance induction nor were necessary for tolerance induction in reconstituted, irradiated mice. No specific suppressor cell activity could be demonstrated in tolerant mice and normal thymus cells did not restore specific reactivity to tolerant spleen cells in mixed cell transfer experiments. Thus, the state of tolerance induced in reconstituted irradiated mice given 0.5 mg DNP-BGG intravenously has properties typical of a B-lymphocyte clonal deletion-type mechanism. This was true both when tolerance was induced in the presence or absence of thymus cells. It is thus clear that the state of tolerance induced in the reconstituted irradiated mice is distinctly different from that induced in intact mice by the same procedure. These findings suggest that the outcome of antigen presentation is determined not only by the nature, physical state, and dose of the antigen, but also by the detailed structural organization of the immune system. Thus, any theory of tolerance based simply upon the interaction of antigen with antigen receptors on lymphoid cells would clearly be inadequate to explain the difference between intact and reconstituted mice. In the reconstituted mice, the immune system is obviously disordered immediately after cell transfer. Under these conditions a B-cell clonal deletion-type tolerance appears to be induced by relatively low doses of antigen both in the presence and absence of T lymphocytes. With time (2 wk) after reconstitution with B and T lymphocytes the structural integrity of the lymphoid system is re-estabished and injection of 0.5 mg soluble DNP-BGG now leads to a state of depressed responsivenes which has properties similar to that induced by this antigen in intact adult mice. This was not the case for mice reconstituted with B cells in the absence of T lymphocytes. Thus, it would appear that re-establishment of the normal microenvironment of the lymphoid system only takes place in the presence of thymus-derived cells.

Varying results have been reported with regard to the effect of T lymphocytes on the induction of tolerance in B cells. While it is clearly possible to induce B-cell tolerance in the absence of T lymphocytes (23–25), some workers have suggested that the presence of T cells tends to protect B lymphocytes from tolerance induction (26–30). In the present studies it is apparent that B-cell tolerance can be induced in reconstituted mice which were thymic deficient. In addition, the degree of B-cell tolerance was not influenced by the presence of T lymphocytes during tolerance induction. The failure to obtain B-cell tolerance in intact mice cannot therefore be simply explained by the presence of T cells. It

would appear that the normal structural organization of the intact mouse is critical in avoiding B-cell tolerance induction after antigen exposure. In the reconstituted animal B cells are apparently exposed to antigen under altered local environmental conditions such that a clonal deletion-type tolerance is readily induced and an antibody-mediated unresponsive state therefore cannot be obtained.

Finally, it must be emphasized that in view of the demonstrated differences between tolerance induction in intact and reconstituted mice, it is necessary to use considerable caution when applying results obtained in cell transfer models to the intact animal. Similar caution must undoubtedly be raised in regard to studies in tissue culture where again the normal structural relationships between cellular elements are disrupted. At least some aspects of the cellular interactions involved in controlling the normal in vivo immune response would appear to depend upon the structural integrity of the local microenvironment. Thus, the ease of B-lymphocyte tolerance induction depends not only upon the intrinsic sensitivity of the individual B cells, but also upon the structural organization of the lymphoid tissues. The detailed structure of the lymphoid tissues may well prove to be of great importance in controlling tolerance induction in the intact animal.

Summary

Tolerance can be induced in adult mice by a single intravenous injection of 0.5 mg dinitrophenylated bovine gamma globulin. The cellular mechanism of the unresponsive state is different depending upon whether the tolerance is induced in normal intact adult mice or in reconstituted, irradiated mice. The tolerant state induced in intact mice is characterized by a high avidity of the residual antibody-forming cells in partially tolerant animals and a prompt reversibility on cell transfer. The overall properties of this unresponsive state are consistent with the hypothesis that it is mediated by the production of small amounts of high affinity antibody in response to the tolerance-inducing injection of antigen. In contrast, the unresponsiveness induced in reconstituted, irradiated mice by the same procedure was characterized by a low avidity of the residual antibody-forming cells in partially tolerant animals and stability on transfer of spleen cells from unresponsive into irradiated recipients. No suppressor cell activity was detected and mixed cell transfer studies were consistent with the view that this unresponsive state represented a B-lymphocyte clonal deletion. The presence or absence of T lymphocytes in the population of cells used for reconstituting the irradiated recipients did not effect the ease of tolerance induction or the cellular mechanism of the tolerant state which was produced. If irradiated mice reconstituted with B and T lymphocytes were rested for 2 wk before tolerance induction then a reversible "high affinity"-type tolerance is obtained such as is typical of normal intact animals. Restoration of a "normal" response to the tolerance-inducing injection of antigen is dependent upon the presence of thymus cells in the population of cells used for reconstitution. It is suggested that the structural integrity of the lymphoid tissue is critical in determining whether B cells will be rendered tolerant after exposure to antigen in vivo.

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