IMMUNOLOGICAL TOLERANCE TO A HAPTEN*

I. Induction and Maintenance of Tolerance to Trinitrophenyl with Trinitrobenzene Sulfonic Acid

By JOHN M. FIDLER‡ and EDWARD S. GOLUB§

(From the Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907)

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The state of immunological unresponsiveness[1] is a widely studied but little understood phenomenon. In the majority of cases, the tolerogenic antigen (tolerogen) is a complex molecule with many determinants, and is often capable of inducing immunity as well as tolerance (1). This is obviously the case with such antigens as heterologous erythrocytes, where the timing and duration of treatment determine the tolerogenicity of the antigen (2). With serum proteins, both immunogenic and tolerogenic forms are often present together (3, 4), further complicating matters. Even with potentially tolerogenic preparations of such materials, the induction of unresponsiveness depends upon the mode of administration.[2] The use of tolerogens with a single determinant would greatly simplify the investigation of the immune system as a whole and aid in the understanding of the cellular basis of immunological tolerance.

Havas (5) and Borel (6) have produced tolerance to a hapten by treatment of mice with haptenated serum proteins and Feldman (7) has induced tolerance to a hapten by using haptenated flagellin. We describe here and characterize the induction of tolerance by the injection of free hapten (8). This method has been investigated in terms of cell-mediated immunity and tolerance (9). While utilizing a hapten, this method of tolerization dispenses with the complication of large and complex carrier molecules. Use of the free hapten as a tolerogen has provided a simple method for the study of tolerance induction, maintenance,

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[1] The terms unresponsiveness and tolerance will be used interchangeably.

[2] Incorporated into incomplete Freund's adjuvant, soluble human gamma globulin (HGG)[3] induces immunity in adult BDF_1 mice, while inducing tolerance when given in the soluble fluid form (unpublished observations).

[3] *Abbreviations used in this paper:* B cell, thymus-independent bone marrow-derived antigen-sensitive cells; BSS, balanced salt solution; HA, hemagglutination titer; HGG, human gamma globulin; HRBC, horse erythrocytes; ME, 2-mercaptoethanol; PFC, plaque-forming cells; SRBC, sheep erythrocytes; T cells, thymus-derived antigen-reactive cells; 1,3,5-TNB, 1,3,5-trinitrobenzene; TNBS, trinitrobenzene sulfonic acid; TNP, 2,4,6-trinitrophenyl; TNP-glycine, trinitrophenyl glycine.

and termination in both the whole animal and at the cellular level. It has allowed us to investigate tolerance induction in B and T cells[4] as well as a possible T cell inhibitory substance in tolerance (10).

Materials and Methods

Mice.—BDF_1 [$(C57BL/6 \times DBA/2)F_1$] mice were from our breeding colony using breeding stocks obtained from the Jackson Laboratory, Bar Harbor, Maine, and were used at 8–12 wk of age. Mice were housed five to a cage and were maintained on Wayne Lab Blox (Allied Mills, Inc., Chicago, Ill.) and chlorinated (11 ppm) and acidified (pH 2.8) water ad lib. (11). Female mice were used unless otherwise noted. In individual experiments, all mice were generally born within the same 1 wk span.

Antigen.—Sheep erythrocytes (SRBC) from the same animal and horse erythrocytes (HRBC) were obtained in Alsever's solution from the Colorado Serum Company, Denver, Colo. SRBC heavily conjugated with 2,4,6-trinitrophenyl (TNP-SRBC) were utilized for immunization to the TNP hapten, and were prepared essentially by the method of Kettman and Dutton (12). In all cases TNP-SRBC were used immediately after preparation because of the high degree of instability. Lightly conjugated HRBC and SRBC were prepared essentially by the method of Rittenberg and Pratt (13). Lightly conjugated TNP-RBC were stable for at least 4 days. The standard immunizing dose for both primary and secondary response challenges of SRBC and TNP-SRBC was 2×10^8 erythrocytes (0.1 ml of 10% erythrocytes by volume in balanced salt solution [BSS] [14]) injected intraperitoneally.

Tolerogen.—Trinitrobenzene sulfonic acid (TNBS; Eastman Kodak Co., Rochester, N. Y.) was prepared for injection by dissolving 100 mg in 10 ml of 0.15 M NaCl plus 0.029 g of $NaHCO_3$. Each injection consisted of 0.5 ml of TNBS solution (5 mg of TNBS). Injections were intraperitoneal. In all cases where concentrations of TNBS other than 5 mg in 0.5 ml of saline were used, the amount and concentration of $NaHCO_3$ were altered appropriately.

Tolerogen Analogues.—Trinitrophenyl glycine (TNP-glycine; Sigma Chemical Co., St. Louis, Mo.) was prepared for injection by dissolving 34.0 mg of TNP-glycine plus 0.014 g of $NaHCO_3$ in 10 ml of 0.15 M NaCl. Each injection consisted of 1.0 ml of TNP-glycine solution (containing 3.4 mg of TNP-glycine), an amount that was equimolar in the TNP moiety with the standard 5 mg dose of TNBS. 1,3,5-trinitrobenzene (1,3,5-TNB; Eastman Kodak Co.) was prepared for injection by dissolving 20.7 mg of 1,3,5-TNB in 5 ml of 0.15 M NaCl that was 1% in Na_2SO_3 to aid in dissolving the material. Individual injections consisted of 0.5 ml of 1,3,5-TNB solution (containing 1.88 mg of 1,3,5-TNB), an amount that was equimolar in the TNP moiety with the standard 5 mg dose of TNBS. 1,3,5-TNB was obtained with water added. In calculations, 10% water was arbitrarily chosen as the degree of dilution.

Assay of Plaque-Forming Cells.—Antibody-forming cells were 19S plaque-forming cells (PFC) determined by the slide modification of the Jerne hemolytic plaque technique (15). PFC to TNP were determined by utilizing lightly conjugated TNP-HRBC. Horse cells exhibit less than 1% cross-reactivity with SRBC in the mouse. In our assay system, fewer than 1% of the TNP-HRBC PFC can be attributed to HRBC-directed PFC (unpublished observations) and PFC directed against TNP are not corrected for HRBC-directed PFC. PFC in each individual spleen to both the hapten and carrier were always determined at the same time. Indirect (7S, IgG) PFC were detected by using guinea pig complement (Hyland Laboratories, Los Angeles, Calif.) that was 1:200 in rabbit anti-mouse IgG (16, 17). The number of indirect (facilitated) PFC recorded is the difference between the direct unfacilitated PFC count and that revealed

[4] Chiscon, M. O., J. M. Fidler, and E. S. Golub. Immunological tolerance to a hapten. III. Induction of tolerance in B and T cells and their interaction in the response to hapten-carrier. Manuscript in preparation.

by using the amplifying antiserum. Primary response assays were conducted on day 5 after the injection of immunogen (unless otherwise indicated), while secondary response assays were conducted on day 4 after the second challenge with antigen.

Serum Collection.—Mice were bled from the lateral tail vein, and the separated serum stored at −15°C. Freezing was found to have no effect upon the 2-mercaptoethanol (ME)-resistant titer.

Serum Titrations.—Serum titrations were performed with a Takatsy microtiter apparatus (Cooke Engineering Co., Alexandria, Va.). Suspensions of thrice washed indicator erythrocytes (SRBC, HRBC, or lightly conjugated TNP-HRBC) 1.0% by volume in BSS were used. In all serum titration assays of the anti-TNP response, all sera were assayed against SRBC, TNP-HRBC, and HRBC. There was generally no detectable hemagglutination (HA) titer to the HRBC carrier in sera assayed against TNP-HRBC. The ME-resistant HA titers are 7S IgG (18, 19) while the ME-sensitive titers, as determined by subtracting the 7S titer from the total titer, represent the 19S IgM titers (20, 21).

RESULTS

Effect of Treatment with TNBS.—The injection of TNBS results in a state of immunological tolerance that is specific for the TNP hapten. This was demonstrated by treating groups of mice with TNBS (5 mg in saline) and challenging with an intraperitoneal injection of 2×10^8 TNP-SRBC after an appropriate interval. Fig. 1 and Table I show the results obtained with a 1 h interval between TNBS treatment and hapten-carrier challenge. No anti-TNP serum antibody nor PFC response was obtained. The anticarrier SRBC response was normal.

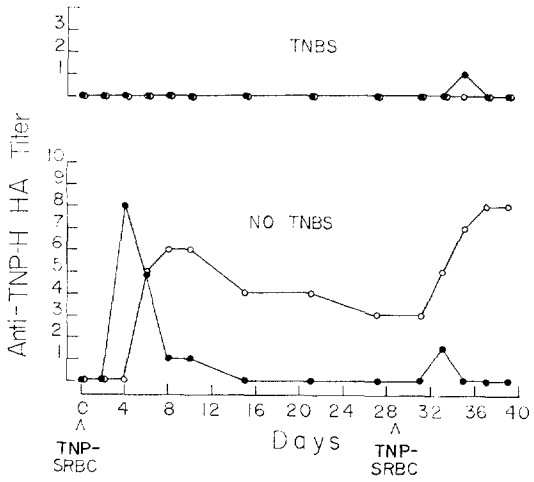

FIG. 1. Effect of TNBS treatment upon the anti-TNP serum antibody response to TNP-SRBC. Anti-TNP HA titer of treated (upper graph) and untreated control (lower graph) mice. Treated mice received 5.0 mg of TNBS in 0.5 ml of saline at −1 h. All mice were challenged with 2×10^8 TNP-SRBC i.p. on days 0 and 29 as indicated. 19S (●) and 7S (○) anti-TNP antibody HA titers were determined as described in Materials and Methods. Titers are from a pool of serum from five mice per group. Abscissa: days after initial TNP-SRBC challenge injection; ordinate: $\log_2$ anti-TNP HA titer.

TABLE I

Effect of TNBS Treatment upon the Immune Response to TNP-SRBC

Treatment	Anti-TNP PFC/10^6	Anti-SRBC PFC/10^6
TNBS*		
−1 hr	0 ± 0§	120.2 ± 20§
None‡	49.8 ± 5.2	127.8 ± 10.9

* An i.p. injection of 5.0 mg of TNBS in 0.5 ml of saline was given 1 h before an i.p. challenge injection of 2×10^8 TNP-SRBC.

‡ No treatment other than challenge with 2×10^8 TNP-SRBC was given.

§ Values represent the mean of five mice per group ± SE. Mice were assayed 5 days after challenge.

In addition to having no effect upon the anticarrier responses, TNBS had no significant effect upon the serum antibody or PFC response to unconjugated SRBC since the serum antibody and PFC response to SRBC in TNBS-treated and untreated mice was the same. Similarly, treatment of mice with TNBS from 1 to 4 days before an injection of SRBC had no effect upon the anti-SRBC response (data not shown). Furthermore, the injection of TNBS did not result in detectable PFC to TNP 5 days later, indicating that TNBS is purely tolerogenic.

Effect of Concentration of TNBS on the Induction of Tolerance to TNP-SRBC.—In order to further characterize the tolerogenic effects of TNBS, a dose-response experiment was conducted. Groups of 5–10 mice were treated with doses of TNBS ranging from 0.1 to 10 mg in 0.5 ml of saline. The control group received saline. 2 days later a challenge injection of 2×10^8 TNP-SRBC was given, and the anti-TNP and anti-SRBC PFC responses were assayed 5 days later. As seen in Fig. 2, a control level anti-TNP response was obtained in the group receiving 0.1 mg of TNBS. As the dose of TNBS was increased, the anti-hapten response decreased until 0.4 ± 0.2 and 0 PFC/10^6 were obtained with 5 and 10 mg of TNBS, respectively. The anticarrier SRBC responses were slightly reduced in comparison to the control at the three highest doses, 2.5, 5, and 10 mg of TNBS. It appears, therefore, that 5 mg is an appropriate dose of TNBS for use in the induction of tolerance to TNP, while having little effect upon control anticarrier responses and eliciting no antibody response itself. There was high mortality in the group receiving 10 mg of TNBS (80%), while no mortality was observed in the remaining groups during this and succeeding experiments.

Effect of Chemical Nature of TNP Moiety upon Tolerance Induction.—In order to investigate the role played by chemical reactivity of the tolerogen in tolerance to TNP, groups of mice were treated with various compounds in amounts equimolar in the TNP moiety with the standard 5 mg TNBS dose. A challenge injection of 2×10^8 TNP-SRBC was given 2 days later, and the direct PFC

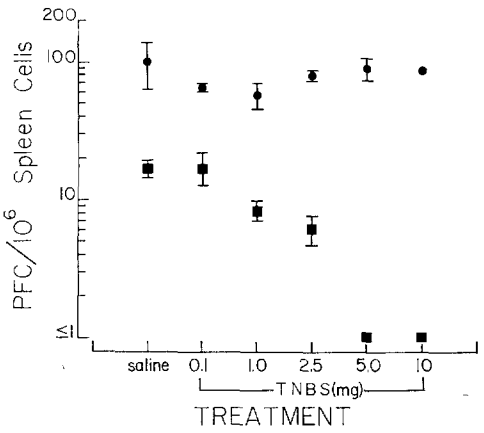

FIG. 2. Dose-response effect of TNBS upon the PFC response to TNP-SRBC. Anti-TNP (■) and anti-SRBC (●) PFC response of mice treated with various doses of TNBS ranging from 0 (control) to 10.0 mg. Mice were challenged with an i.p. injection of 2×10^8 TNP-SRBC 2 days after TNBS treatment, and were assayed for PFC 5 days later. Values represent the mean PFC response of five mice per group ± SE with the exception of group F (receiving 10 mg of TNBS), which is the response of the lone survivor. The anti-TNP responses of groups E and F were 0.4 ± 0.2 and 0, respectively. Abscissa: treatment of mice; ordinate: anti-TNP and anti-SRBC PFC/10^6 responses.

response was assayed 5 days later. The results are shown in Table II. As previously demonstrated, TNBS treatment induces tolerance to TNP. TNP-glycine did not reduce the response to either hapten or carrier. The response of mice receiving 1,3,5-TNB was reduced compared with the control group; however, giving TNP in this free but nonreactive form did not result in tolerance induction. It appears, therefore, that the virtue of TNBS being a free hapten is not sufficient to induce tolerance to TNP, and that the reactivity of the SO_3^- group may be the necessary feature.

Duration of TNBS-Induced Tolerance.—In order to investigate the extent and duration of TNBS-induced tolerance to TNP, groups of 5–30 mice were treated with TNBS followed by a challenge injection of 2×10^8 TNP-SRBC after an appropriate interval. The responses to hapten and carrier were assayed 5 days later. The results are shown in Fig. 3 *a*. With the shortest intervals between TNBS treatment and hapten-carrier challenge (<1 day), complete tolerance to TNP was obtained, and the anticarrier SRBC responses were at control levels. As the interval between tolerogen and hapten-carrier challenge was lengthened the anti-TNP response increased gradually. By 1 mo, the antihapten response had risen to approximately the control level. It is apparent that tolerance to TNP induced by TNBS treatment is of finite duration, is hapten specific, and does not affect the anticarrier response.

Effect of TNBS after TNP-SRBC Challenge.—The effect of TNBS treatment

TABLE II

*Effect of Reactivity and "Free" Form of Hapten in the Induction of Tolerance to TNP**

Group and treatment	TNP		SRBC	
	PFC/10^6	PFC/spleen	PFC/10^6	PFC/spleen
A				
TNBS	0.1‡ ± 0.1	12 ± 10	77 ± 18	6,834 ± 1,907
B				
TNP-glycine	33 ± 12	2,384 ± 740	161 ± 39	11,532 ± 2,323
C				
1,3,5-TNB§ in 1% Na_2SO_3	8 ± 1	646 ± 122	93 ± 14	7,800 ± 1,541
D				
1% Na_2SO_3	35‖	3,150	201	18,060
E				
Control	16 ± 3	1,840 ± 201	103 ± 7	10,758 ± 1,476

* Groups of five mice were treated with a variety of materials containing the TNP moiety. Treatment was an i.p. injection of 0.5 ml of saline containing an amount of material equimolar with the standard 5 mg dose of TNBS in terms of the TNP moiety.

‡ Values represent the mean day 5 PFC/10^6 or PFC/spleen response against TNP or SRBC of five mice per group ± SE.

§ 1% Na_2SO_3 in saline was used as the diluent for 1,3,5-TNB because of solubility difficulties.

‖ Values for group D represent the responses of a pool of five spleens.

upon an ongoing immune response to TNP-SRBC was investigated. Groups of 5–16 mice were challenged with an injection of 2×10^8 TNP-SRBC followed by the standard dose of TNBS after an interval of 1 h to 4 days. The PFC responses to hapten and carrier were determined 5 days after the TNP-SRBC immunization. These results are presented in Fig. 3 *b*. Tolerance to TNP was produced in mice given TNBS 1 h to 3 days after hapten-carrier challenge. With a 4 day interval between immunogen and tolerogen, however, a significant anti-TNP PFC response was observed. In this case, TNBS was given 24 h before the PFC assay, indicating that TNBS does not mask the immune response to the hapten by binding to PFC. Thus TNBS can induce specific tolerance to TNP in mice which are in the induction phase of the response to a hapten-carrier conjugate without altering the anticarrier response.

Effect of Secondary Injection of Hapten-Carrier in TNBS-Tolerant Animals.— The effect of TNBS treatment before a primary challenge of TNP-SRBC on the response to a subsequent injection of hapten-carrier was investigated. Groups of five to nine mice were given 5 mg of TNBS in 0.5 ml of saline followed after an interval of from 1 h to 14 days by a primary challenge injection of TNP-SRBC. All mice received a second injection of TNP-SRBC 28 days later and were assayed 4 days later for both direct and indirect PFC (see Materials and Methods). The results are shown in Figs. 4 *a* and 5 *a*. Essentially control-level

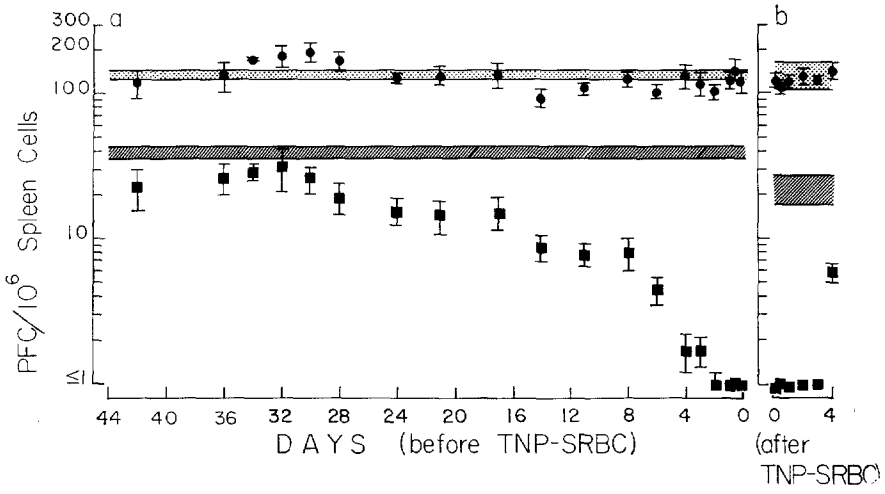

FIG. 3. Duration of TNBS-induced tolerance. Anti-TNP (■) and anti-SRBC (●) PFC response of mice treated with 5.0 mg of TNBS in 0.5 ml of saline at a wide variety of intervals (*a*) ranging from 1 h to 42 days before or (*b*) ranging from 1 h to 4 days after an i.p. challenge injection of 2×10^8 TNP-SRBC. PFC were determined 5 days later. Values represent the mean of the PFC response of 5–30 mice ± SE. The horizontal shaded bars represent the SE range (mean ± SE) of the anti-SRBC (upper bar) and anti-TNP (lower bar) responses of (*a*) 31 or (*b*) 16 untreated control mice to a challenge injection of 2×10^8 TNP-SRBC. (*a*) and (*b*) were run as two separate experimental procedures. Abscissa: time before TNP-SRBC challenge that TNBS treatment was given; ordinate: anti-TNP and anti-SRBC PFC/10^6 responses.

direct and indirect secondary anticarrier responses were obtained but there were no indirect anti-TNP PFC in response to the secondary injection of hapten-carrier conjugate. The direct antihapten response was very low (<5 PFC), less than the control direct responses.

The secondary response of mice given TNBS after the initial TNP-SRBC challenge was investigated. Groups of seven to nine mice were immunized with 2×10^8 TNP-SRBC and given TNBS after an interval of 12, 24, or 48 h. All mice received a secondary challenge of TNP-SRBC 28 days after the initial injection, and were assayed for the PFC response to TNP and SRBC 4 days later. As seen in Figs. 4 *b* and 5 *b*, tolerance to TNP was observed in all treated groups, while the anticarrier responses were at control levels. Thus, similar to TNBS treatment before the initiation of the primary response, treatment after the initiation of a primary response results in tolerance to a secondary challenge given 28 days later.

It is important to note that the antihapten responses of treated mice to a secondary injection of hapten-carrier conjugate are in some cases lower than the primary response of similarly treated mice (compare groups given TNBS with intervals before primary TNP-SRBC challenge of 6, 8, and 14 days be-

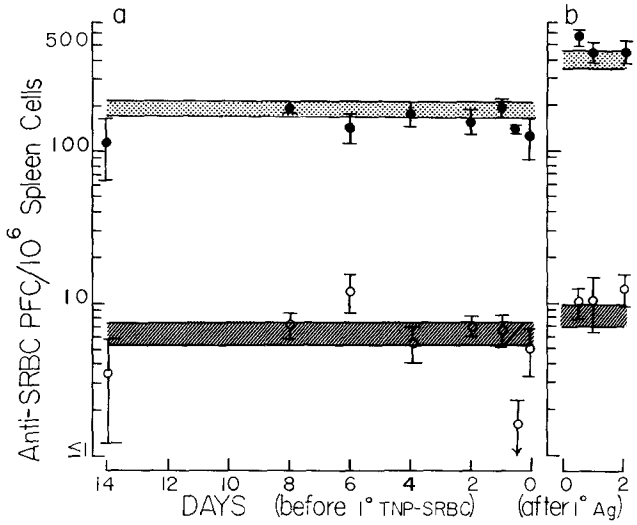

FIG. 4. Effect of TNBS upon the anti-SRBC PFC response to a secondary challenge with TNP-SRBC. Anti-SRBC PFC response of mice treated with TNBS at a variety of intervals ranging from (*a*) 1 h to 14 days before or (*b*) 12–48 h after a primary injection of 2×10^8 TNP-SRBC. 28 days later a second injection of TNP-SRBC was given, and the secondary response was assayed 4 days thereafter. Values represent the mean of the anti-SRBC PFC response of 5–9 mice ± SE. Open figures represent direct PFC responses, and closed figures are indirect PFC responses. The horizontal shaded bars represent the control direct (lower) and indirect (upper) PFC responses. Abscissa: time after TNBS treatment that 2×10^8 TNP-SRBC primary challenge injection was given; ordinate: anti-SRBC PFC/10^6 responses.

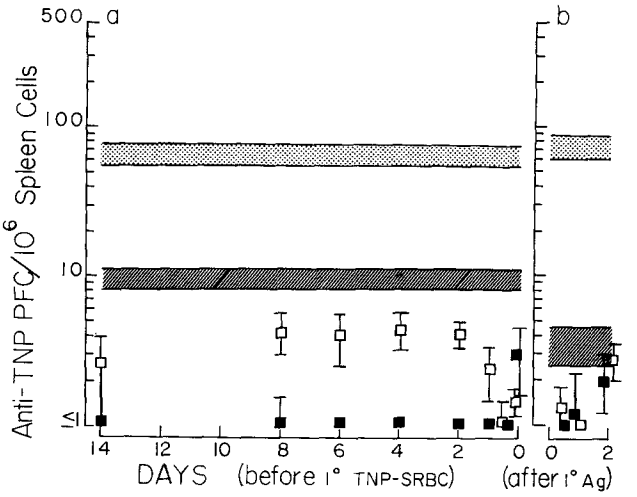

FIG. 5. Effect of TNBS upon the anti-TNP response to a secondary challenge with TNP-SRBC. For details, see legend for Fig. 4.

tween Figs. 3 and 4). Apparently, mice which were in the process of escaping from tolerance when initially challenged had been induced to reenter the tolerant state by the TNP-SRBC challenge and this apparent state of tolerance persisted through the 28 day secondary hapten-carrier challenge. This seems to be the case even where a significant antihapten response to the initial challenge was obtained.

Reentry into the Tolerant State by Mice Escaping from Tolerance.—In order to test more precisely the possibility that animals escaping from tolerance could be induced to reenter the tolerant state, the following experiment was conducted. Groups of 10 mice were given TNBS injections (5.0 mg in 0.5 ml of saline). One-half of the mice in each group were given a challenge injection of TNP-SRBC at an interval after TNBS treatment that varied with the group (2–14 days). All mice were challenged with TNP-SRBC 28 days after the day of the initial challenge. A primary response assay (direct PFC) was conducted on those mice receiving only one hapten-carrier injection, while a secondary response assay (indirect PFC) was performed on the mice having received two injections. The data from this experiment are summarized in Table III. By comparing those mice treated with TNBS at the same interval it is apparent that there is a decrease in the anti-TNP responsiveness of TNBS treated mice given a day 0 hapten-carrier challenge. It appears that the initial challenge injection of TNP-SRBC resulted in the induction of a longer-lasting state of tolerance.

DISCUSSION

The experiments reported here show that treatment of mice with a nonimmunogenic preparation of free reactive hapten leads to the induction of a state of tolerance to the hapten. This is determined by the lack of response to the haptenic moiety when administered as an immunogenic hapten-carrier conjugate. The tolerance produced is specific for the hapten, since the anticarrier responses are essentially unaltered. The unresponsiveness induced by TNBS treatment is a dose-dependent phenomenon becoming less complete with low doses of tolerogen. The tolerance is of a definite length both in the induction phase and in the duration of the established unresponsive state. Tolerance can be maintained and extended both by hapten-carrier challenge and by the administration of additional tolerogen, and may be reentered once escape has been initiated.

TNBS is a purely tolerogenic form of the TNP moiety, apparently owing its tolerogenicity at least partially to the presence of a reactive sulfonic acid group which binds in vitro to lysine groups of proteins. The tolerance induced by TNBS is hapten (TNP)-specific, and is observed not only at the serum antibody level, where antigen could conceivably remove antibody from the circulation, but also at the cellular level (PFC). Serum antibody and PFC responses to the carrier are unaffected by the tolerization by TNBS. However, in a later paper,[4]

TABLE III

*Effect of Antigen Challenge on Tolerance Induction**

Group	TNBS interval	TNP-SRBC day 0§	TNP-SRBC day 28§	Anti-TNP D‖	PFC/10^6 I¶	Anti-SRBC D‖	PFC/10^6 I¶
	days‡						
I A	2	−	+	27**± 6		196**± 31	
B	2	+	+	2.6 ± 0.8	0.7 ± 0.4	5.9 ± 0.9	185 ± 52
II A	4	−	+	32 ± 10		184 ± 31	
B	4	+	+	4.3 ± 1.2	0.6 ± 0.4	5.5 ± 1.5	176 ± 33
III A	6	−	+	29 ± 4		174 ± 5	
B	6	+	+	3.9 ± 1.5	0.5 ± 0.2	12 ± 3	142 ± 31
IV A	8	−	+	26 ± 6		134 ± 30	
B	8	+	+	4.2 ± 1.3	1.0 ± 0.5	7.3 ± 1.4	194 ± 18
V A	14	−	+	23 ± 7		120 ± 28	
B	14	+	+	2.5 ± 1.3	0.3 ± 0.3	3.5 ± 2.3	113 ± 49
VI A	Control	−	+	38 ± 8		126 ± 10	
B	Control	+	+	12 ± 2	47 ± 9	8.4 ± 1.4	208 ± 36

* TNBS (5.0 mg in 0.5 ml of saline) was given followed in an interval of from 2–14 days by a challenge to one-half of each group of 2×10^8 TNP-SRBC. All mice were challenged with 2×10^8 TNP-SRBC 28 days later. Mice receiving one injection of hapten-carrier were assayed for a primary response and those receiving two injections were given a secondary response assay.

‡ The interval between TNBS and the initial injection of hapten-carrier. Control mice received no TNBS.

§ +, 2×10^8 TNP-SRBC injection given on the day indicated; −, no injection given on the day indicated.

‖ D-direct PFC/10^6 response.

¶ I-indirect PFC/10^6 response (see Materials and Methods).

** Values represent the mean of five mice ± SE.

we will show that thymus-derived cells from TNBS-tolerant animals can reduce the anticarrier response under certain experimental conditions.

It is unlikely that masking of PFC by TNBS is responsible for the lack of TNP-specific PFC. When TNBS was given on day 4 after TNP-SRBC challenge, a significant response was obtained, while shorter intervals (from 1 h to 3 days) resulted in complete tolerance to TNP. This shows that tolerance to TNP may be induced during the induction phase of the antibody response and also presumably while the antibody response is in progress, i.e., on day 3. The result with a 4 day interval indicates that after a certain point in the response PFC have been generated which cannot be tolerized or blocked by TNBS.

The injection of TNBS could result in the trinitrophenylation of serum pro-

teins, which are retained in the circulation for several days after the treatment. Additionally, within 1 h after treatment, TNBS or conjugation products are being excreted in the urine. It is very unlikely that TNBS in the free, native form makes contact with more than the few antigen-sensitive cells which are presumably present in the peritoneal cavity. Affinity labeling is therefore not likely to be the mode of action of the tolerogen. It is possible that, in conjugating to various constituents of serum and peritoneal fluid, a situation similar to that investigated by Havas (5) and Borel (6) exists. Rather than conjugation of serum proteins in vitro, haptenation takes place in vivo. Serum from TNBS-treated mice contains both trinitrophenylated albumins and globulins (unpublished observations). Neither treatment of spleen cells with TNP serum (serum on day 1 after TNBS treatment) in vitro followed by adoptive transfer to lethally X-irradiated recipients nor pretreatment of intact mice with TNP serum had any effect upon the response to a subsequent TNP-SRBC challenge. Of course, variables such as haptenation ratios and possible alterations in serum constitution cannot be controlled in vivo as with in vitro conjugation.

In addition to trinitrophenylation of serum constituents, mouse erythrocytes taken from mice 1 day after standard TNBS treatment were found by HA titration of a standard reference anti-TNP antiserum to be conjugated with TNP (unpublished observation). The injection of these cells or in vitro heavily or lightly conjugated TNP-mouse erythrocytes into syngeneic or allogeneic recipients failed to induce tolerance.[5]

The response to certain antigens is dependent upon cellular cooperation between thymus-derived antigen-reactive cells (T cells) and thymus-independent bone marrow-derived antigen-sensitive cells (B cells). The mechanism of cooperation is still unclear. According to the cell interaction model of humoral immunity, tolerance in either the B or T cell population or both will result in a state of systemic tolerance in the animal in question. In the cell cooperation theory of hapten-carrier immunity, carrier-specific T cells aid the hapten-specific B cells in some manner in the production of antihapten antibody. In such a case, tolerance in either cell type would also lead to a state of tolerance to the hapten. Using HGG, Chiller et al. (22) have demonstrated tolerance in both the B and T cell lines, with T cells entering tolerance more quickly, at a lower dose of tolerogen, and for a much longer duration than the B cell line. In the TNBS-TNP system there is reason to believe that tolerance is induced in the hapten-specific B cell line.[4] Presumably, the long-lasting antihapten tolerance in delayed hypersensitivity (23) is due to unresponsiveness at the T cell level. It is likely that TNBS induces tolerance in the T cell line as well as the B cell line. If tolerance is induced in the TNP-specific T cell line, the differences in humoral anti-TNP responsiveness would be virtually undetectable according to the model. This is so because the T cells specific for the carrier (SRBC) would fulfill a

[5] Fidler, J. M., and E. S. Golub. Manuscript in preparation.

carrier-specific function and aid the hapten-specific B cells in the response to TNP. Essentially, therefore, according to the hapten-carrier theory of cell cooperation only tolerance in the hapten-specific B cell line would be detectable using a hapten-carrier system of humoral immunity. Tolerance in the hapten-specific T cell line would be masked by the cooperative efforts of the carrier-specific T cells. However, a T cell inhibitor substance may be induced by TNBS.[4]

The state of tolerance to TNP induced by TNBS treatment is of limited duration. Escape from TNBS-induced tolerance can be postponed by a challenge with a hapten-carrier conjugate which under normal circumstances would be optimal for the induction of immunity. This extension and in some cases reversion to the tolerant state after a significant level of responsiveness had been regained is dependent upon the protocol of maintenance challenges in terms of duration and characteristics of the escape phenomenon and will be the subject of the next paper in this series.[6]

SUMMARY

Treatment of mice with a nonimmunogenic preparation of free reactive hapten, trinitrobenzene sulfonic acid (TNBS), leads to the induction of a state of tolerance to the hapten, 2,4,6-trinitrophenyl (TNP). This is determined by the lack of response to the haptenic moiety in an immunogenic hapten-carrier conjugate (TNP-SRBC) as assayed both by serum antibody titrations and the hemolytic plaque assay. The tolerance produced is specific for the hapten, since the anticarrier responses are essentially unaltered compared with the control values. The unresponsiveness induced by TNBS treatment is a dose-dependent phenomenon, becoming less complete at lower doses of TNBS. The tolerance is of a definite length, both in its induction phase and in the duration of the established unresponsive state. Tolerance can be maintained and extended, and may also be reentered once escape has been initiated.

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