NONANTIGENICITY AND IMMUNOLOGIC TOLERANCE: THE ROLE OF THE CARRIER IN THE INDUCTION OF TOLERANCE TO THE HAPTEN*

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The phenomenon of immunologic tolerance has been known for more than 20 yr, yet its mechanism remains obscure and difficult to study. One reason for this is that substances able to induce tolerance generally also induce an immune response, thus confusing the issue. If a "pure" tolerance-inducing substance were available, it would provide a basis for isolating and analyzing the phenomena of tolerance and immunity as separate events. In this paper we will show that a hapten (dinitrophenyl [DNP])[1] bound to a nonimmunogenic carrier meets the requirement of a pure tolerogen in that it can induce tolerance without causing antibody synthesis. This extends our preliminary observation that a hapten bound to a nonimmunogenic protein carrier induces tolerance to the same hapten bound to an immunogenic protein carrier (1). The data show that the key to this capability rests with the carrier moiety of the hapten-carrier conjugate.

Materials and Methods

Animals.—6–7-wk old male (C57BL/6J × DBA/2)F_1 (hereafter referred to as BDF_1), C57BL/6J, and BALB/c mice were used. All animals were obtained from Jackson Laboratories, Bar Harbor, Maine.

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[1] *Abbreviations used in this paper:* Alb, albumin; BDF_1, (C57BL/6J × DBA/2)F_1; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DNFB, 1-fluoro-2,4-dinitrobenzene; DNP, dinitrophenyl(ated); DNPS, 2,4-dinitrophenyl sulfonic acid; DNP-serum, DNP-C57BL/6; εACA, εaminocaproic acid; HGG, human gamma globulin; HRBC, horse red blood cells; IgG, 7S immunoglobulin; IgM, 19S immunoglobulin; KLH, keyhole limpet hemocyanin; MGG, mouse gamma globulin; MSA, mouse serum albumin; PFC, plaque-forming cells; RGG, rabbit gamma globulin; SRBC, sheep red blood cells; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNP, trinitrophenyl.

Haptens.—2,4-Dinitrophenyl sulfonic acid (DNPS), twice recrystallized (Eastman Kodak Co., Rochester, N. Y.), 1-fluoro-2,4-dinitrobenzene (DNFB) (Eastman Kodak Co.), and picryl sulfonic acid (2,4,6-trinitrobenzene sulfonic acid, TNBS) (Nutritional Biochemicals Corp., Cleveland, Ohio), twice recrystallized, were used.

Protein Carriers.—

Keyhole limpet hemocyanin (KLH): Obtained from Pacific Bio Marine Supply Co., Venice, Calif., and prepared according to the method of Campbell (2).

Mouse serum (C57BL/6J): Purchased from Jackson Laboratories, Bar Harbor, Maine.

Mouse Serum Fractions.—19S immunoglobulin (IgM) was obtained by chromatographic separation of C57BL/6J serum on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). 7S immunoglobulin (IgG) and albumin (Alb) were prepared by starch block electrophoresis of C57BL/6J serum. The purity of these three fractions was tested by immunoelectrophoresis using a rabbit anti-whole mouse serum. Rabbit gamma globulin (RGG). (Pentex Biochemical, Kankakee, Ill.) and human gamma globulin (HGG) (Pentex Biochemical) were further purified by diethylaminoethyl (DEAE) chromatography. Bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co., Chicago, Ill.

Synthetic Antigens.—α-DNP-lysine $(lys)_5$, α-DNP-$(lys)_{\overline{8.4}}$, and α-DNP-$(lys)_{31}$ were a gift of Dr. Stuart Schlossman.

Preparation of Conjugates.—DNPS was bound to protein carriers according to the method of Eisen (3). The preparations of DNP-C57BL/6 serum (hereafter referred to as DNP-serum) used to induce tolerance, unless otherwise mentioned, contained 14–23 moles of DNP/mole of serum (1×10^5 was arbitrarily chosen as the average molecular weight of serum protein). To study the dose relationship between hapten and serum protein carrier, the conjugates of DNP-serum used ranged from DNP_1- to DNP_{61}-serum. In the case of DNP-KLH, either DNPS or DNFB was used and the molar ratios of DNP:KLH ranged from DNP_{29}-KLH to DNP_{246}-KLH (assuming the mol wt of KLH to be 8×10^5). TNBS was bound to KLH or to isogeneic mouse serum according to the method of Rittenberg (4) (TNP_{101}-KLH, TNP_{47}-serum).

Immunization.—This was always done at 7 wk of age. When the primary immune response was tested by the hemolytic plaque assay, a single intraperitoneal injection of 1 mg of either DNP_{246}-KLH or DNP_{101}-KLH was given. When DNP_{62}-KLH was used, each mouse was given 0.2 mg. During the course of the experiments it was found that challenging doses of 0.2 mg/mouse and 1 mg/mouse of DNP-KLH elicited similar immune responses. Therefore, 0.2 mg of DNP-KLH was chosen as the immunizing dose. Furthermore, there were no differences in splenic plaque-forming cells (PFC) in mice challenged with preparations of DNP-KLH varying from 62 moles/mole to 246 moles/mole. All other DNP conjugates, when used for immunization, were given in a dose of 0.2 mg/mouse. When trinitnophenyl $(TNP)_{101}$-KLH was used for eliciting a primary response, the dose was also 0.2 mg/mouse. All challenges with DNP or TNP conjugates were administered intraperitoneally in complete Freund's adjuvant (CFA).

When the antigenicity of the various DNP conjugates was tested, groups of BDF_1 or BALB/c mice were injected intraperitoneally three times at intervals of 2 wk with DNP conjugates in CFA in a dose of 0.05–0.15 mg/mouse. Serum from each mouse was collected by three to four repeated bleedings (at 2-day intervals) from the tail starting 3 days after the last antigenic challenge and pooled. In some experiments animals were immunized simultaneously with DNP-KLH and 0.1 ml of a 10% suspension of washed horse red blood cells (HRBC) (Baltimore Biological Laboratories, Cockeysville, Md.).

Induction of Tolerance.—This was done by a single intravenous injection of 0.3–5.0 mg/mouse of DNP-serum.When synthetic polypeptide conjugates were used, 4–5 daily intraven-

ous injections of 0.25 mg/mouse were given. For all other conjugates the dose was 0.2 mg/mouse.

Spleen Cell Suspension.—Spleens were excised, weighed, and minced with scissors. The fragments were gently pressed through a tantalum gauze into 5 ml of tissue culture media (TCM 858, Difco Laboratories, Detroit, Mich.). Nucleated cell counts were made for each spleen suspension by using a hemocytometer.

Hemolytic Plaque Assay.—The method described by Rittenberg using TNP-coated sheep red blood cells (SRBC) (Colorado Serum Co., Denver, Colo.) for the detection of direct plaque-forming cells was used (5).

Passive Hemagglutination.—A modification of the micromethod of Heller et al. was used (6). DNP_{60}-BSA was used to coat the tanned SRBC.

Radioactive Antigen-Binding Immunoassay.—A modification of Farr's technique, described by Green et al., was used (7). DNP-ϵ-aminocaproic acid (ϵACA)-^{3}H was a gift of Dr. Stuart Schlossman. In preliminary experiments, various concentrations of DNP-ϵACA-^{3}H (5×10^{-6} M to 5×10^{-9} M) and various dilutions of the antisera (1/10–1/800) were used to determine the amount of antigen that provided the most sensitive assay of murine anti-DNP antibody. These were found to be 5×10^{-8} M of DNP-ϵACA-^{3}H (specific activity of 1 μCi/5×10^{-8} M) and a dilution of serum of 1/100. To relate the percentage of antigen binding to actual amounts of anti-DNP antibody, the assay was calibrated with sera containing known amounts of anti-DNP antibody. This disclosed that the lower limit of sensitivity of the assay was 0.002 mg of antibody at 30% binding. 30% antigen binding was chosen as the lower limit for reliable results, since the 95% confidence limit for nonspecific binding was 24%. The binding values of serum from normal (nonimmunized) animals ranged from 0–8%.

Statistical Analysis.—Statistical analysis was done according to Student's t test. For PFC, the geometric mean for each group was calculated.

RESULTS

Induction of Immunological Tolerance to DNP by DNP-Serum.—At various times before or after challenge with DNP-KLH, different groups of BDF_1 mice were given a single intravenous injection of DNP_{14}-serum. The immune response of these animals to DNP was assayed 5 days after challenge with DNP-KLH. The results are shown in Fig. 1. Tolerance was induced in less than 24 hr, and lasted between 2 and 3 wk. The immune response of the group injected with DNP-serum 3 wk before the challenge with DNP-KLH was not different from that of the control group ($P > 0.05$). When DNP-serum was administered 1 day after immunization with DNP-KLH, tolerance of DNP developed; however, tolerance was not found when DNP-serum was given 3 or 4 days after challenge with DNP-KLH. Since in the latter cases the DNP-serum was given 48 or 24 hr before the assay for PFC, the results support our previous observation that DNP-serum does not "mask" the immune response by binding to antibody-forming cells (1).

Since tolerance of DNP induced by DNP-serum was lost after 3 wk, two groups of mice were given 2.5 mg of DNP_{14}-serum. 3 wk later one group received a second injection of 2.5 mg of DNP_{14}-serum, and the other group received nothing. At that time both groups were challenged with DNP-KLH. The number of PFC in the group injected twice with the tolerogen was 3.4 ± 0.6

PFC/10^6 spleen cells, whereas the group injected once had 99.7 ± 23.3 PFC/10^6 spleen cells. It thus appeared that tolerance of DNP could be maintained by repeated doses of the tolerogen.

The Dose of Hapten or Carrier Required to Induce Tolerance to DNP.—In the first series of experiments, the amount of the carrier was kept constant (2.5 mg of serum protein/mouse) and the amount of the hapten was varied. In the

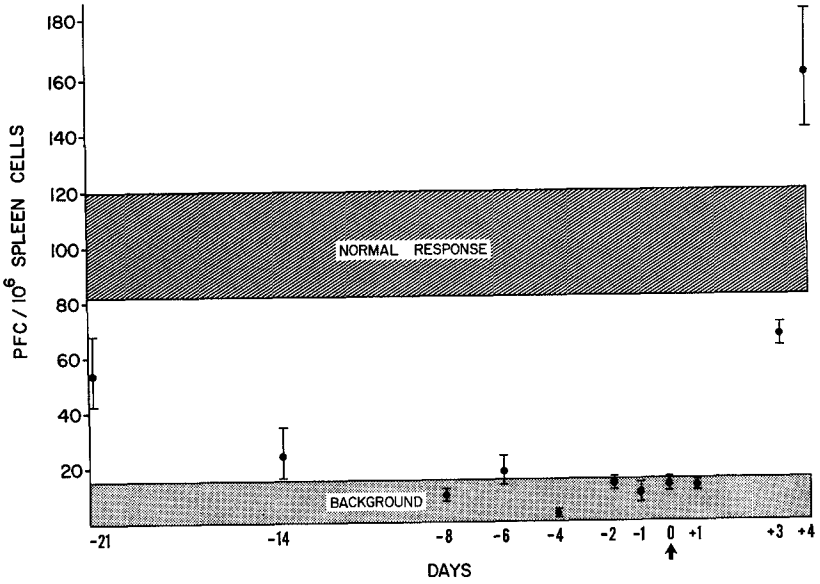

FIG. 1. *Induction and duration of immunological tolerance to DNP-serum.* The upper shaded area represents the number of direct PFC ± SE to DNP in 14 control mice challenged with 1 mg of DNP_{189}-KLH intraperitoneally in CFA. The lower shaded area represents the number of direct PFC ± SE to DNP in groups of mice (6–10 in each) immunized with 1 mg of KLH intraperitoneally in CFA. Each point represents the geometrical mean ± SE of the number of direct PFC to DNP obtained in mice injected intravenously with a single dose of 2.5 mg of DNP_{14}-serum/mouse at various days before or after challenge with 1 mg of DNP_{189}-KLH.

second series of experiments, the amount of the hapten was kept constant (14 moles of hapten/100,000 mol wt protein) and the amount of the carrier (mouse serum) was varied. In all cases the experimental animals were given a single injection of DNP-serum 8 days before challenge with DNP-KLH. The results (Fig. 2) show that when DNP was bound to whole serum, at least 7 moles of hapten/100,000 mol wt of serum protein were necessary to induce tolerance of DNP. A dose of 0.6 mg/mouse of whole isogeneic serum (the carrier) was necessary to induce tolerance.

Hapten Specificity of Tolerance.—Several attempts were made to coat sheep

red cells with DNP, either by coating them directly (8, 9) or by coating them with DNP bound to a protein carrier (10) in order to measure anti-DNP PFC in animals immunized with DNP-KLH. The results were inconsistent and the number of direct PFC detectable was always about 8–10-fold lower than that obtained with TNP-SRBC. These methods were therefore abandoned and for technical efficiency TNP was used to coat the target sheep red cells. In view of the fact that the immune response was tested in a cross-reacting system, several questions arose concerning both immunity and the specificity of

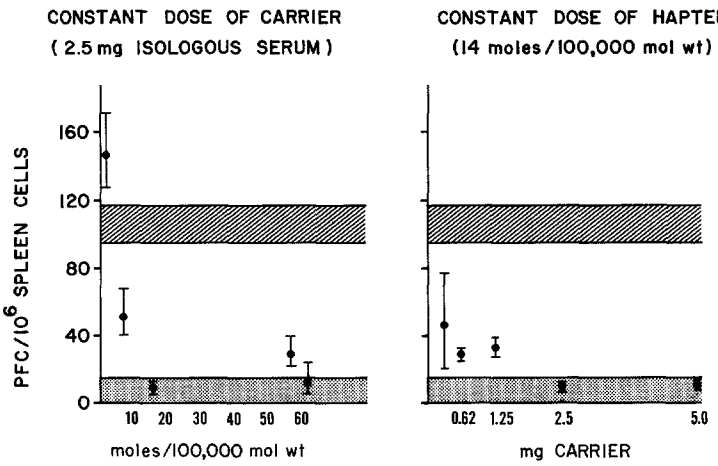

FIG. 2. *Hapten carrier (isogenic serum) relationship in induction of tolerance by DNP-serum.* Left panel: Dose of carrier (2.5 mg of serum protein/mouse) was kept constant. The degree of substitution of DNP varied (DNP_1-serum to DNP_{61}-serum). Right panel: Dose of hapten (14 moles/100,000 mol wt serum protein) was kept constant. The amount of carrier varied (0.3–5.0 mg/mouse). The upper shaded area represents the normal primary immune response; the lower shaded bar, the background response in BDF_1 mice immunized with the carrier (KLH) alone. Each point represents the geometrical mean of the result in groups of six to eight experimental mice that were pretreated with a single injection of DNP-serum intravenously 8 days before challenge with DNP_{189}-KLH. The vertical bars represent 1 SE.

tolerance. (*a*) Would immunization with TNP-KLH instead of DNP-KLH result in a greater number of direct PFC to TNP? (*b*) Do all the direct PFC elicited by DNP-KLH cross-react with TNP on the target SRBC? When preparations of DNP_{62}-KLH and TNP_{101}-KLH were used to immunize mice, similar numbers of direct PFC were obtained when the assay was carried out using TNP-SRBC (Fig. 3). Since direct assay for DNP-PFC is unsatisfactory, the possibility remains that the TNP assay does not reveal all PFC responding to DNP. However, the number of PFC elicited by DNP-KLH was the same as the one elicited by TNP-KLH, suggesting that most of the anti-DNP antibody cross-reacts with TNP-SRBC.

The next series of questions concerns the hapten specificity of the induction of tolerance: Would tolerance to a hapten be obtained in a homologous system? The results show that animals injected intravenously with TNP-serum and then challenged with TNP-KLH were tolerant to TNP-SRBC (Fig. 3). Thus, we were not suppressing only the anti-DNP antibody cross-reacting with TNP. The results also show that, when the immune response was tested in a cross-reacting system (i.e. challenged with DNP-KLH and tested with TNP-SRBC), tolerance is induced equally well by DNP-serum or TNP-serum. Finally, the question arose as to whether hapten specificity can be revealed when the immune response is tested in a non–cross-reacting system. Tolerance in animals

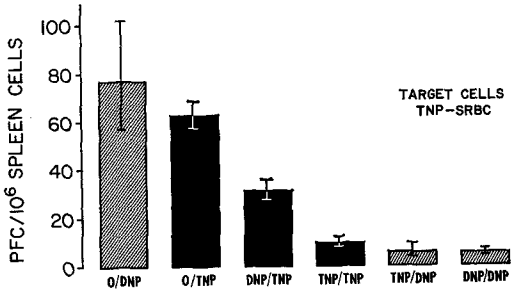

FIG. 3. *Hapten specificity (DNP and TNP) of the immune response and tolerance.* Shaded columns represent groups of BDF_1 mice (8–10/group) challenged with 0.2 mg of DNP_{62}-KLH/mouse. Black columns represent groups of mice (8–10/group) challenged with 0.2 mg of TNP_{101}-KLH/mouse. At the bottom of the column is indicated which haptens were used: those before the bar represent the hapten bound to isogeneic serum conjugate used 2.5 mg/mouse to induce tolerance (DNP_{58}-serum, TNP_{47}-serum), and those after the bar represent the hapten-KLH conjugate used to challenge the animal. All animals were challenged with DNP or TNP-KLH immediately after pretreatment intravenously with tolerogen. In all experiments the direct PFC were tested with target SRBC coated with TNP. The vertical bar represents 1 SE.

treated with TNP-serum and challenged with TNP-KLH was more profound ($P > 0.001$) than in animals treated with DNP-serum and challenged with TNP-KLH. This may reflect the immunochemical specificity of the tolerance, since the target SRBC in both cases was TNP-SRBC.

The Role of Isologous Serum Proteins in the Induction of Tolerance to DNP.— Thus far, we have seen that DNP bound to whole serum can induce tolerance of DNP. In order to determine their roles as carriers in this system, purified IgM, IgG, and albumin were prepared and conjugated to DNP. The final preparations had a similar degree of hapten substitution, and they were compared for their capacity to induce tolerance both on a weight (Table I) and a molar basis (Table II). IgG was superior as a carrier for the induction of tolerance to both of the other proteins. Another experiment was done to

determine if lightly substituted albumin would be more tolerogenic than DNP_{18}-albumin. A group of five mice was injected intravenously with 0.2 mg of DNP_9-albumin and challenged with DNP-KLH in CFA. The geometrical mean of PFC/10^6 spleen cells was (134.9 ± 25.7), which is not different from

TABLE I

*Induction of Tolerance to DNP by DNP Bound to Isogeneic Protein Carriers**

No. of mice	Tolerogen	Direct PFC/10^6 (± SE)	*P*
BDF_1			
10	None	95.1 (17.2)	
8	DNP_{12}-IgG	4.1 (0.5)	<0.01‡
8	DNP_{14}-IgM	41.0 (8.9)	<0.001§
11	DNP_{18}-Alb	31.4 (3.3)	<0.001§
C57BL/6			
4	None	102.5 (15.3)	
6	DNP_{12}-IgG	5.2 (1.7)	<0.001‡
7	DNP_{23}-Alb	99.7 (23.3)	<0.001§

* Background PFC/10^6 spleen cells in nonimmunized BDF_1 and C57BL/6 mice were 4.0 (±0.7) and 2.1 (±0.17), respectively. All mice were challenged with 0.2 mg of DNP_{62}-KLH in CFA intraperitoneally. Experimental mice received 0.2 mg of the tolerogen intravenously immediately before being challenged with the antigen intraperitoneally.

‡ Compared to immunized control mice.

§ Compared to DNP-IgG experimental mice.

TABLE II

*Induction of Tolerance to DNP by DNP Bound to Isogeneic Protein Carriers**

No. of BDF_1 mice	Tolerogen	Direct PFC/10^6 (± SE)	*P*
10	None	95.1 (17.2)	
9	DNP_{12}-IgG	4.9 (0.3)	<0.001‡
4	DNP_{14}-IgM	16.9 (7.0)	<0.001§
9	DNP_{18}-Alb	32.0 (4.3)	<0.001§

* Background PFC/10^6 spleen cells in nonimmunized BDF_1 mice were 4.0 (±0.7). All mice were challenged with 0.2 mg of DNP_{62}-KLH in CFA intraperitoneally. Experimental mice received 1.4×10^{-6} M of the tolerogen intravenously immediately before being challenged with antigen intraperitoneally.

‡ Compared to immunized control mice.

§ Compared to DNP-IgG pretreated experimental mice.

the response of mice given only DNP-KLH. Thus, the difference between IgG and albumin cannot be attributed to a difference in their degree of substitution with DNP.

Since the duration of tolerance induced by 2.5 mg/mouse of DNP-serum was less than 3 wk, it was of interest to determine if smaller doses of DNP-IgG

would induce tolerance of longer duration. A group of seven mice was pretreated with 0.5 mg/mouse of DNP-$_7$IgG 23 days before the challenge with DNP-KLH. No response to DNP was detectable in these animals (mean PFC/10^6 spleen cells, 3.0 ± 0.9). Thus a single injection of 0.5 ml of lightly substituted DNP_7-IgG is as effective to maintain tolerance than two injections of 2.5 mg of DNP_{14}-serum given 3 wk apart.

The Role of Heterologous Protein Carriers in the Induction of Tolerance to DNP.—Different groups of BDF_1 mice were treated with DNP-HGG, DNP-RGG, DNP-BSA, or DNP-KLH. The results (Fig. 4) indicate that the induc-

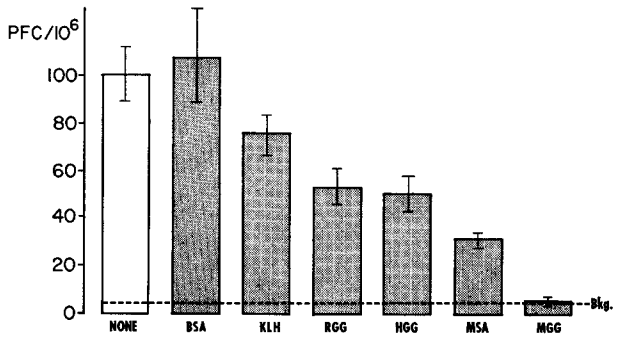

FIG. 4. *Induction of tolerance to DNP with DNP bound to heterologous protein carriers.* White column represents the geometrical mean of the number of direct PFC ± SE in control mice immunized with 0.2 mg of DNP_{62}-KLH. The broken line represents the geometrical mean (including SE) of the number of direct PFC in animals immunized with 0.2 mg of KLH only. The hatched columns represent the geometrical mean of the number of direct PFC ± SE in groups of mice (4–6/group) pretreated intravenously with DNP bound to different heterologous protein carriers: DNP_{21}-BSA, DNP_{29}-KLH, DNP_{22}-HGG (for comparison the results obtained with DNP_{12}-mouse IgG and DNP_{18}-mouse albumin are given). All animals were challenged intraperitoneally with DNP-KLH immediately after pretreatment with the different DNP carrier conjugates. The vertical bar represents 1 SE.

Bkg, background.

tion of tolerance to the hapten is carrier dependent. Isogeneic carriers had a greater ability to induce tolerance to the hapten than any of the foreign carriers tested. When the various conjugates were emulsified in CFA and tested for their ability to initiate an immune response to DNP, only DNP-KLH and DNP-HGG had this ability. No correlation between the capacity of the conjugates to immunize and their ability to induce tolerance was found (Fig. 5).

The Role of Synthetic Polypeptide Carriers in the Induction of Tolerance to DNP.—Three synthetic homopolymers of lysine coupled with DNP [α-DNP-$(lys)_5$, α-DNP-$(lys)_{\overline{8.4}}$, α-DNP-$(lys)_{31}$] were tested in the following way for their ability to induce tolerance to DNP. Three groups of BDF_1 mice were challenged with 0.2 mg of DNP_{62}-KLH/mouse in CFA intraperi-

toneally. Starting 3 days before this, each mouse received four daily intravenous injections of one of the DNP-homopolymers (0.25 mg/mouse, for a total of 1 mg/mouse). A fourth group of mice was pretreated in the same way with DNPS not bound to a carrier, and thereafter immunized with 0.2 mg of

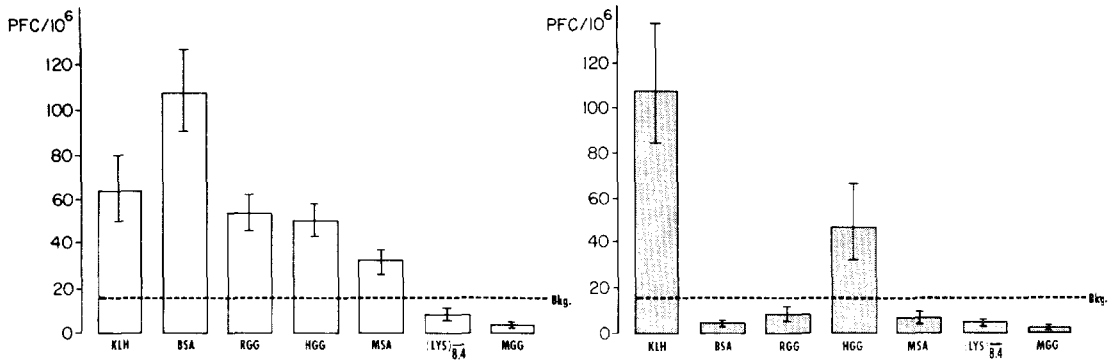

FIG. 5. *Comparison of the carrier with its immunogenic or tolerogenic effect.* Left panel: White columns represent the geometrical mean of the number of direct PFC ± SE of groups of mice (4–6/group) pretreated intravenously with various DNP conjugates, 0.2 mg/mouse, and thereafter immunized with 0.2 mg/mouse of DNP_{62}-KLH. Right panel: Hatched columns represent the geometrical mean of the number of direct PFC ± SE of groups of mice (4–5/group) that were immunized intraperitoneally with various DNP conjugates. The broken line represents geometrical mean of the direct PFC in animals immunized intraperitoneally with KLH alone. The vertical bars represent 1 SE.

TABLE III

Role of Synthetic Polypeptide Carrier in the Induction of Tolerance to DNP

Tolerogen	No. of mice tolerant	PFC/10^6 (± SE)	
		Tolerant*	Nontolerant*
—	—	—	100.0 (11.1)
DNP	0/10	—	122.2 (18.6)
α-DNP-$(lys)_5$	3/10	27.7 (10.6)	91.1 (5.4)
α-DNP-$(lys)_{8.4}$	7/10	7.9 (2.8)	90.4 (17.9)
α-DNP-$(lys)_{31}$	7/10	18.3 (5.8)	96.5 (16.3)

* Tolerant and nontolerant animals were separated by the 95% confidence limit of the control immunized animals, i.e., animals which were not within the 95% confidence limit of the control were said to be tolerant.

DNP_{62}-KLH/mouse. A fifth and control group of mice was not pretreated before immunization. The results are summarized in Table III. Three out of 10 mice treated with α-DNP-$(lys)_5$, seven out of 10 treated with α-DNP-$(lys)_{8.4}$, and seven out of 10 treated with α-DNP-$(lys)_{31}$ acquired tolerance of DNP. A sixth group of four mice were injected intravenously with 1 mg of

ϵ-DNP-$(lys)_1$ before being challenged with DNP-KLH. This failed to induce tolerance (mean PFC/10^6 100.8 $\pm$ 28.0).

The antigenic specificity of the tolerance to DNP was examined in the case of α-DNP-$(lys)_{\overline{8.4}}$. Mice pretreated with the homopolymer conjugate for 4 days were challenged simultaneously with DNP-KLH in CFA and HRBC in saline. A control group of mice was immunized with DNP-KLH and HRBC, but did not receive the α-DNP-$(lys)_{\overline{8.4}}$. The results are presented in Table IV, which shows that immunologic tolerance in the experimental mice was specific for α-DNP-$(lys)_{\overline{8.4}}$.

Immunogenicity of the Tolerogen.—The immunogenicity of the different tolerance-inducing substances used in these experiments was tested by: (*a*)

TABLE IV

Antigen Specificity of Tolerance to DNP

No. of mice	Tolerogen	Antigen		PFC/10^5 ($\pm$ SE)	
		DNP-KLH	HRBC	DNP (PFC)	HRBC (PFC)
3	—	+	+	68.6 (5.4)	145.7 (13.7)
4	α-DNP-$(lys)_{\overline{8.4}}$	+	+	15.1 (2.5)	150.5 (24.9)

TABLE V

Inability of the Tolerogens to Induce a Primary Response to DNP

No. of mice	Challenge	PFC/10^6 ($\pm$ SE)
9	DNP_{62}-KLH	99.9 (11.1)
6	None	4.0 (0.6)
5	α-DNP-$(lys)_{8.4}$	4.2 (0.6)
5	DNP_{23}-MGG	1.9 (0.5)
4	DNP_{12}-MSA	6.3 (2.5)

their ability to induce direct PFC when injected in CFA and (*b*) by their ability to induce the formation of circulating antibodies when injected repeatedly in CFA into BDF_1 and BALB/c mice. Since the results in both strains were similar, only the results obtained in BDF_1 mice are mentioned. The results are summarized in Table V and Fig. 6 and 7.

An inability to induce a primary response to DNP was a common feature of all the tolerogenic conjugates when the immune response was tested by the hemolytic plaque assay (Table V). However, the various tolerogens differed in their ability to provoke an immune response when injected repeatedly. When tested by the modified Farr technique the sera of most of the animals had no anti-DNP antibody (Fig. 6). However, when the microhemagglutination technique was used, the sera of mice given DNP-serum and DNP-C57BL/6J albumin contained anti-DNP antibodies in moderately high titers. No antibody was detected in the sera of mice injected with polylysine conjugates, and

low titers of antibody were found in 2/5 animals hyperimmunized with DNP-C57BL/6J IgG (Fig. 7).

DISCUSSION

These results show that when mice receive a hapten (DNP) bound to a nonimmunogenic carrier, they are unable to respond to the same hapten when it is bound to an immunogenic carrier (KLH). The unresponsiveness induced by treatment with the nonimmunogenic hapten carrier conjugate has all the characteristics of immunologic tolerance: it has a definite induction time; it is transient, but can be maintained by additional injections of the tolerogen; it is dose dependent; the unresponsiveness is highly specific for the hapten. Further

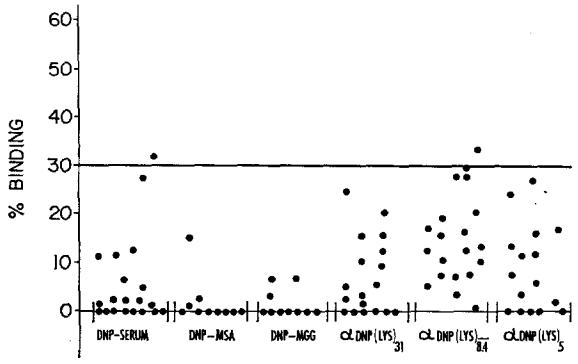

FIG. 6. *Radioactive immunoassay according to a modification of Farr's technique.* 30% binding should be considered the limit of the sensitivity of the technique, which can detect 0.002 mg of antibody. Each sample was analyzed in duplicate for each group of 10 mice (except for the groups immunized with DNP-MSA and DNP-mouse gamma globulin (MGG), which have five mice each).

more, as we will show elsewhere, the tolerance of DNP can be transferred or abrogated by lymphoid cells.[2]

It is widely held that tolerance can be induced only by immunogenic substances. This notion has been previously challenged by Schechter et al., who found that rabbits pretreated with poly-DL-alanine, which by itself is nonimmunogenic, are unresponsive to that polypeptide when it is bound either to HSA or ribonuclease (11). Ordinarily, conjugates of poly-DL-alanine with those carriers are immunogenic in the rabbit. Similar results were obtained by Maurer et al. and Roelants and Goodman by using the nonimmunogenic polyglutamic acid to induce tolerance (12, 13). Poly-DL-alanine was sub-

[2] Borel, Y., U. Jehn, and D. T. Golan. Cellular cooperation in the induction of tolerance to DNP. In preparation.

sequently found to be immunogenic when certain conditions of immunization were used (14), and in our experiments DNP-isogeneic serum, which was nonimmunogenic under conditions where it was tolerogenic, was weakly immunogenic when administered repeatedly in CFA. The difference between immunogenicity and nonimmunogenicity may thus be more apparent than real, and reflect only the mode of immunization and the sensitivity of the techniques used to reveal immunity.

With these limitations in mind, it seems that defined conjugates of DNP, such as α-DNP-$(lys)_5$, α-DNP-$(lys)_{\overline{8.4}}$, and α-DNP-$(lys)_{31}$ are not immunogenic in mice. Using two modes of immunization and three different methods of antibody detection, we found no anti-DNP antibodies despite repeated administration of these conjugates in complete Freund's adjuvant. These results confirm the work of Pinchuck and Maurer (15). What is of importance

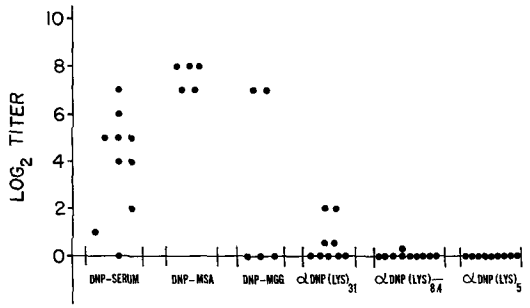

FIG. 7. *Passive hemagglutination test using* DNP_{60}*-BSA-coated SRBC.* Each point represents individual results of each sample tested.

is that, although these substances were not immunogenic, they were tolerogenic. With the reservation that nonimmunogenicity may merely be an operational definition, this result implies that immunogenicity is not an obligatory requirement for the induction of immunologic tolerance.

Collotti and Leskowitz, who studied tolerance in terms of delayed hypersensitivity in guinea pigs, concluded that immunogenicity is a requirement for the induction of tolerance (16). In other work,[3] we found that DNP-$(lys)_5$, which is nonimmunogenic in guinea pigs, failed to induce tolerance in terms of delayed hypersensitivity to DNP-$(lys)_{\overline{8.4}}$, which is immunogenic. Several reasons may account for this apparent discrepancy. First, the mechanism of suppression of delayed hypersensitivity and antibody formation, which are different immunologic phenomena, may be different. Second, the suppression of delayed hypersensitivity and antibody formation may, under certain conditions, be mediated by different antigenic determinants on the substance

[3] Borel, Y., and S. Schlossman. Unpublished observation.

inducing tolerance. Third, the difference between the two animal species used and between their relative degrees of immunological maturity might also account for this discrepancy.

The ability to induce tolerance of an antigen with a nonantigenic substance may permit separation of the phenomenon of tolerance from that of immunity. Other data showing that the part of the antigen against which antibody reacts in vitro is not the same as the part of the antigen responsible for the induction of tolerance in vivo, also support the notion that these immunologic phenomena can be dissociated from each other (17). It is conceivable that tolerance and immunity involve different systems of cellular recognition. Whether the tolerogen and antigen compete for the same or different receptors on a single cell or on different cells is not known.

Sela has suggested that the part of the antigen molecule that is most important for antibody formation is not necessarily the most important for the induction of tolerance (18). According to him, the latter property of the molecule may be strongly influenced by its carrier moeity. Our data illustrate this point. The antibody produced by animals immunized with DNP conjugates is directed primarily at DNP; however, DNP alone or DNP bound to one lysine is ineffective in inducing tolerance to DNP. By contrast, DNP bound to $(lys)_5$, $(lys)_8$ and $(lys)_{31}$ are tolerogens. In further contrast, DNP bound to KLH is an immunogen but a poor tolerogen. Here we see a clear example of the influence of the carrier on the induction of tolerance. In the case of a simple hapten such as DNP, we can conclude that its carrier determines its immunological properties: lacking a carrier, DNP is immunologically inert; when conjugated to immunogenic carriers such as KLH or HGG, it is immunogenic; when conjugated to nonimmunogenic carriers, like α-DNP-$(lys)_{\overline{8.4}}$ or isologous IgG, it is tolerogenic. Among the nonimmunogenic or poorly immunogenic carriers, isogeneic IgG appears to be the most effective, not only to induce tolerance to DNP but also to maintain it.

An inverse relation between antigenicity and tolerogenicity has been suggested (13). This is supported by our finding that a carrier, to which an individual is naturally tolerant, has a greater ability to induce tolerance to the hapten than foreign carriers. It has also been found that animals rendered tolerant to a carrier cannot respond to a hapten attached to it (19). However, this apparent inverse relation between immunogenicity and tolerogenicity of the conjugate does not appear to be a general phenomenon as shown in Fig. 5.

It is of interest that among the various carriers tested, IgG was the most tolerogenic carrier. A similar finding was reported by Havas (20). Whether other serum proteins will also show this ability is not yet known. What accounts for the difference among these molecules is not yet clear. Whatever the explanation, these data illustrate the importance of the selection of the carrier

when attempting to induce tolerance to its haptenic moiety. This might explain why other investigators were only partially successful in inducing tolerance to a hapten by treatment with these haptens conjugated to mouse serum albumin (MSA), BSA, or BGG (21, 22).

It might not be fortuitous that a molecule known to be an antibody functions also in the induction of tolerance. Whether or not this property of IgG is merely coincidental is presently under investigation. It is conceivable that IgG plays some role in the induction or maintenance of tolerance.

These findings have many implications, not only in approaching the understanding of tolerance, but also in advancing a clinically feasible solution to the induction of tolerance. For example, it is not difficult to envision the induction of tolerance of tissue antigens by treatment of a prospective allograft recipient with purified histocompatibility antigens coupled to nonimmunogenic carriers. Whatever the results of such experiments will be, induction of tolerance with a nonimmunogenic substance allows the study of the cellular mechanism of this immunologic phenomenon in the absence of the technically undesirable immune response.

SUMMARY

Treatment of adult mice with dinitrophenyl (DNP) bound to isogeneic serum resulted in a specific inability to respond to DNP after challenge with DNP-keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant. The unresponsiveness to the hapten had all the characteristics of immunologic tolerance: it had a definite induction time; it was transient but could be maintained by additional injections of the tolerogen; it was antigen specific and dose dependent. In addition, the induction of tolerance to DNP is dependent on the nature of the carrier.

Two main conclusions can be drawn from these data: DNP conjugates of three homopolymers of lysine were found to be nonimmunogenic in mice, yet tolerogenic. Thus, antigenicity is not necessary to induce tolerance.

Among the various carriers tested, isogeneic 7S immunoglobulin (IgG) was found to be the most effective to induce and maintain tolerance to the hapten. This suggests that IgG may have a function other than its usual role as an immunoglobulin.

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