

SUPPRESSION OF THE ANTI-DNP IgE RESPONSE WITH TOLEROGENIC CONJUGATES OF DNP WITH POLYVINYL ALCOHOL

I. Specific Suppression of the Anti-DNP IgE Response¹

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The capacity of $B_6D_2F_1$ mice to produce anti-DNP reagenic (IgE) antibodies and anti-DNP plaque-forming cells as a result of immunization with DNP₃-OA in presence of A1(OH)₃ was specifically suppressed by treatment of mice—before or after immunization—with tolerogenic conjugates consisting of the hapten coupled to polyvinyl alcohol (PVA) with m.w. of 10,000 or 14,000. The suppression was dose-dependent, and the effectiveness of the suppression increased with increasing epitope density (DNP_n-PVA; $n = 0.5$ to 4.3). Thus, although a single dose of 0.1 mg of DNP_{1.4}-PVA₁₄ depressed significantly an ongoing anti-DNP IgE response, complete suppression of this response was achieved with 1 mg of the conjugate; significantly lower amounts of the conjugate were needed to dampen the *de novo* anti-DNP IgE response. This mode of suppression was uniquely hapten specific, since it did not affect the immune response of the host to the carrier. Further evidence for the specific unresponsiveness to DNP of the mice treated with DNP-PVA conjugates was provided by the finding that although mice immunized to DNP₃-OA in the presence of *Bordetella pertussis* died of anaphylaxis on i.v. challenge with 2 mg of DNP₃-M γ G or OA, mice that had been immunosuppressed did not show any discomfort on challenge with DNP₃-M γ G, but died within 30 min after i.v. administration of OA. The unresponsive state of the spleen cells of mice that had been rendered tolerant to DNP was maintained even after cell transfer into x-irradiated syngeneic mice. It was also demonstrated that i) the interaction of DNP-specific B₆ cells with OA-specific T cells resulted in a marked anti-DNP IgE response, whereas the B cells of mice that had been treated with DNP_n-PVA cooperated poorly with OA-specific T cells and produced only a low level of anti-DNP IgE antibody; ii) the B cells of tolerized mice did not affect the cooperation of B and T cells of immune mice in the production of IgE responses.

It appears that there exists an inverse relationship between the immunogenicity and tolerogenicity of hapten-carrier conjugates. Thus, although haptens attached to highly immunogenic carriers elicit a marked anti-hapten response, animals tolerized to the carrier or animals genetically unresponsive to the carrier give little or no response when the hapten is presented on that carrier. In support of this generalization, one may cite the findings that

presentation of haptens on nonimmunogenic carriers, such as isologous serum proteins (1, 2), isologous red blood cells (3), nonimmunogenic or weakly immunogenic polypeptides (4, 5), polysaccharides (6, 7), carboxymethyl cellulose (8), and polyvinyl pyrrolidone (9), induced partial or complete hapten-specific tolerance.

Recently, it was shown in this and other laboratories that the formation of IgE antibodies to haptens, such as the benzylpenicilloyl (BPO)² group, that is the major immunogenic determinant in penicillin allergy (10), and the 2,4-dinitrophenyl (DNP) group, could be readily suppressed in mice (11–14), rats (15), and dogs (16) by treatment of these animals with conjugates consisting of the corresponding hapten coupled covalently to the isologous γ -globulins (γ G), i.e., mouse γ G, rat γ G, and dog γ G, respectively. The most important finding of these investigations was that immunologically specific suppression of IgE antibodies to either of these haptens could be induced readily by this regimen not only in normal animals, but also in sensitized animals; i.e., this treatment abrogated the primary as well as ongoing IgE responses.

Studies have been also conducted in recent years in many laboratories with a view to developing regimens that would lead to the immunosuppression of IgE antibodies to high m.w. antigens. For this purpose, the antigens were modified by denaturation with urea (17), photo-oxidation (18), or by coupling to nonimmunogenic polymers, such as the polymer of D-glutamic acid and D-lysine (D-GL) (19), isologous γ -globulins (20), polyethylene glycol (PEG, HO-(CH₂CH₂O)_n-CH₂CH₂OH) (21, 22), or the monomethoxy derivative of PEG (23, 24).

It is important to note that although PEG conjugates of common allergens, such as ovalbumin (OA) or the high m.w. constituents of ragweed and Timothy grass pollens, proved to be tolerogenic, DNP-PEG conjugates were shown in this laboratory to be devoid of the capacity to suppress the production of anti-hapten IgE antibody responses in normal or in sensitized mice. Hence, a search for synthetic polymers, isomeric or analogous to PEG, capable of acting as tolerogenic carriers for haptens was initiated in an attempt to unravel the molecular features that endow a hydrophilic carrier with immunosuppressive property.

In the present study was explored the possibility of inducing specific suppression of the immune response to DNP by administration of conjugates of DNP with the hydrophilic and nonimmunogenic polymer, polyvinyl alcohol (PVA, HO-(CH₂CHOH)_n-CH₂CH₂OH). The choice of this carrier was based on the fact that PEG and PVA consist, respectively, of the isomeric units, (CH₂CH₂O) and (CH₂CHOH). Indeed, the results to be reported here demonstrate that administration of DNP-PVA conjugates, even with an average epitope density as low as 0.5 to 1, resulted in an immunologically specific suppression not only of IgE antibodies, but also of antibodies belonging to other classes of immunoglobulins. Moreover, these tolerogenic conjugates were capable of

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² Abbreviations used in the text: BPO, benzylpenicilloyl group; PVA, polyvinyl alcohol; OA, ovalbumin; M γ G, isologous mouse γ -globulins; D-GL, copolymer of D-glutamic acid and D-lysine; PEG, polyethylene glycol; PCA, passive cutaneous anaphylaxis; ASC, extract of *Ascaris suum*; MYC, mycobacterium; BGG, bovine γ -globulins; HA, passive hemagglutination; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetyl group; PVP, poly-(N-vinylpyrrolidone).

suppressing the anti-hapten responses in both normal and sensitized animals.

MATERIALS AND METHODS

Animals. Inbred 6- to 10-wk-old (C57BL/6 × DBA/2)_{F1} mice (designated as B₆D₂F₁) were purchased from The Jackson Laboratory, Bar Harbor, ME. For the passive cutaneous anaphylaxis (PCA) assays, random-bred hooded rats, supplied by Bio-Breeding Laboratories Ltd., Ottawa, were used.

Preparation of amino-derivatives of PVA (PVA-(NH₂)_x). For the sake of brevity, the 2 preparations of PVA, purchased from Aldrich Chemicals, Milwaukee, WI, with average m.w. of 10,000 and 14,000, will be referred to hereafter as PVA₁₀ and PVA₁₄, respectively. (All PVA preparations are produced by hydrolysis of the corresponding polyvinyl acetates; the degrees of hydrolysis of the PVA₁₀ and PVA₁₄ preparations obtained for this study were 88 and 100%, respectively.) To a solution of PVA₁₀ or PVA₁₄ (10 g) in 200 ml of distilled water was added 5 g of cyanogen bromide (Eastman Kodak, Rochester, NY). The pH of the reaction mixture was maintained at 11 by dropwise addition of 2N NaOH. Twenty minutes later, 10 g of 1,6-hexanediamine was added to the mixture, and it was stirred for 48 hr at room temperature. The reaction mixture was then dialyzed exhaustively against distilled water and lyophilized. The exact number of NH₂ groups grafted onto a molecule of PVA was not determined, since it was deemed more important to establish the average number of hapten molecules coupled per PVA molecule, as shown below.

Preparation of DNP_n-PVA. To a solution of 50 ml of 0.4 M Na₂CO₃ containing 0.5 g of PVA-(NH₂)_x was added 0.5 g of sodium 2,4-dinitrobenzene sulfonate (Baker Chemical Co., Phillipsburg, NJ), and the mixture was maintained at 37°C with constant stirring for 3 days. A small amount of precipitate, formed during this reaction time, was separated by centrifugation at 18,000 rpm in a Sorvall RC2-B centrifuge for 20 min and then dissolved in distilled water, dialyzed exhaustively, and lyophilized. The supernatant was passed through a column (4 × 60 cm) of Sephadex G-25 gel to remove any unreacted hapten. The effluent containing the conjugate was dialyzed against distilled water and lyophilized. The degree of substitution, *n*, i.e., the average number of DNP groups coupled per PVA molecule, was determined spectrophotometrically by taking the molar absorption coefficient for DNP as 17,400 at 360 nm. It was thus established that the degrees of substitution of the conjugates present in the precipitate and supernatant of the reaction mixture were 2.4 and 1.5, respectively. As indicated below, it appears that this relatively low degree of substitution was due to the partial precipitation of PVA-NH₂, that was subsequently diminished by using the lower concentration of Na₂CO₃ at 0.2 M.

The other conjugates were prepared according to the above method with small modifications. Thus, for the preparation of DNP_{3.1}-PVA₁₀, the concentrations of the reagents were identical to those used above, except that 0.2 M Na₂CO₃ was used in the reaction mixture, which was maintained for only 2 days at room temperature; under these conditions, no precipitate was formed. The reaction conditions for the preparation of DNP_{4.3}-PVA₁₄ were identical to those used for the preparation of DNP_{3.1}-PVA₁₀, except that the amount of PVA₁₄(NH₂)_x used in the reaction mixture was increased to 0.75 g. The epitope density of PVA₁₄ conjugates was higher than that of the PVA₁₀ derivatives, since the content of amino groups of PVA₁₄(NH₂)_x was higher than that of PVA₁₀(NH₂)_x.

Preparation of hapten-protein conjugates. The DNP₃-OA and DNP₁₈-BGG conjugates were synthesized as reported earlier (11). The 2,4-dinitrophenylated extract of *Ascaris suum* (DNP-ASC), that contained 6.5×10^{-8} M

DNP/mg ASC, was prepared by reacting 46 mg of ASC with 24 mg of the sodium salt of 2,4-dinitrobenzene sulfonate in the presence of 46 mg of Na₂CO₃ in a total volume of 5.5 ml of distilled water at 37°C for 2 hr.

Immunization and measurement of reaginic and hemagglutinating antibody responses. For optimal anti-DNP and anti-OA IgE responses, mice were injected i.p. with the standard, low dose of 1 µg of DNP₃-OA suspended with 1 mg of freshly prepared aluminum hydroxide in 0.5 ml of phosphate-buffered saline (PBS); this dose, when administered by the i.p. route, will be referred to hereafter as the sensitizing dose of DNP₃-OA. Mice were also sensitized with 10 µg of DNP-ASC in presence of 1 mg of A1(OH)₃ in 0.5 ml of PBS for the induction of anti-DNP and anti-ASC IgE antibodies. Groups of 4 mice were treated in an identical manner, and the sera of mice within each group were pooled for determination of the reaginic and hemagglutinating antibody responses.

The average reaginic titers were measured by the PCA assay in outbred hooded rats (11). The end point of the titration was taken as the reciprocal of the highest dilution of each serum resulting in a reaction of 5 mm in diameter. The PCA titers are reported as averages of 2 determinations in 2 different rats.

The presence of anti-DNP and anti-OA antibodies belonging to immunoglobulin classes other than reagins was demonstrated by the passive hemagglutination (HA) procedure, utilizing conjugates of sheep red blood cells (SRBC) with DNP₁₈-BGG or OA, that were prepared by cross-linking with glutaraldehyde as described by Avrameas *et al.* (25).

Adoptive cell transfer. This procedure consisted of the transfer of spleen cells from sensitized and tolerized mice into x-irradiated (650 R) syngeneic recipients and injection of the recipients with the standard sensitizing dose of antigen in presence of A1(OH)₃ within 4 hr after cell transfer.

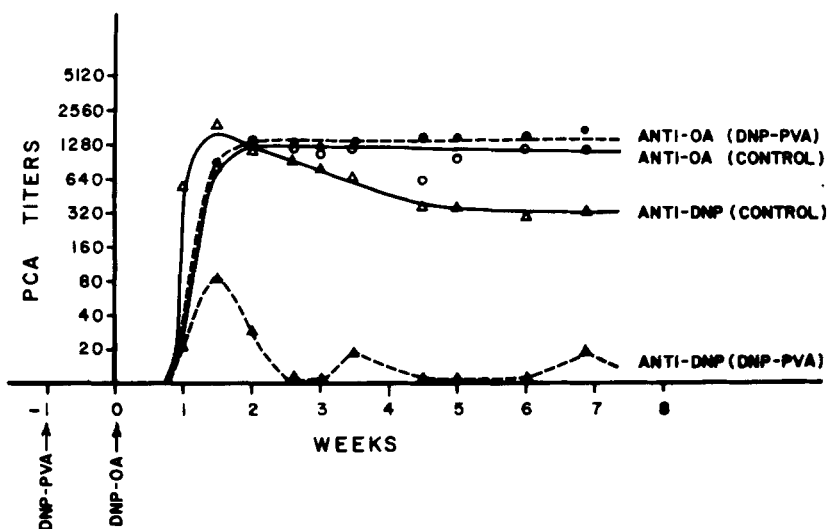
RESULTS

Suppression of anti-DNP IgE responses with DNP_{3.4}-PVA₁₀. To test the capacity of DNP_{3.4}-PVA₁₀ to suppress the induction of the anti-DNP IgE response, a single i.p. dose of 1 mg of DNP_{3.4}-PVA₁₀ was injected into normal mice 1 wk before sensitization with the standard dose of 1 µg of DNP₃-OA in the presence of 1 mg of A1(OH)₃. As is evident from the results illustrated in Figure 1, the anti-DNP IgE response was profoundly suppressed, in relation to both its level and duration, in mice treated with this DNP-PVA conjugate. Moreover, the suppression was antigen specific as demonstrated by the fact that the anti-OA IgE response was virtually unaffected.

Abrogation of an ongoing anti-DNP IgE response with DNP_{3.4}-PVA₁₀. The possibility of using the above immunosuppressive approach also for the abrogation of an ongoing anti-DNP IgE response was explored by injection of 1 mg of DNP_{3.4}-PVA₁₀ into mice that had received a sensitizing dose of DNP₃-OA 14 days earlier. Thus, as substantiated by the data plotted in Figure 2, it is clear that the anti-DNP IgE response was abrogated readily after injection of the tolerogen. However, these mice continued to produce high levels of anti-OA IgE antibodies, that were indistinguishable from those produced by the control mice.

Effect of dose of DNP_{1.4}-PVA₁₄ on suppression of the anti-DNP IgE response in normal and primed mice. Normal mice and immune

Figure 1. Suppression of the induction of anti-DNP IgE responses with DNP_{3.4}-PVA₁₀. All mice received a sensitizing dose of 1 µg of DNP₃-OA in the presence of 1 mg of A1(OH)₃. Mice in the test group received an additional i.p. injection of 1 mg of DNP_{3.4}-PVA₁₀ 7 days before sensitization. The IgE responses specific for the DNP determinants (Δ—Δ) and for the carrier (○—○) are expressed in terms of PCA titers.



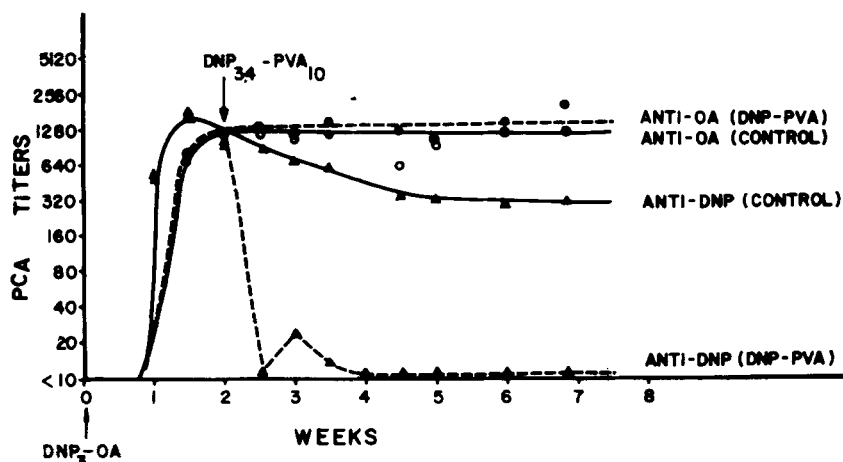


Figure 2. Abrogation of an ongoing anti-DNP IgE response with DNP_{3.4}-PVA₁₀. All mice were sensitized with 1 μ g of DNP₃-OA in presence of 1 mg of Al(OH)₃. Mice in the test group received an additional i.p. injection of 1 mg of DNP_{3.4}-PVA₁₀ 14 days after sensitization. The IgE responses specific for the DNP determinant and the carrier are represented by triangles and circles, respectively.

mice, which had received a sensitizing dose of DNP₃-OA 34 days earlier, were administered i.p. varying doses of the tolerogen 1 day before being injected with DNP₃-OA in presence of Al(OH)₃. It is evident from the upper diagram of Figure 3 that treatment of normal mice with as low a dose of DNP_{1.4}-PVA₁₄ as 1 μ g resulted in an 18-fold reduction of the anti-DNP IgE antibodies without affecting at all the anti-OA IgE response. Moreover, as would be expected, the suppression of the anti-DNP IgE response in sensitized mice required higher doses of the tolerogen. Thus, as shown in the lower diagram of Figure 3, a single dose of 100 μ g of DNP_{1.4}-PVA₁₄ induced a significant degree of suppression in sensitized mice, and complete abrogation of their anti-DNP IgE response was achieved with a dose of 1 mg. By contrast, injection of free PVA (either PVA₁₀ or PVA₁₄) even at a dose as high as 10 mg did not affect the IgE responses; these negative results are not shown in Figure 3.

Effect of time of injection of DNP_{4.3}-PVA₁₄ on reaginic antibody formation. Mice were injected i.p. with the tolerogen (1 mg per mouse) at various intervals either before or after the primary immunization with DNP₃-OA in Al(OH)₃, and their sera were collected at the peak of the reaginic antibody response and assayed for PCA activity. From the results listed in Table I, it is obvious that the primary anti-DNP IgE response was markedly dampened at different times of injection of the tolerogen and that the immunosuppressive effect was more pronounced when the tolerogen was injected close to the time of immunization, i.e., within the interval between day -7 and day +4. Moreover, it is important to note that the tolerogenic effect of DNP_{4.3}-PVA₁₄ was sustained for a long period (for at least 6 wk); i.e., the immunosuppressed mice, including those that had received the injection of the tolerogen on day -14, remained unresponsive to the injection of the second sensitizing dose of DNP₃-OA on day 28.

Suppression of anti-hapten plaque forming cell (PFC) response to TNP-SRBC with DNP_{1.7}-PVA₁₄. For testing the effect of DNP_{1.7}-PVA₁₄ on the production of antibodies belonging to classes other than IgE, mice received a single i.p. injection of various doses of this conjugate 1 day before their immunization with an i.v. dose of 5×10^8 TNP-coupled sheep red blood cells (TNP-SRBC). Both direct and indirect anti-hapten PFC responses were assayed with TNP-coupled horse red blood cells (TNP-HRBC) by the modified Jerne hemolytic plaque assay (26, 27) 6 days after immunization. As is evident from the data given in Table II, both IgM and IgG PFC responses were progressively suppressed with increasing doses of DNP_{1.7}-PVA₁₄.

In experimental data not shown here, it was found that treatment of mice with DNP-PVA conjugates suppressed also the formation of circulating anti-hapten antibodies elicited by DNP₃-OA, which were demonstrated by HA. Moreover, this treatment suppressed the anti-hapten PFC response to DNP-Ficoll, that is a T-independent antigen. This finding indicated that B cells were tolerized even without the participation of T cells by treatment with DNP-PVA.

Tolerogenicity of DNP-PVA₁₀ conjugates with different epitope densities in sensitized mice. The effect of the epitope density on the tolerogenicity of conjugates of DNP with PVA was studied by

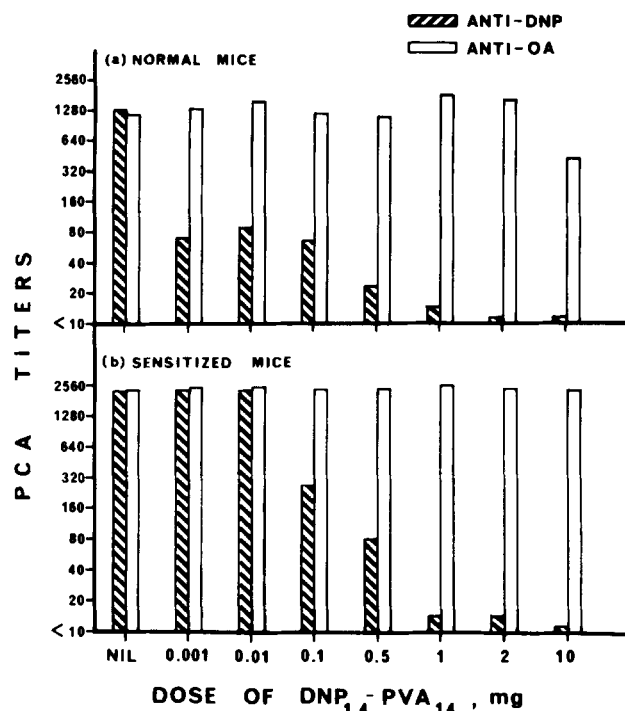


Figure 3. Effect of dose of DNP_{1.4}-PVA₁₄ on suppression of the anti-DNP IgE response in normal and primed mice. Normal mice, or mice sensitized 34 days earlier, received i.p. varying doses of the tolerogen 1 day before injection of a sensitizing dose of DNP₃-OA. The primary and secondary IgE responses were determined on days 14 and 7, respectively, after sensitization.

injecting i.p. 1 mg of different conjugates varying in their degree of substitution into mice that had received a sensitizing dose of DNP₃-OA 42 days earlier. The mice received 2 additional injections of the sensitizing dose of DNP₃-OA 1 and 4 wk after the injection of the conjugates, and their IgE antibody titers were determined 7 days after the 2nd and the 3rd injections of DNP₃-OA. The results shown in Table III demonstrate that complete suppression of the anti-DNP IgE response was achieved by injection of 1 mg of DNP-PVA₁₀ conjugates with an average epitope density of 1 or more. On the other hand, the conjugate with an epitope density significantly lower than 1, i.e., DNP_{0.5}-PVA₁₀, only partially suppressed the anti-DNP IgE response. However, as stated earlier, the injection of free PVA did not have any effect whatsoever on either the anti-DNP or anti-OA IgE response; this finding thus provides additional evidence for the specificity of the tolerogenic effect of hapten-PVA conjugates.

Hapten-specificity of the target cells immunosuppressed by DNP_{2.4}-PVA₁₀. The results of the preceding sections demonstrate that injection of mice with DNP-PVA conjugates dampened specifically the anti-DNP antibody responses, from which it may be

TABLE I
Effect of time of injection of DNP₃-PVA₁₄ on reaginic antibody formation

Time of Treatment ^a	PCA Titers			
	Primary		Secondary	
	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
Day -14	90	1580	70	3160
Day -10	210	1580	220	3800
Day -7	30	1280	150	2950
Day -3	30	1320	30	2400
Day -1	50	1280	50	2630
Day 0	30	1280	20	4170
Day 1	90	1510	80	6610
Day 4	70	1380	90	3980
Day 7	190	2340	330	5750
Day 14 ^b			70	5890
Day 21 ^b			180	6460
Control group	1050	1320	1510	3090

^a The mice were treated with the tolerogen on the same day (i.e. day 0), or before, or after i.p. immunization with DNP₃-OA in Al(OH)₃. The primary IgE responses were determined on day 14 after immunization. For the induction of secondary responses, all mice were sensitized with DNP₃-OA on day 28 and the PCA titers were determined 7 days later. The control group of mice received only 2 sensitizing doses of DNP₃-OA on days 0 and 28.

^b The primary anti-DNP and anti-OA IgE responses of these 2 groups (before the injection of the tolerogen) were identical to those of the control groups, i.e. 1,050 and 1,320, respectively.

TABLE II
Suppression of anti-hapten PFC responses to TNP-SRBC

Dose ^a of DNP _{1,7} -PVA ₁₄ (μg)	PFC/Spleen ^b		% Suppression	
	IgM	IgG	IgM	IgG
Nil	6,100	37,000	0	0
1	5,600	34,000	8.2	8.1
10	5,000	33,000	18.0	10.8
100	2,500	33,000	59.0	10.8
1,000	2,400	18,000	60.7	51.4
2,000	2,300	11,000	62.3	70.3

^a Various doses of DNP_{1,7}-PVA₁₄ were administered i.p. into mice 1 day before immunization with an i.v. dose of 5×10^6 TNP-SRBC.

^b The direct and indirect anti-hapten PFC responses were assayed 6 days after immunization with TNP-SRBC.

inferred that the DNP-specific B cells were the target cells that were immunosuppressed by these conjugates. To test this inference, an experiment was designed to examine the effect of DNP-PVA conjugates on the production of anti-DNP IgE antibodies that were elicited by conjugates of DNP with different immunogenic carriers. For this purpose, mice were injected with 1 mg of DNP_{2,4}-PVA₁₀ 2 days before receiving a sensitizing dose of DNP₃-OA (Expt. 1) or DNP-ASC (Expt. 2) and 4 wk later were administered a sensitizing dose of the alternate carrier conjugate, i.e., DNP-ASC (Expt. 1) or DNP₃-OA (Expt. 2), respectively. From the results given in Table IV, it is obvious that the anti-DNP IgE response was suppressed by the single injection of DNP_{2,4}-PVA₁₀ regardless of the nature of the carriers of the immunizing antigens. Hence, these results support the view that the DNP-specific B cells are either eliminated or inactivated by treatment with DNP-PVA conjugates. However, it is to be stressed that these results may also be attributed to the induction of hapten-specific suppressor cells, as has been shown in experiments reported below.

Maintenance of unresponsiveness in adoptive cell transfer system. The donor mice were immunized with a sensitizing dose of DNP₃-OA 49 days before the cell transfer. As indicated in Table V, at various times before cell transfer the donors had received an additional i.p. injection of 1 mg of DNP_{2,4}-PVA₁₀, and their spleen cells (3×10^7) were transferred into x-irradiated (650 R) recipients, which were also given a sensitizing dose of DNP₃-OA within 4 hr after cell transfer. The results shown in Table V clearly demonstrate that although the splenic cells of donors that had received DNP_{2,4}-PVA₁₀ 2 or more days before transfer into x-irradiated animals remained unresponsive, the cells of donors that had received the tolerogen 5 hr or 1 day before transfer were not significantly suppressed. Hence, one may conclude that the treatment of mice with DNP_{2,4}-PVA₁₀ requires at least 2 days to render the cells unresponsive.

Evidence for tolerization of B₁ cells with DNP_{1,7}-PVA₁₄. The results of the experiment illustrated in Table VI demonstrate that the anti-DNP IgE response could be produced in the adoptive transfer system by the cooperation of hapten-primed B cells and carrier-primed T cells. For this experiment, DNP-specific primed B cells, designated as B_{DNP(p)}, were obtained by treatment with anti- θ serum and complement (C) of spleen cells from mice that had been immunized 120 days previously with 10 μg of DNP-ASC and 1 mg of Al(OH)₃. The DNP-specific tolerized B cell population, designated as B_{DNP(t)}, was obtained by the treatment with anti- θ serum and C of spleen cells of mice, which had been sensitized with DNP-ASC and tolerized by an i.p. injection of 2 mg of DNP_{1,7}-PVA₁₄, respectively, 120 and 7 days before sacrifice. The carrier-primed T cells, designated as T_{OA(p)}, were isolated by passage over a nylon wool column of spleen cells of mice that had been immunized with 1 μg of OA in the presence of 1 mg of Al(OH)₃ 90 days before sacrifice. Different combinations of these cell populations were transferred into x-irradiated (650 R) recipients, which received also 10 μg of DNP₃-OA in 0.5 ml saline. As is evident from the data given in Table VI, the cooperation of B_{DNP(p)} and T_{OA(p)} cells resulted in the marked production of both anti-DNP and anti-OA

TABLE III
Tolerogenicity of DNP_n-PVA₁₀ conjugates with different epitope density in sensitized mice

Groups	Compound Injected ^a	PCA Titers ^b			
		Secondary		Tertiary	
		Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
I	PBS	3470	3470	5120	6160
II	PVA ₁₀	3310	3470	5750	6610
III	DNP _{0.5} -PVA ₁₀	830	3470	3390	5390
IV	DNP ₁ -PVA ₁₀	<10	3160	<10	6610
V	DNP ₄ -PVA ₁₀	<10	3530	<10	7090
VI	DNP _{3.1} -PVA ₁₀	<10	3390	<10	6160

^a All mice were sensitized with DNP₃-OA 42 days before the i.p. injection of 0.5 ml PBS, or 1 mg of free PVA₁₀, or 1 mg of DNP_n-PVA₁₀ conjugates of varying epitope density. All mice received 2 sensitizing doses of DNP₃-OA 1 and 4 wk after the injection of the preparations listed above.

^b The IgE responses were determined by PCA assay 7 days after administration of the 2nd and 3rd sensitizing doses of DNP₃-OA.

TABLE IV
Suppression of DNP-specific B₁ cells by DNP_{2,4}-PVA₁₀

Groups	Treatment ^a		PCA Titers			
	Day	Compound	Day	Anti-DNP	Anti-OA	Anti-ASC
Experiment 1 Control	-2	Saline				
	0	DNP ₃ -OA	7	<10	<10	N.T. ^b
			14	1700	1820	N.T.
	28	DNP-ASC	35	3470	3160	20
			42	2690	830	70
Test	-2	DNP-PVA				
	0	DNP ₃ -OA	7	<10	<10	N.T.
			14	20	1740	N.T.
	28	DNP-ASC	35	<10	1700	10
			42	<10	760	80
Experiment 2 Control	-2	Saline				
	0	DNP-ASC	7	<10	N.T.	<10
			14	300	N.T.	100
	28	DNP ₃ -OA	35	3540	80	370
			42	3500	480	320
Test	-2	DNP-PVA				
	0	DNP-ASC	7	<10	N.T.	<10
			14	<10	N.T.	80
	28	DNP ₃ -OA	35	<10	20	240
			42	20	810	80

^a In experiment 1, all mice received sensitizing doses of DNP₃-OA and DNP-ASC on days 0 and 28, respectively. The test mice received an additional injection of 1 mg of DNP_{2,4}-PVA₁₀ on day -2. In experiment 2, all mice were immunized with sensitizing doses of DNP-ASC and DNP₃-OA on days 0 and 28, respectively. The test mice received the additional injection of 1 mg of DNP_{2,4}-PVA₁₀ on day -2.

^b N.T., not tested, since the animals were not immunized with the corresponding carriers.

^b The PCA titers refer to the reaginic antibodies in sera of mice 7 days after immunization.

capable of also abrogating readily an ongoing anti-DNP IgE response in sensitized mice. Moreover, treatment with DNP-PVA conjugates of immunized mice suppressed effectively not only the anti-DNP IgE response, but also antibodies to the hapten belonging to other classes of immunoglobulins, that were detected by the hemagglutination reaction and the PFC assay (Table II). Thus, a single injection of 0.1 mg was sufficient to induce a marked depression of the anti-DNP IgE response, which was completely abrogated on increasing the dose to 1 mg (Fig. 3); for a marked suppression of anti-hapten PFC responses, the dose of DNP-PVA had to be increased to 2 mg (Table II). The time of injection of DNP-PVA conjugates did not appear to be critical, since, as documented by the data of Table I, suppression of the anti-hapten response was achieved by a single injection of the tolerogen either before (days -14 to -1), after (days 1 to 21), or at the time of sensitization (day 0). The specificity of the suppression by hapten-PVA conjugates is also born out by the fact that free PVA even at a dose as high as 10 mg did not lead to any detectable depression of the immune response. Furthermore, as is evident from the results given in all figures and tables, the injection of hapten-PVA conjugates did not affect the anti-OA IgE response, which constituted an "internal control" for the hapten-specificity of the conjugates.

The results presented in Table IV indicate that after injection of DNP-PVA conjugates the anti-DNP IgE response remained suppressed regardless of the nature of the carrier molecule of the immunizing antigen used before or after the injection of the DNP-PVA conjugates. Hence, these results suggest that administration of hapten-PVA conjugates may lead to the elimination or inactivation of hapten-specific B lymphocytes. In fact, this suggestion is supported by the results shown in Table VI, which demonstrate that hapten-specific tolerance was induced in the B₆ cells of mice that had been treated with DNP-PVA. Moreover, the experimental data illustrated in the accompanying article (29) clearly demonstrate that hapten-specific suppressor T cells were generated and that these cells had the capacity of actively suppressing the spleen cells of sensitized animals to mount a secondary IgE response on adoptive transfer.

The finding that hapten-specific suppressor cells were generated by DNP-PVA conjugates is reminiscent of the studies of Kishimoto and his colleagues (30, 31). These authors demonstrated that injection of conjugates of DNP with mycobacterium (DNP-MYC) induced DNP-reactive T cells, which on exposure to conjugates of DNP even with unrelated carriers released a suppressor factor capable of abrogating selectively the IgE antibody response not only to DNP, but also to all other determinants; i.e., the suppression induced by DNP-MYC was IgE class pan-specific but antigen nonspecific. Hence, the suppression induced by DNP-PVA conjugates differs in at least 2 aspects from that of DNP-MYC; viz, i) the suppression by DNP-PVA conjugates was highly hapten specific and did not affect IgE antibody responses to other antigenic determinants, and ii) DNP-PVA conjugates suppressed not only the anti-DNP IgE response, but also anti-DNP antibodies belonging to classes other than IgE (Table II).

In relation to the possibility of generating hapten-specific suppressor cells with hapten conjugates of nonimmunogenic carriers, it is to be noted that von Specht *et al.* (9) reported that conjugates of NIP (4-hydroxy-3-iodo-5-nitrophenylacetyl group) with the synthetic polymer, poly(*N*-vinylpyrrolidone) (PVP), induced suppression of both primary and secondary anti-DNP IgG and IgM responses and that the suppression of the antibodies belonging to the IgG class was more effective. Similarly, in recent experiments conducted in collaboration with von Specht, the present authors demonstrated (32) that a single injection of NIP₂-PVP suppressed the anti-NIP IgE response in mice by the activation of hapten-specific suppressor cells. Similar hapten-specific suppression has been recently induced in the authors' laboratory with DNP conjugates of 2 other synthetic polymers, i.e., polyacrylic acid and the copolymer of ethylene and maleic acid (33). However, it is to be stressed that in spite of these findings suggesting that hapten conjugates of a wide spectrum of nonimmunogenic and water soluble synthetic polymers may lead to the abrogation of hapten-specific immune responses, not all hydrophilic polymers function as tolerogenic carriers. Thus, as stated in the *Introduction*, DNP_{1,5}-PEG conjugates had proved to be devoid of the capacity to induce

hapten-specific suppression, and more recently, it was found in this laboratory that conjugates of DNP with gelatin were also incapable of inducing hapten-specific tolerance. Similarly, Watanabe and his colleagues (34) had reported that DNP-Ficoll conjugates suppressed only partially the primary anti-DNP IgE response without exerting any tolerizing effect on the secondary response.

With respect to the possible mechanism underlying the suppression induced by DNP-PVA conjugates, the results given in Table V demonstrate that the spleen cells of mice that had been treated with the tolerogen 2 to 14 days before adoptive cell transfer remained unresponsive in the irradiated recipients even on further exposure to sensitizing doses of DNP₃-OA. Moreover, as substantiated by the findings presented in Table V, the spleen cells of immune mice required *in vivo* contact with the tolerogen for a minimum of 2-3 days in order to become suppressed, since the cells of these animals did not show any impairment of their capacity to mount either anti-DNP or anti-OA IgE responses, if they were transferred earlier than 2 days after treatment with the tolerogen into x-irradiated recipients. This evidence, therefore, would support the inference that the suppression induced by hapten-PVA conjugates was not due to the carry-over of the tolerogen with the cells of the donors used in the adoptive cell transfer experiment; additional support for this interpretation is provided by the experimental data reported in the accompanying paper (29).

In addition to the suppression of the formation of circulating anti-hapten IgE antibodies, the results listed in Table VII demonstrate that the administration of DNP-PVA conjugates resulted also in the systemic desensitization of previously immunized animals, which would otherwise undergo anaphylactic shock on i.v. challenge with a polyvalent hapten conjugate such as DNP₃-M γ G. Hence, the mode of suppression described in this paper appears to have the potential for clinical application for the specific suppression of IgE antibodies mediating immediate type hypersensitivity diseases to small molecules such as those encountered in drug allergies. Indeed, as reported elsewhere (35), the suppression of IgE antibodies specific to the BPO group was achieved in sensitized mice by the administration of BPO-PVA conjugates.

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