CELLULAR IMMUNOLOGY 15, 321–335 (1975)

Induction of Primary and Inhibition of Secondary Antibody Response to Hapten by Hapten Conjugates of Type III Pneumococcal Polysaccharide[1]

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Received June 20, 1974

Tri- or dinitrophenylated pneumococcal polysaccharide type III (TNP- or DNP-SIII)) induced a primary 19S anti-TNP response without generating immunological memory to the hapten in LAF_1 mice. Hapten–hemocyanin (TNP-KLH) or hapten conjugates of *B. abortus* organisms (DNP-BA) induced both 19S and 7S primary responses and memory to the hapten. Spleen cells from mice immunized with TNP-KLH or DNP-BA did not give adoptive memory responses upon challenge with hapten-SIII and, in fact, were inhibited from responding to their homologous hapten conjugates by simultaneous injection of hapten-SIII. Incubation of TNP-KLH-primed spleen cells for as short as 5 min at 0°C with 10 μg of TNP-SIII per milliliter virtually abolished their ability to give 19S and 7S memory responses to TNP-KLH upon transfer into irradiated recipients. It is suggested that a difference in avidity and/or number of anti-TNP receptors per cell between virgin and primed B cells may be an important factor in determining whether the cells will be stimulated or inhibited by exposure to hapten-SIII. Another factor may be a difference between virgin and memory cells in their requirement for T-cell help.

INTRODUCTION

Immunization with hapten coupled to a polysaccharide carrier which, by itself, induces thymus-independent immune responses, may give rise to anti-hapten antibody (1, 2). The mechanism of cellular interaction involved, if any, is unclear, but it has been suggested that direct mitogenic activity of these carriers for B cells is of prime importance in inducing T-cell independent responses (3, 4). The anti-hapten response may have similar properties as the response to its carrier in that it is limited to antibody production of the 19S class (1, 2) and deficient in the formation of immunological memory (5). On the other hand, hapten conjugates of nonimmunogenic molecules such as a copolymer of D-glutamic acid and D-lysine (D-GL) (6–8), syngeneic γ-globulin (9, 10), and syngeneic red cells

[1] Supported by Grant AI-3076 from the United States Public Health Service and in part by Grant GFB13592 AI from the National Science Foundation.

[2] Supported by USPHS Training Grant No. GM000127.

[3] Recipient of National Institutes of Health Training Grant No. 5GM01668-11 from the National Institute of General Medical Science.

do not appear to induce immune responses, but on the contrary may induce tolerance to the hapten (11).

It has been suggested that "nonimmunogenic" hapten-carrier conjugates might have important applications in the abrogation of ongoing but unwanted humoral responses (12). It would be of interest if hapten conjugates could be found which specifically abolish some but not all facets of the immune response to the hapten. For example, B-cell tolerogens such as the dinitrophenylated (DNP) D-GL inhibit antibody production while leaving intact T-cell functions with specificity for the hapten such as delayed and contact hypersensitivity (13, 14). Another promising approach to this problem was initiated by Mitchell *et al.* (15), who demonstrated interference with the 7S antibody memory response to DNP-hemocyanin through injection of the DNP-lysine conjugates of pneumococcal polysaccharide type III (DNP-SIII).

The present studies were undertaken to determine (i) whether the trinitrophenyl–lysine conjugates of SIII (TNP-SIII) could induce an immune response to TNP with generation of immunological memory, and (ii) whether or not the inhibition of 7S memory expression observed by Mitchell *et al.* (15) also encompassed 19S memory and could be obtained by *in vitro* incubation of memory cells.

MATERIALS AND METHODS

Animals and Immunizations

Male young adult LAF_1 mice (Jackson Labs, Bar Harbor, ME) were used throughout. Donor mice were immunized by intravenous injection of 0.3 mg TNP_{18}-KLH usually with 10 μg *E. coli* endotoxin (Difco Laboratories, Inc., Detroit, MI). In a few experiments donor mice received 0.1 ml of a 0.3% (v/v) suspension (equivalent to 2×10^8) of killed *Brucella abortus* (BA) organisms. Whenever the interval prior to sacrifice of donor mice exceeded 2–4 months, they received a second dose intraperitoneally. Donors were used no earlier than 1 month after their last priming injection. Immunizations with TNP and DNP conjugates of SIII or of BA were always by intravenous injection of antigen in dosages as described in the tables. On the day prior to transfer, recipient mice received 630–750 R ^{137}Cs γ-irradiation from a gammator M (Radiation Machinery Corp., Parsippany, NJ).

Antigens

DNP_{30}-SIII and TNP_{110}-SIII were prepared by conjugation to SIII of ϵ-DNP–L-lysine HCl (Sigma Chemical Co., St. Louis, MO) or ϵ-TNP–L-lysine HCl (Nutritional Biochemicals Corp., Cleveland, OH), respectively, by the cyanogen halide method of Axen and Ernback (16) as described by Mitchell *et al.* (15). Trinitrophenylated sheep erythrocytes (TNP-SE) were prepared according to the method of Rittenberg and Pratt (17). TNP-BA and DNP-BA were prepared by the method of Little and Eisen (18). Briefly, trinitrobenzene–sulfonic acid (TNBS) was recrystallized from the commercial preparation (Eastman Kodak Co., Rochester, NY) by cooling from hot 1.0 *N* HCl and washing with cold HCl. To 20 mg of recrystallized TNBS or of dinitrobenzene–sulfonic acid (Eastman Kodak Co., Rochester, NY) dissolved in 2.0 ml of a 2% K_2CO_3 solution were added 2.0 ml of packed BA (U.S. Department of Agriculture) with stirring. Stir-

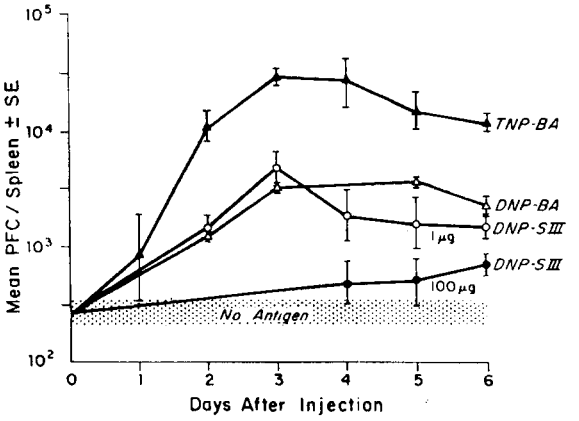

FIG. 1. Primary anti-TNP responses of LAF_1 mice expressed as PFC/spleen at various days after injection of DNP-BA (△——△) and TNP-BA (▲——▲) or 1 μg (○——○) and 100 μg (●——●) DNP-SIII. Each point represents the mean ± SE for 2–9 mice. Shaded area denotes the mean PFC level for normal spleen ± SE measured with TNP-sheep erythrocytes. The numbers of PFC/spleen against sheep erythrocytes were subtracted from all values.

ring was continued for an additional 2 hr at room temperature and overnight at 0°C. The mixture was dialyzed successively against distilled water, 0.1 *M* $KC_2H_3O_2$, and finally 0.15 *M* NaCl.

Dinitrophenyl groups were conjugated to human gamma globulin (HGG) and keyhole limpet hemocyanin (KLH) (Mann Laboratories, Orangeburg, NY) (18). The extent of coupling of DNP to HGG, KLH, and SIII was estimated from the molar extinction coefficient, 17,400, at 360 nm with 100,000 as the unit of molecular weight for the protein and polysaccharide carriers (15). The degree of TNP conjugation was estimated using the molar extinction coefficient, 15,400, at 346 nm.

SIII was prepared as described previously (19), and the amount of polysaccharide present in each of our hapten–polysaccharide conjugates was determined by the phenol–sulfuric acid reaction of Dubois *et al.* (20).

Cell Transfers

The spleens of donor mice were teased into a cell suspension in Medium 199 (Microbiological Associates Inc., Bethesda, MD) and washed twice. Cells were either injected intravenously into irradiated recipients with antigen or were incubated with antigen *in vitro* prior to transfer as described under Results.

Assays

Numbers of antibody-forming cells per spleen to TNP were enumerated by localized hemolysis in gel of TNP-SE (17). As a control, spleen cell suspensions were also plated against uncoated SE and the values obtained subtracted from those for PFC against TNP-SE. Rabbit anti-mouse Ig was used for development of indirect PFC (21). The numbers of 7S PFC were calculated by subtracting the direct PFC from the indirect PFC.

Sera from recipient mice were titrated for anti-TNP and anti-KLH by passive hemagglutination. Indicator cells were tanned cells (22) to which had been adsorbed 0.05 mg TNP_{13}–ovalbumin or KLH per milliliter of 2.5% tanned SE.

RESULTS

Primary Response to Hapten Presented on the BA and SIII Carriers

Initial studies of responses to a single injection of DNP-BA, TNP-BA, DNP-SIII, and TNP-SIII showed that all induced a rise in the number of PFC per spleen with anti-TNP specificity, but with large differences in effectiveness between the SIII and the BA carrier. Figure 1 shows the responses to DNP-BA and TNP-BA. Peak PFC/spleen values were reached on day 3, and the number of antibody-forming cells remained high for several days. TNP-BA always gave a much higher response than three different preparations of DNP-BA, and at the dose used was also more effective than either TNP-KLH or DNP-KLH given as a single intravenous dose of 100 μg. The mean PFC/spleen on day 5 after TNP-KLH was 4175 as compared to 583 for DNP-KLH.[4] The high immunogenicity of BA conjugates might be due to their probable content of endotoxin. Indeed, immunization with DNP-KLH, given together with 50 μg of *E. coli* endotoxin, resulted in a response at least as high as that to DNP-BA. Although the majority of the PFC detected through day 6 after injection of KLH or BA conjugates were 19S-producing, a significant percentage of animals also showed 7S antibody-producing cells on day 6 of the primary response. The response to DNP- or TNP-SIII, on the contrary, did not show a significant 7S component at any time.

Peak PFC/spleen values after an optimal (1 μg) dose of DNP-SIII were reached on day 3 and declined thereafter (Fig. 1). Ten micrograms of DNP-SIII induced a similar response, but that to 100 μg was much lower (Fig. 1). Control LAF_1 mice injected with 0.1 ml of 10^{-5} or of 10^{-6} *M* TNP-lysine containing 1 μg unconjugated SIII did not show any splenic PFC above background 4 days later. The response to TNP-SIII was slightly higher than to DNP-SIII but otherwise was similar. Mean PFC/spleen values on days 4–5 were 3550 after 1 μg, 3218 after 10 μg, and 724 after 100 μg of TNP-SIII.

Experiments in which immunization with DNP-SIII was attempted 1 week after a previous tolerogenic or immunogenic dose of SIII were difficult to interpret, since both dose levels of SIII used appeared to lower somewhat the subsequent response to DNP-SIII.

Secondary Response in Vivo to Haptens Presented on BA and SIII Carriers

Mice reinjected with 1 μg DNP-SIII 3 weeks after a primary injection of 1, 10, or 100 μg DNP-SIII did not show a secondary response. Direct PFC/spleen values measured 6 days later were no different from those in unchallenged mice and were similar to those seen 5 days after a primary injection (Expt 1, Table 1). 7S PFC were absent. DNP-BA induced a slightly higher response comparable to the one seen on day 6 after a primary injection of this antigen. There was no

[4] In this experiment all PFC determinations were made against TNP-SE rather than DNP-SE. That higher PFC values after immunization with TNP as compared to DNP conjugates were not due to the method of detection used was shown in unpublished observations with Dr. E. Goidl and Dr. G. W. Siskind, Department of Medicine, Cornell University Medical Center, New York, in which PFC detected with DNP-SE as indicator cells were also more numerous after immunization with TNP conjugates. Thus, TNP conjugates appear to have higher immunogenicity for LAF_1 mice than do DNP conjugates.

TABLE 1

INABILITY OF DNP-SIII TO INDUCE IMMUNOLOGICAL MEMORY TO DNP

Priming antigen	Challenging antigen	Mean log PFC/spleen ± SE (geometric mean)			
		Expt 1[a]		Expt 2[a]	
		Direct	Indirect	Direct	Indirect
DNP-SIII (1 μg)	DNP-SIII[b]	3.25 ± 0.09 (1760)	3.39 ± 0.15 (2425)		
	DNP-BA	3.52 ± 0.10 (3290)	3.53 ± 0.17 (3405)	3.35 ± 0.13 (2255)	3.36 ± 0.16 (2270)
	TNP-BA			4.45 ± 0.07 (28,150)	4.65 ± 0.13 (44,500)
	None	3.18[c] (1510)	3.11[c] (1300)		
DNP-SIII (10 μg)	DNP-SIII	3.36 ± 0.07 (2270)	3.19 ± 0.13 (1555)		
	DNP-BA	3.66 ± 0.10 (4535)	3.67 ± 0.12 (4670)	3.30 ± 0.12 (2010)	3.29 ± 0.07 (1940)
	TNP-BA			4.38 ± 0.25 (24,200)	4.56 ± 0.22 (36,480)
	None	3.18[c] (1520)	3.20[c] (1570)		
DNP-SIII (100 μg)	DNP-SIII	3.19 ± 0.12 (1555)	3.23 ± 0.08 (1710)		
	DNP-BA	3.45 ± 0.07 (2785)	3.46 ± 0.06 (2850)		
	None	3.18[c] (1510)	3.16[c] (1460)		
DNP-BA	DNP-SIII	3.58 ± 0.06 (3830)	3.64 ± 0.06 (4410)		
	DNP-BA	3.69 ± 0.08 (4930)	4.06 ± 0.09 (11,450)		
	None	3.14[c] (1370)	3.35[c] (2230)		

[a] Interval between injections was 21 days (Expt 1) or 14 days (Expt 2). Numbers of mice in each group varied from 4 (Expt 1) to 2 or 3 (Expt 2). Determination of PFC/spleen was done on day 6 (Expt 1) or day 5 (Expt 2) after challenge.

[b] Challenge dose was 1 μg DNP-SIII in all experiments.

[c] Recipients' spleens in these groups were pooled prior to assay for PFC.

tolerance to DNP in mice previously injected with 100 μg DNP-SIII since they were still able to respond to DNP-BA, although possibly somewhat less than normal mice. In Expt 2, mice reinjected with DNP-BA and TNP-BA 2 weeks after priming with DNP-SIII gave similar results. Responses comparable to those

TABLE 2

INABILITY OF DNP-SIII TO PRIME FOR AND TO ELICIT AN ADOPTIVE SECONDARY 7S RESPONSE

Donors primed with	Recipients challenged with	Mean log PFC/spleen ± SE (geometric mean)			
		Expt 5[a]		Expt 6[a]	
		Direct	Indirect	Direct	Indirect
DNP-BA	DNP-BA	2.65 ± 0.05 (442)	3.68 ± 0.09 (4810)	3.11 ± 0.04 (1290)	3.70 ± 0.08 (5200)
	DNP-SIII[b]	1.76 ± 0.45 (57)	1.81 ± 0.18 (65)	2.75 ± 0.14 (563)	2.68 ± 0.18 (479)
	DNP-KLH[b]			3.07 ± 0.13 (1160)	3.11 ± 0.14 (1305)
	None	2.36 ± 0.16 (229)	2.26 ± 0.08 (182)	2.93[c] (851)	2.94[c] (870)
DNP-SIII (1 μg)	DNP-BA	3.28 ± 0.13 (1905)	3.21 ± 0.06 (1620)		
	DNP-SIII	3.19 ± 0.19 (1550)	3.05 ± 0.09 (1130)		
	None	3.26[c] (1840)	3.08[c] (1190)		
DNP-SIII (100 μg)	DNP-BA	3.22 ± 0.08 (1660)	2.87 ± 0.18 (742)		
	DNP-SIII	2.85 ± 0.21 (709)	2.79 ± 0.14 (617)		
	None	3.08[c] (1200)	ND[d]		

[a] Interval between priming and transfer was 16 weeks for the DNP-BA-primed spleen cells and 4 weeks for DNP-SIII-primed cells in Expt 5, and 3 weeks for DNP-BA-primed spleen cells in Expt 6. Irradiated recipients received 3–3.5 × 10^7 cells (Expt 5) or 2 × 10^7 cells (Expt 6) together with the challenging antigen. Receipients' spleens were assayed for PFC 7 days later (4–5 mice per group).

[b] All challenges with DNP-SIII employed 1 μg DNP-SIII except for Expt 5 (donors primed with DNP-BA), where the dose was 50 μg DNP-SIII. Dose of DNP-KLH was 100 μg.

[c] Recipients' spleens in these groups were pooled prior to assay for PFC.

[d] Not determined.

after a primary injection of these antigens were found on day 5 after challenge, and a 7S component was detectable as also seen after the primary injection.

In contrast, mice primed with DNP-BA gave a secondary 7S anamnestic response to DNP which was not demonstrable after challenge with DNP-SIII. There was a slightly higher 19S response to DNP-SIII in these mice than in those primed with DNP-SIII.

Adoptive Secondary Responses to DNP-BA and DNP-SIII

Since it was conceivable that the presence of antibody to the SIII carrier molecule in the sera of primed mice was inhibiting the secondary response to

TABLE 3

ABILITY OF DNP-BA AND DNP-HGG TO ELICIT AN ADOPTIVE SECONDARY RESPONSE FROM DNP-KLH-PRIMED SPLEEN CELLS

Recipients challenged with	Mean log PFC/spleen ± SE (geometric mean)		
	Expt 3[a] Direct	Expt 4[a]	
		Direct	Indirect
DNP_{26}-KLH[b]	3.21 ± 0.08 (1623)	3.38 ± 0.09 (2380)	4.40 ± 0.04 (25,200)
DNP-BA	3.12 ± 0.04 (1320)	3.26 ± 0.09 (1830)	3.81 ± 0.22 (6460)
DNP_5-HGG	2.92 ± 0.05 (832)	3.28 ± 0.04 (1895)	3.70 ± 0.04 (5020)
DNP_{49}-HGG	1.26 ± 0.43 (18)	3.01 ± 0.21 (1025)	3.20 ± 0.08 (1585)

[a] Spleen cells were taken 2 weeks (Expt 3) or 3 weeks (Expt 4) after injection of 1 mg DNP_{26}-KLH with or without 10 μg *E. coli* endotoxin. Irradiated recipients received 10^7 (Expt 3) or 2×10^7 (Expt 4) spleen cells together with the challenging antigens. Recipients' spleens were assayed for PFC 6 days (Expt 3) or 7 days (Expt 4) later (4–5 mice per group).

[b] Challenge dose of antigen, 0.2 mg DNP-KLH or 0.1 mg DNP-HGG.

these conjugates, adoptive secondary responses after transfer of 2–3 × 10^7 spleen cells from immunized mice were studied. Table 2 shows the results of these experiments. Cells taken 4 weeks after injection of DNP-SIII did not respond to either DNP-BA or DNP-SIII, giving similar PFC values in recipient spleens with or without antigenic challenge at the time of transfer. DNP-BA-primed spleen cells showed definite 7S and weak 19S anamnestic responses to DNP-BA but none to DNP-SIII.

These data implied that DNP-SIII was unable to generate memory cells to DNP and, thus, behaved like SIII alone, which is known to induce 19S responses without generating detectable memory (22). However, the possibility existed that DNP-SIII had generated memory cells but was unable to challenge them and that DNP-BA could not challenge this memory to DNP-SIII because of the difference in carrier. Although BA itself is a largely thymus-independent antigen with respect to challenge of memory cells (23), the DNP-BA conjugate might not be equally thymus independent. We therefore tested the capacity of DNP-BA to challenge memory cells generated by DNP-KLH. Spleen cells from DNP-KLH-primed mice were taken 2 weeks (Expt 3, Table 3) or 3 weeks (Expt 4, Table 3) after immunization and challenged upon transfer (2×10^7 cells/mouse) with DNP-KLH, DNP-BA, or DNP-HGG conjugates. DNP-BA was much more efficient than DNP-HGG in challenging these memory cells, although it induced a lower 7S response than the homologous DNP-KLH conjugate. It should, therefore, have been capable of challenging memory cells to DNP-SIII if such cells had been present.

Ability of DNP-SIII and TNP-SIII to Block Adoptive 19S and 7S Memory Responses

The previous experiments showed that DNP-SIII neither generated nor challenged DNP memory cells. We next examined the ability of these conjugates to block the responsiveness of memory cells *in vivo*. Table 4 shows that, when hapten-BA- or hapten-KLH-primed memory cells were transferred with the homologous antigen and varying doses of the hapten-SIII conjugates, both 19S and 7S memory cells did not respond to the homologous conjugate. Even as little as 0.5–1 μg of hapten-SIII resulted in significant inhibition of both responses. In two of the experiments in which this comparison could be made the 19S responses appeared slightly less sensitive to inhibition than the 7S responses (Expts 11 and 12, Table 4).

When passive hemagglutination titers to TNP of the recipients' sera were compared with values for PFC/spleen, agreement between these two measures of immune responsiveness of the transferred cells was noted. In experiments in which the PFC/spleen values indicated an effective block of the response, the anti-TNP titers of the sera were also greatly reduced, while a less effective block of the PFC/spleen response resulted in a smaller reduction in titer. For example, in three typical experiments, the control recipient groups had mean $\log_2$ serum titers ranging from 10.0 to 10.8, the groups which did not receive antigen had mean $\log_2$ titers of 2.0–2.7, and the mice receiving TNP-KLH with 5–50 μg TNP-SIII had mean $\log_2$ titers of 2.3–3.3. Mice receiving a lower dose of the blocking antigen (0.5–1 μg) had titers of 7.4–8.7.

Further studies showed that a simple preincubation with hapten-SIII for as short as 5 min at 0°C, followed by washing of the cells and transfer into irradiated recipients together with the homologous conjugate, was sufficient to block partially the response of TNP memory cells (Table 5). Excellent correlation was again found between results obtained for PFC per spleen and for anti-TNP titers in recipients' sera (Table 6). Incubation at 0°C was no less effective than at 37°C, and prolonging the incubation to 60 min did not increase the degree of inhibition (Table 5), nor did increasing the concentration of DNP-SIII to 200 μg/ml (45% vs 41%). Although the results were somewhat variable, TNP-SIII was more effective than DNP-SIII, particularly at the 10 μg/ml level, possibly because of the much higher degree of conjugation of SIII with TNP than with DNP. While only one experiment was included in Table 5, good inhibition was obtained at this level of TNP-SIII in several additional experiments. It was noted that both 19S and 7S memory cells were sensitive to the blocking effect of preincubation. Preincubation with 40 μg DNP-SIII/ml, followed by washing, was slightly less effective in blocking the subsequent response to TNP-KLH than the simultaneous injection of 0.5 μg of DNP-SIII (Expt 12, Tables 4 and 5).

Additional experiments (not in the tables) showed that incubation of TNP-KLH primed cells with 100 μg/ml of either DNP_5-HGG or DNP_{49}-HGG, followed by washing had no detectable blocking effect on the subsequent adoptive response to TNP-KLH.

Controls for Specificity and Suppressor Effects

Specificity of the phenomenon was established by an experiment in which cells primed to burro erythrocytes were exposed for 1 hr at 0°C to 40 μg TNP-SIII/ml.

TABLE 4

PERCENT OF RESPONSE TO HOMOLOGOUS ANTIGEN REMAINING AFTER INJECTION OF BLOCKING ANTIGEN

Homologous antigen challenge[a]	Blocking antigen[b] (μg)	% (PFC/spleen)								
		Expt 7[c]	Expt 8		Expt 9[c]	Expt 10[c]	Expt 11		Expt 12	
		7S	19S	7S	7S	7S	19S	7S	19S	7S
+	None	100 (637)	100 (1660)	100 (1790)	100 (1339)	100 (1501)	100 (16,932)	100 (14,904)	100 (2891)	100 (35,329)
+	5–50	<1	4	3	3	<1				
+	0.5–1	3	20	37		<1	53	16	41	4
−	1–50	<1	10	<1	<1	<1				
−	None	<1	1	<1	<1	<1	16	<1	19	2

[a] The homologous challenging antigen in Expts 7 and 10 was DNP-BA, in Expt 8 it was TNP-BA, and in Expts 9, 11, and 12 it was TNP-KLH (0.1 mg).

[b] The blocking antigen in Expts 7 and 10–12 was DNP-SIII, and in Expts 8 and 9 it was TNP-SIII.

[c] In these experiments the 19S values are not included because the 19S responses to homologous antigen were not significantly above the values for cells injected without antigen.

TABLE 5

PERCENT OF SECONDARY RESPONSE TO TNP-KLH REMAINING AFTER INCUBATION OF PRIMED CELLS WITH DNP- OR TNP-SIII

Cells incubated with (μg/ml)	Incubation time (min)	% (PFC/spleen)[a]											
		Expt 11		Expt 12		Expt 13		Expt 14		Expt 15		Expt 16	
		19S	7S	19S	7S	19S	7S	19S	7S	19S	7S	19S	7S
Medium only	60	100 (16,932)	100 (14,904)	100 (2891)	100 (35,329)	100 (3289)	100 (26,389)	100 (3529)	100 (28,321)	100 (9671)	100 (13,922)	100 (1356)	100 (1902)
TNP-SIII (40)	60					75	23	35	3				
	5					23	8						
TNP-SIII (10)	5									12	18		
DNP-SIII (40)	60			28	9							40	61
DNP-SIII (10–20)	60	34	83									52	81
Medium only[b]	60	16	<1	19	2	18	<1	11	<1	3	<1	4	<1

[a] Cells were preincubated at 2×10^7 cells/ml with or without DNP- or TNP-SIII at 0°C as indicated. They were washed twice prior to injection of $1–2 \times 10^7$ cells per recipient with 0.1 mg TNP-KLH. PFC/spleen in recipients were determined 7 days after transfer.

[b] Control cells, not challenged with TNP-KLH after transfer.

TABLE 6

INHIBITORY EFFECT OF PREINCUBATION WITH TNP-SIII ON ADOPTIVE ANTI-TNP BUT NOT ON ANTI-KLH RESPONSES OF TNP-KLH-PRIMED SPLEEN CELLS

Cells incubated with[a]	Cells injected with	Expt 14			Expt 17		
		Geometric mean PFC/spleen	Mean $\log_2$ hemagglutination titer		Geometric mean PFC/spleen	Mean $\log_2$ hemagglutination titer	
			anti-TNP	anti-KLH		anti-TNP	anti-KLH
Medium only	TNP-KLH	31,850	10.8	13.3	25,365	9.6	15.6
TNP-SIII	TNP-KLH	1,514	2.8	14.3	4,562	4.8	7.0
Medium only	Medium only	606	2.7	7.0	677	4.0	17.7

[a] Cells were preincubated at 2×10^7 cells/ml with or without 40 μg/ml TNP-SIII for 60 min at 0°C. They were washed twice prior to injection of 1×10^7 (Expt 14) or 2×10^7 (Expt 17) cells per recipient with 0.1 mg TNP-KLH. PFC/spleen vs TNP and serum antibody titers were determined 7 days after transfer.

The adoptive memory response of these cells, approximately 6000 7S PFC/spleen, was totally unaffected. Furthermore, while serum titers to TNP of recipients were reduced in parallel with numbers of PFC per spleen, the preincubation of donor cells with the blocking antigen had no detectable effect on the response to KLH, the carrier protein, as determined by serum antibody titers (Table 6).

Also, transfer of a combination of unincubated TNP-KLH memory cells with an equal number of such cells after incubation with TNP-SIII and washing gave similar responses to those of unincubated cells showing both the absence of suppressor activity and of excess TNP-SIII with the incubated cells.

DISCUSSION

The results indicate that DNP-SIII, like DNP–levan (2), induces a primary response to DNP in mice which is dose dependent in the sense that high doses (100 μg) give a lower response than low doses (0.5 μg). The response to the hapten on the SIII molecule as a "carrier" appears to take on the properties of that to the carrier in two significant respects: (i) 19S but no 7S antibody is detectable in the serum, as is also the case after injection of optimal doses of SIII alone; and (ii) immunological memory, that is, a higher response to a second injection than to the first, is absent as with SIII (23). This lack of immunological memory was seen both upon challenge of the intact "primed" mice and in adoptive immune responses. It was not due to an inhibition by serum antibody, since higher doses of the conjugates could not overcome this defect and, on the contrary, made the situation worse. The lack of memory was not only due to the shown inability of DNP-SIII to challenge 7S memory cells, but also to a lack of 7S memory formation after priming, since memory was equally lacking upon challenge of DNP-SIII-primed cells with either DNP-BA or with DNP-SIII.

Although the thymus-independent responses to polysaccharide antigens are frequently ascribed to the ability of these antigens to induce mitotic activity in B lymphocytes (3), the lack of memory formation characterizes the major

deficiency of such responses and is probably due to insufficient proliferation of the B cells. Allogeneic effect (25, 26), antilymphocyte serum (27, 28), and coupling to a thymus-dependent carrier (29, 30) may all result in significant increases of the response to SIII and other T-independent antigens in the mouse, in some cases accompanied by the appearance of 7S serum antibody (26, 28) and memory (31). It remains to be determined whether enhanced proliferation of the B lymphocytes, possibly in germinal centers, constitutes the major difference between the full-blown humoral immune response with resulting 19S+7S antibody and memory, and the response to "thymus-independent" antigens lacking 7S antibody and memory. In nude, thymusless mice the response to SIII is normal or supranormal (27) as compared to littermate controls, even though all germinal center formation in nude mice is lacking. It should be noted, of course, that even in primary responses of nude and other mice to SIII a minor component of 7S antibody-forming cells does occur (31).[5] In the absence of T help the proliferation induced by polysaccharide antigens in B lymphocytes may drive all the antigen-sensitive precursors into terminal differentiation without establishment of a memory B-cell line, as suggested by recent studies of Howard and co-workers (32).

The inability of TNP-SIII and DNP-SIII to challenge memory cells to TNP-KLH was not simply due to the absence of interaction between the B memory cells and TNP-SIII. On the contrary, the hapten-SIII conjugates interacted so well with the TNP-memory cells that they blocked the response to the homologous antigen. This almost instantaneous abolition of responsiveness in TNP memory cells is in apparent contrast with the ability of these conjugates to induce primary responses to TNP. In view of the observed dose effect in the primary response, where 100 μg induced a lower response than did 1 μg while 1 μg completely inhibited the secondary response to homologous antigen, a quantitative difference between primary and secondary responses suggests itself. It seems likely that such a difference between primary and memory responses is caused by the affinity of the cells for the antigen (TNP group) which is influenced by (i) the numbers of antibody molecules on the surface of the cell (IgM 7S monomers or 7S IgG molecules), and (ii) the avidity of these antibody molecules. It is well known that serum antibody attains a higher average avidity with time after immunization (33), and it is less sure but likely that this property is shared by IgM and IgG (34, 35). Higher affinity of the cells for antigen would result in the concentration of much more antigen on their surface, resulting in a situation akin to the one seen with high conjugates of DNP–polymeric flagellin (36), or with antibody–antigen complexes (37, 38) which have been shown to facilitate induction of tolerance *in vitro*, a tendency attributed to the need for a certain degree of lattice formation on the surface of the cells (39). It has also been suggested by Coutinho *et al.* (24) that high concentrations of B-cell mitogens on the cell surface may in themselves be inhibitory rather than stimulatory for B-cell mitotic activity.

The present results are consistent with (i) a higher avidity of both the IgM and IgG antibody on memory cells and (ii) a complete lack of proliferation and differentiation once the cells have interacted with too high a concentration of hapten-SIII. Other explanations are also possible. It is conceivable that a relative lack of

[5] Similar findings have recently been obtained in chickens (M. D. Grebenau and G. J. Thorbecke, unpublished observations), in which low doses of DNP-SIII or of SIII alone induced some mercaptoethanol-resistant serum antibody, but no memory.

competition for antigen between B cells and macrophages in the primary response results in the stimulation of some B cells by antigen via a macrophage surface, whereas in the secondary response the higher affinity of the cells overrides such an intermediary role for macrophages and the direct interaction between the antigen and B cells causes tolerance. This implies the need for macrophages in thymus-independent responses, which so far has not been established *in vitro* (40).

Tolerance in both unprimed and primed B cells has been induced *in vivo* and *in vitro* by a variety of hapten conjugates to nonimmunogenic carriers (6, 11). Under special circumstances soluble hapten conjugates may interfere with the induction of a response *in vitro* by macrophage-bound hapten conjugates to the same immunogenic carrier (41). The avidity of the receptor on the B cell has been implicated in the sense that higher avidity always leads to a higher sensitivity to tolerance induction or to "blocking" (33, 42). In some such *in vitro* systems it was shown that several hours of incubation at 37°C were needed (43, 44). In the present study 5–15 min at 0°C sufficed, but further studies are required to determine whether or not a simple block of the receptors induced *in vitro* is followed by cellular changes at 37°C after transfer into the animal before unresponsiveness is established. Studies on the cellular mechanism and reversibility of this induction of tolerance in memory cells will be the subject of another publication (45).

One striking similarity between induction of tolerance to hapten–polysaccharide conjugates and hapten conjugates to syngeneic substances is that they either do not involve T cells at all or are relatively inefficient at inducing T help via a new antigenic determinant (46). Nude mice which lack T cells can be rendered tolerant at the B-cell level with relative ease, even to thymus-dependent antigens (47). Fidler and Golub (48) also showed recently that free hapten (TNBS), which probably conjugates in the animals to produce TNP conjugates of syngeneic proteins, may induce tolerance in the absence of added T cells in B cells from fetal liver.

The absence of suppressor activity in incubated, blocked cell populations is clearly shown in the present studies. It appears, therefore, that the absence of T help, rather than the presence of T suppressor cells, is crucial in the induction of tolerance in this system and that the sensitivity of B cells to such induction is dependent on the affinity of the cells for the antigen. It can be postulated that a high concentration of antigen directly bound to the B-cell surface induces tolerance or blocking, which is overcome if the cells are made to proliferate greatly by T help. Such a proliferation alone would be expected to dilute the antigen on the surface of B cells and cause a lowering of its concentration to immunogenic levels. This interpretation of tolerance induction at the B-cell level is supported by the recent suggestion of Benson and Borel (49) that tolerant cells are able to circulate with antigen bound to their surface. This might explain the continued block of receptors and interference with T help by carriers such as SIII which are nonimmunogenic for T cells.

In view of recent observations that B cells form caps and strip their surface free of Ig within 1 hr after incubation with antigen (50), an explanation is needed why blocked cells should keep the antigen on their surface for a time. Perhaps the multipoint surface interaction with antigen can initiate a state similar to the one described by Edelman and co-workers (51) after interaction of cells with tetrameric concanavalin A involving resistance to cap and patch formation by anti-Ig.

Such changes on the cell surface might be reflected intracellularly by variations in mediator levels such as cyclic AMP, an increase which may cause inhibition of the immune response (52) and may be important as a signal for induction of tolerance (53).

ACKNOWLEDGMENTS

We are deeply indebted to Dr. A. Nisonoff, Department of Biochemistry, University of Illinois Medical Center, for generous gifts of TNP–protein conjugates. We also wish to thank Dr. C. E. Watson of the U.S. Department of Agriculture, Albany, NY for providing us with the *B. abortus* used in these studies. In addition, we wish to express our appreciation to Melvin Bell and Pedro Sanchez for their excellent technical assistance.

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