

## IMMUNITY AND TOLERANCE TO A HAPTEN (NIP) COUPLED TO AN ISOLOGOUS CARRIER (MOUSE GAMMA GLOBULIN)\*

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Animals injected with haptens coupled to carriers can make hapten-specific immunologic responses. In general, the strength of the anti-hapten response is proportional to the immunogenicity of the carrier; haptens on highly immunogenic carriers give marked anti-hapten responses, but animals made tolerant to the carrier, or animals genetically unresponsive to the carrier, make little or no anti-hapten responses when the hapten is presented on that carrier (1-5). These findings are usually interpreted in terms of the current framework of cellular immunology as follows; B (bone marrow-derived) lymphocytes<sup>1</sup> make anti-hapten antibody only if T (thymus-derived) lymphocytes can recognize the carrier as "foreign" and respond to it (6). (It is apparent, however, that T cell function can be modified by graft-vs.-host reactions [7].)

Haptens on nonimmunogenic carriers are not inert, however, because they can induce hapten-specific tolerance (5, 8, 9). In this case, exposure to a hapten on a nonimmunogenic carrier renders the animal incapable of making an anti-hapten response. Presumably, B lymphocytes recognize the hapten at first, but the T "helper" lymphocytes are not activated since they do not recognize the carrier as foreign. Furthermore, the B lymphocytes must be occupied with this hapten on the nonimmunogenic carrier for some time, since they cannot respond to the hapten when presented later on an immunogenic carrier.

These previous experiments have mainly analyzed serum antibody responses to the hapten. Here, we present a system in which a hapten (NIP) coupled to an isologous carrier (mouse  $\gamma$ -globulin) can provoke a hapten-specific immune response if given in adjuvant, and can elicit hapten-specific tolerance if given in saline.

### *Materials and Methods*

*Animals.*—Adult inbred mice of the strains (ACA  $\times$  CBAF<sub>1</sub>), BALB/c, and LAF<sub>1</sub> were used.

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<sup>1</sup> *Abbreviations used in this paper:* B lymphocytes, bone marrow-derived lymphocytes; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; EACA, epsilon-aminocaproic acid; M $\gamma$ G, mouse gamma globulin; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; OA, ovalbumin; PFC, plaque-forming cells; PHA, phytohemagglutinin; SRBC, sheep erythrocytes; T-<sup>3</sup>H, tritiated thymidine; T lymphocytes, thymus-derived lymphocytes.

*Antigen.*—Mouse immunoglobulin (M $\gamma$ G) was prepared from sera or ascites of BALB/c mice carrying a mineral oil-induced tumor MOPC-21 with  $\gamma$ 1 specificity (a gift from Dr. Howard Grey). The M $\gamma$ G was purified by ammonium sulfate precipitation and Sephadex G-200 filtration (Pharmacia Fine Chemicals, Inc., Piscataway, N. J). The hapten used was 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP). Hapten-protein coupling was accomplished by the method of Brownstone et al. (10) and yielded a ratio of 8–10 NIP per molecule of NIP-M $\gamma$ G, bovine serum albumin (BSA) (Pentex Biochemical, Kankakee, Ill.), or ovalbumin (OA) (Miles Lab., Inc., Elkhart, Ind.).

*Immunization.*—BALB/c mice were immunized intraperitoneally with 400  $\mu$ g of NIP-M $\gamma$ G in complete Freund's adjuvant (CFA) (Difco Laboratories, Inc., Detroit, Mich.).

*Tolerization.*—Tolerization was accomplished by treating (ACA  $\times$  CBA) or LAF<sub>1</sub> mice with 1 mg of soluble NIP-M $\gamma$ G three times weekly for 3–4 wk, intraperitoneally. Untreated mice served as controls. After a rest period of 10 days the tolerized and normal mice were challenged with either 0.2 mg of NIP-OA (CFA) in the footpads of the hind legs or with 0.4 mg of NIP-M $\gamma$ G (CFA), intraperitoneally.

*In Vitro Stimulation of DNA Synthesis.*—For in vitro study, the stimulation of DNA synthesis by antigen was studied in a manner similar to that of Dutton and Eady (11). 4 million spleen cells from immunized or tolerant mice were cultured with different concentrations of antigen in 1 ml of RPMI-1640 media (Grand Island Biological Co., Grand Island, N. Y.) containing 5% heat-inactivated rat serum, 100 units of penicillin G, and 100  $\mu$ g of streptomycin/ml. 3 days after culturing the cells in triplicate at 37°C in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub> and 95% air), 1  $\mu$ Ci of tritiated thymidine (T-<sup>3</sup>H) (Schwartz/Mann, Div. Becton, Dickinson, & Co., Orangeburg, N. Y.) (6 Ci/mole) was added for 5 hr. DNA synthesis was determined by precipitating trichloroacetic acid-insoluble material on glass fiber filters (Whatman GF/C grade) and by counting in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.).

*In Vitro Blocking of DNA Synthesis.*—4 million spleen cells from mice immunized, as before mentioned, were incubated for 3 hr at 37°C with different concentrations of NIP-epsilon-amino-caproic acid (NIP-EACA) prepared according to Brownstone et al. (10) before the stimulatory antigen was added. The NIP-EACA was not removed and after addition of antigen, the experiments were performed in the regular manner.

*Serological Tests.*—Sera of mice were tested for anti-NIP antibody by an indirect hemolytic assay (12). Threefold dilutions of sera were made and equal volumes of NIP-coated sheep erythrocytes (SRBC) and complement were added. The tubes were then incubated at 37°C for 30 min. The reciprocal of the dilution that gave complete lysis was considered the titer of that serum. Uncoated SRBC were used as control. The antigen-binding capacity of sera from control and tolerant mice was determined by a modified Farr assay (10). For (ACA  $\times$  CBA) mice anti-NIP response was measured as micrograms of antigen bound per 1 ml of undiluted serum at 50% binding using 10<sup>-6</sup> M BSA-<sup>125</sup>I. The results for LAF<sub>1</sub> mice are per cent antigen precipitated at 1:3 serum dilution using 10<sup>-9</sup> M EACA-N<sup>125</sup>IP.

*Antibody Synthesis In Vitro.*—The number of cells in the spleen forming antibodies against NIP was determined by a modification of the localized hemolysis-in-gel technique (13) using NIP-coated SRBC and uncoated SRBC as controls. The plaque-forming cells (PFC) determined was a total of direct and indirect plaques. Rabbit anti-mouse  $\gamma$ 1 and  $\gamma$ 2a antisera (a gift from Dr. Roy Woods, Immunoglobulin Reference Center, Meloy Inc., Falls Church, Va.) were used to develop indirect plaques.

## RESULTS

*Immunogenicity of NIP-M $\gamma$ G.*—The immunogenicity of NIP-M $\gamma$ G was determined by immunizing mice with this antigen in CFA according to the protocol indicated in Materials and Methods. 2–4 wk after immunization the number of spleen cells forming antibody was enumerated. The serum hemolytic

TABLE I  
*Stimulation of Primed Mouse Spleen Cells by Hapten-Isologous Carrier*

Antigen in vitro	$\mu\text{g}/\text{Culture}$	Exp. 1	Exp. 2	Exp. 3
		<i>cpm/culture <math>\pm</math> SE</i>		
0	—	1421 $\pm$ 399	1001 $\pm$ 221	613 $\pm$ 94
NIP-M $\gamma$ G	100	5575 $\pm$ 1346	14,170 $\pm$ 392	15,822 $\pm$ 2365
NIP-M $\gamma$ G	500	22,558 $\pm$ 899	18,968 $\pm$ 1269	
M $\gamma$ G	100	1759 $\pm$ 126	961 $\pm$ 216	
M $\gamma$ G	500	1584 $\pm$ 42	1140 $\pm$ 225	
NIP-EACA	10			672 $\pm$ 34
NIP-EACA	150			470 $\pm$ 95

BALB/c female mice were immunized i.p. with NIP-M $\gamma$ G (CFA). 2-4 wk later,  $4 \times 10^6$  spleen cells were cultured with antigens for 3 days and pulsed with  $1 \mu\text{Ci T-}^3\text{H}$  for 5 hr. Results are expressed as counts per minute per culture. (Mean of triplicates  $\pm$  standard error.)

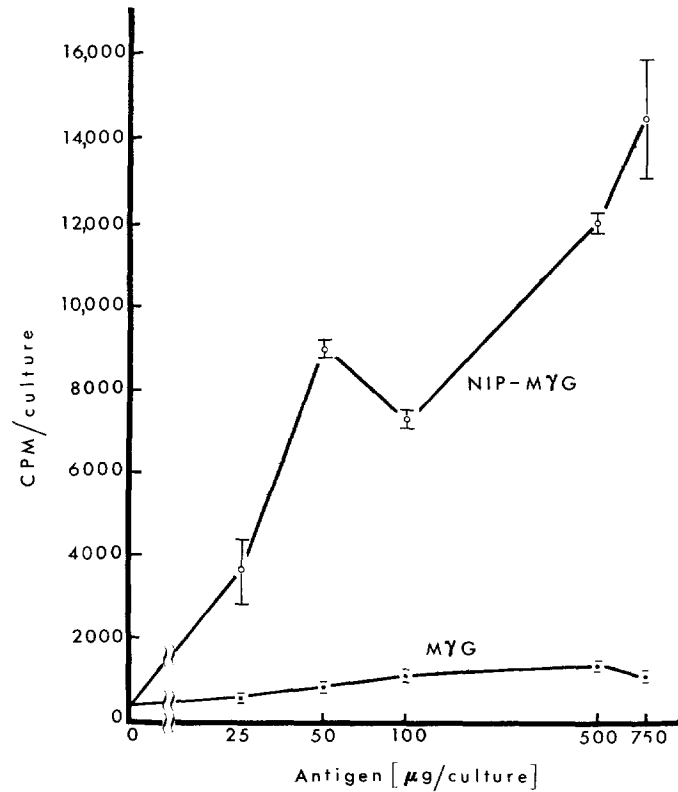


FIG. 1. Uptake of thymidine- $^3\text{H}$  in vitro by cells from mice primed with NIP-M $\gamma$ G (CFA).  $4 \times 10^6$  spleen cells from primed mice were cultured for 3 days with varying concentrations of NIP-M $\gamma$ G or M $\gamma$ G. Thymidine- $^3\text{H}$  was added for last 5 hr of culture. Results are expressed as counts per minute (CPM) per culture tube and graphed as mean  $\pm$  SE of triplicates.

titers of these mice were also determined. Spleen cells from these mice produced an average of 500 NIP-specific hemolytic plaques per spleen. (Nonimmunized mice had no NIP-PFC by this technique.) The sera had a mean hemolytic titer of  $1:243 \pm 102$  using complement-dependent lysis of NIP-SRBC. Control sera were negative by this method. In vitro cellular studies shown in Table I and Fig. 1 indicate that NIP-M $\gamma$ G was effective in triggering secondary antigen-dependent DNA synthesis in spleen cells, while M $\gamma$ G alone had little or no stimulatory effect. NIP-EACA did not stimulate by itself, indicating that the triggering mechanism involved more than interaction with one NIP determinant coupled to a single amino acid. Therefore, the hapten coupled to the carrier was stimulatory although neither the hapten nor the carrier alone initiated DNA synthesis.

TABLE II  
*Hapten Inhibition by NIP-EACA of In Vitro Stimulation by NIP-M $\gamma$ G*

NIP-M $\gamma$ G	NIP-EACA	Exp. 1		Exp. 2	
		cpm/Culture	Per cent inhibition	cpm/Culture	Per cent inhibition
$\mu\text{g}/\text{Culture}$	$\mu\text{g}/\text{Culture}$		%		%
0	0	613 $\pm$ 94	—	304 $\pm$ 33	—
500	0	15,822 $\pm$ 2365	—	4148 $\pm$ 475	—
500	1	13,640 $\pm$ 784	14.3	4561 $\pm$ 149	0
500	10	12,537 $\pm$ 799	21.6	3159 $\pm$ 288	25.7
500	50	9670 $\pm$ 760	40.5	2648 $\pm$ 244	39.0
500	100	8360 $\pm$ 1402	50.9	2379 $\pm$ 300	46.0
500	150	9622 $\pm$ 910	40.6		

Spleen cells from mice primed with NIP-M $\gamma$ G (CFA) as in Table I were cultured for 3 hr with NIP-EACA before NIP-M $\gamma$ G was added for the remainder of the 3 days. Cultures and results are expressed as in Table I.

*The Specificity of the In Vitro Response for the NIP Determinant.*—To determine how much of the in vitro response could be attributed to the hapten, NIP-EACA was used as a blocking agent, this being more efficient in blocking a hapten-specific reaction than the hapten alone (10). The results of such experiments (Table II) indicate that almost 50% of the response could be blocked with a concentration of 100  $\mu\text{g}$  of NIP-EACA. This inhibition was hapten-specific because at a similar concentration the hapten had no inhibitory effect on phytohemagglutination (PHA) stimulation or on the in vitro response of cells primed to and stimulated by an irrelevant antigen (ovalbumin).

*Tolerance to NIP-M $\gamma$ G.*—To further substantiate the hapten-specific response, mice were made tolerant to the hapten by pretreatment with soluble NIP-M $\gamma$ G followed by challenge with immunogenic NIP-BSA (CFA) or

NIP-OA (CFA). The results in Table III indicate that the mice pretreated with soluble NIP-M $\gamma$ G had a markedly reduced antibody response. The tolerance weakened as the interval of time lengthened after challenge with NIP-heterologous carrier. The hapten-specific tolerance in these experiments is emphasized by the fact that these mice had a normal response to the heterologous carrier (i.e. BSA) to which the challenge NIP was coupled.

TABLE III  
*Effect of Pretreatment with NIP-M $\gamma$ G on the Immune Response to NIP*

Mouse strain (No. of mice)	Pretreatment	Challenge antigen (CFA)	Time after chal- lenge	Mean anti-NIP $\pm$ SE	Mean anti-BSA $\pm$ SE
			<i>days</i>		
ACA $\times$ CBA					
Control (6)	None	NIP-BSA	15	1.56 $\pm$ 0.397	19.03 $\pm$ 3.78
Tolerant (8)	NIP-M $\gamma$ G	NIP-BSA	15	0.03 $\pm$ 0.034	20.16 $\pm$ 3.26
Control (6)	None	NIP-BSA	22	11.10 $\pm$ 2.34	0.95 $\pm$ 0.14
Tolerant (8)	NIP-M $\gamma$ G	NIP-BSA	22	0.07 $\pm$ 0.023	0.95 $\pm$ 0.08
Control (6)	None	NIP-BSA	32	16.08 $\pm$ 2.079	1.72 $\pm$ 0.17
Tolerant (8)	NIP-M $\gamma$ G	NIP-BSA	32	0.71 $\pm$ 0.175	1.79 $\pm$ 0.16
LAF <sub>1</sub>					
Control	None	NIP-OA	15	36. $\pm$ 5.48	
Tolerant	NIP-M $\gamma$ G	NIP-OA	15	2.1 $\pm$ 0.8	
Control	None	NIP-OA	21	76. $\pm$ 4.5	
Tolerant	NIP-M $\gamma$ G	NIP-OA	21	25.3 $\pm$ 2.73	

Pretreated mice were injected 3  $\times$  weekly for 4 wk with soluble NIP-M $\gamma$ G. Control mice were not injected. After 10 days rest, all mice were challenged with 0.2 mg of NIP-BSA (CFA) or 0.2 mg of NIP-OA (CFA) in the footpads and the antigen-binding capacity of the serum was detected at intervals thereafter. For ACA  $\times$  CBA mice, anti-NIP response measured as  $\mu$ g bound/ml serum at 50% binding, using  $10^{-6}$  M EACA-N<sup>125</sup>I and anti-BSA response measured as  $\mu$ g bound/ml serum at 50% binding using  $10^{-6}$  M BSA-<sup>125</sup>I. For LAF<sub>1</sub> mice, results are per cent antigen precipitated at 1:3 serum dilution using  $10^{-9}$  M EACA-N<sup>125</sup>I.

Further experiments were done testing hapten-specific tolerance induced by soluble NIP-M $\gamma$ G by challenging *in vivo* with the same antigen in a form known to be immunogenic, namely NIP-M $\gamma$ G in CFA, and then stimulating the cells *in vitro* using NIP-M $\gamma$ G. Untreated LAF<sub>1</sub> mice challenged *in vivo* with NIP-M $\gamma$ G (CFA) had spleen cells capable of responding to NIP-M $\gamma$ G *in vitro* with a dose-dependent increase in T-<sup>3</sup>H uptake as in Table I and Fig. 1, but mice pretreated with soluble NIP-M $\gamma$ G did not show this stimulation of thymidine uptake (Table IV). In neither case did carrier alone stimulate T-<sup>3</sup>H uptake. This lack of stimulation further substantiates an *in vitro* hapten-specific response.

TABLE IV  
*Effect of Pretreatment of Mice with Soluble NIP-M $\gamma$ G on In Vitro Challenge of Cells Immunized with NIP-M $\gamma$ G (CFA)*

Antigen in vitro	$\mu$ g/Culture	Control	Tolerant	Time after challenge
<i>cpm/Culture <math>\pm</math> SE</i>				<i>Days</i>
—	0	1790 $\pm$ 447	1014 $\pm$ 455	17
NIP-M $\gamma$ G	10	4244 $\pm$ 317	1280 $\pm$ 266	
NIP-M $\gamma$ G	50	7175 $\pm$ 575	1208 $\pm$ 348	
NIP-M $\gamma$ G	100	8617 $\pm$ 666	1810 $\pm$ 796	
NIP-M $\gamma$ G	500	15,517 $\pm$ 927	1912 $\pm$ 898	
M $\gamma$ G	100	2033 $\pm$ 10	819 $\pm$ 98	
M $\gamma$ G	500	2421 $\pm$ 398	1001 $\pm$ 68	
PHA		17,399 $\pm$ 1134	22,665 $\pm$ 1021	
—	0	737 $\pm$ 111	995 $\pm$ 96	23
NIP-M $\gamma$ G	500	6334 $\pm$ 225	3516 $\pm$ 630	
M $\gamma$ G	500	1003 $\pm$ 194	1206 $\pm$ 211	
PHA		4159 $\pm$ 454	9247 $\pm$ 429	

LAF<sub>1</sub> mice were untreated (control) or treated 3  $\times$  weekly for 4 wk with 1 mg of soluble NIP-M $\gamma$ G (tolerant). After a 10 day rest, all mice were given i.p. 0.4 mg of NIP-M $\gamma$ G (CFA). Spleen cells were cultured 17 and 23 days later as in Tables I and II and results expressed as cpm  $\pm$  SE/culture.

#### DISCUSSION

The results reported in this paper demonstrate that both hapten-specific immunity and tolerance can be induced in mice using the hapten NIP coupled to isologous mouse gamma globulin. Injection of the antigen (NIP-M $\gamma$ G) mixed with CFA elicits hapten-specific immunity as measured by NIP-specific PFC, specific binding of N<sup>125</sup>IIP by immune serum, and by antigen-driven DNA synthesis in vitro. Hapten-specific tolerance was induced by injecting the antigen in soluble form. Mice treated in this way and challenged with the hapten coupled to M $\gamma$ G or a heterologous carrier had a markedly reduced antibody response to NIP, and their spleen cells did not respond with increased DNA synthesis in vitro when mixed with NIP-M $\gamma$ G.

Hapten-specific immunity and stimulation of primed cells in vitro has been reported previously using guinea pigs immunized with DNP coupled to guinea pig albumin or DNP coupled to heterologous carriers (1, 7, 11, 14). Specific tolerance induced by treatment with DNP coupled to nonimmunogenic carriers such as mouse serum or polypeptides has also been shown (5, 9). However, hapten-specific cell stimulation in vitro invoked by NIP-M $\gamma$ G which can be abolished by inducing NIP-specific tolerance has not been previously demonstrated. We have shown that spleen cells from mice primed with NIP-M $\gamma$ G in CFA can be triggered by the antigen to give a secondary type response in

vitro, as measured by increased DNA synthesis. The concentrations of antigen used in this system for immunization and stimulation are much higher than those used in a different hapten-isologous carrier, i.e., DNP-guinea pig albumin (15). This indicates that our NIP-M $\gamma$ G is not highly immunogenic *in vivo*. Recent experiments have shown that 200  $\mu$ g of NIP-M $\gamma$ G can be immunogenic *in vivo*. 500  $\mu$ g of NIP-M $\gamma$ G *in vitro*, although not optimal, gave much better increment over background than lower concentrations (Fig. 1). Therefore, we chose this as our stimulatory dose *in vitro* in most experiments. The *in vitro* response requires both the hapten and carrier since neither by itself is capable of stimulating the cells. This is similar to the findings of others (7, 11). Although the carrier by itself did not stimulate the cells, the response had a great deal of carrier specificity. This was shown in the experiment in which cells primed to NIP-M $\gamma$ G gave a fivefold response when the stimulatory antigen was NIP-M $\gamma$ G (500  $\mu$ g) and only a twofold response when the stimulatory antigen was NIP-OA (500  $\mu$ g). This finding confirms the fact that the "carrier effect" is not absolute and that hapten coupled to a heterologous carrier can be weakly stimulatory (11).

The participation of the hapten in the *in vitro* response was demonstrated by blocking experiments. Nearly 50% of the *in vitro* DNA response was blocked by the addition of NIP-EACA. This strongly suggests that a significant portion of the response is due to the hapten. This blocking corroborates the findings of others (1, 14), although in our hands significant blocking occurred when free hapten was in 10-fold excess compared with hapten carrier. At higher concentrations NIP-EACA was somewhat toxic for the cells which masked any further blocking that might be obtained. Preliminary experiments indicate that M $\gamma$ G (which had no stimulatory effect) also had no blocking effect.

Final interpretation of these results rests on the identification of the cells involved in responsiveness or unresponsiveness to both NIP and M $\gamma$ G. Although this is not possible at present, it is clear that NIP-M $\gamma$ G (CFA) stimulates the production of hapten-specific antibody (presumably by B lymphocytes) (15) as shown by serum NIP-hemolysins and NIP-PFC in the spleen. According to the current concepts outlined above, hapten-specific antibody production by B lymphocytes requires T cell help (6). In the context of our experiments, immunogenicity is acquired via the coupling of NIP to M $\gamma$ G. This implies that helper cell activity *in vivo* arises from T cell recognition of the NIP-M $\gamma$ G complex (16). A similar situation applies to the *in vitro* stimulation of cells primed to NIP-M $\gamma$ G (CFA) in that antigen-driven DNA synthesis occurs with the complex, NIP-M $\gamma$ G, but not with M $\gamma$ G alone, either in its native form or as M $\gamma$ G "sham-coupled to NIP" (i.e., put through the coupling procedure in the absence of NIP, unpublished observations). Although the bulk of antigen-stimulated DNA synthesis *in vitro* is probably due to responding T cells (because a substantial part of it is abolished by treatment

with anti-theta serum and complement,<sup>2</sup> the participation of NIP-specific B cells is as yet undetermined.

In tolerant systems involving proteins, it is clear that unresponsiveness may inhere in T cells, B cells, or both (17). In our hapten-conjugate system, antibodies capable of binding NIP were scarcely detectable when the tolerant mice were challenged with NIP on either the tolerizing carrier, M $\gamma$ G, or on an unrelated carrier, BSA. (Both tolerant and control mice made good responses to BSA.) Tolerance is therefore directed specifically to the hapten, and the current hypothesis would interpret this to mean hapten-specific blockade of B lymphocytes (18).

The failure of NIP-M $\gamma$ G to stimulate cells from tolerant mice *in vitro* (Table IV) has additional implications, we believe. Since mice immunized to NIP-M $\gamma$ G have cells capable of recognizing NIP-M $\gamma$ G (but not M $\gamma$ G alone), it appears that in tolerant mice these cells are either lacking or are inactivated. Since many of these cells are probably T cells, this implies that mice tolerant to NIP-M $\gamma$ G have B cell tolerance (to NIP) as well as T cell tolerance (to NIP-M $\gamma$ G).

The ability to produce either hapten-specific immune responses or tolerance using "isologous carrier" with or without adjuvant, and the ability to measure these responses at the cellular level, have significant implications for the study of autotolerance and autoimmunity.

#### SUMMARY

A hapten, 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) when coupled to isologous mouse gamma globulin (M $\gamma$ G) elicits a hapten-specific immune response in mice if administered in Freund's complete adjuvant. This response is measurable by the capacity of the sera to bind N<sup>125</sup>IP, by detection of NIP-specific plaque-forming cells (B cells), and by *in vitro* secondary type antigen-driven DNA synthesis (T cells and probably B cells). The *in vitro* response requires both the hapten and carrier since neither by itself is capable of stimulating the spleen cells. This same antigen gives rise to hapten-specific tolerance when given in the soluble form. Mice pretreated with soluble NIP-M $\gamma$ G and challenged with NIP coupled to a heterologous carrier give a normal antibody response to the carrier but have barely detectable levels of antibody to NIP. Spleen cells from mice made tolerant to NIP-M $\gamma$ G do not respond *in vitro* with increased DNA synthesis. This implies that thymus-derived cells as well as bone marrow-derived cells are involved in hapten-specific tolerance.

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<sup>2</sup> Moorhead, J. W., C. S. Walters, and H. N. Claman. Unpublished data.



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