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 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 γ-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 X CBAF1), BALB/c, and LAF1 were used.

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Abbreviations used in this paper: B lymphocytes, bone marrow-derived lymphocytes; BSA, bovine serum albumin; CFA, complete Freund’s adjuvant; EACA, epsilon-amino-caproic acid; MyG, mouse gamma globulin; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; OA, ovalbumin; PFC, plaque-forming cells; PHA, phytohemagglutinin; SRBC, sheep erythrocytes; T-3H, tritiated thymidine; T lymphocytes, thymus-derived lymphocytes.
Antigen.—Mouse immunoglobulin (M'yG) was prepared from sera or ascites of BALB/c mice carrying a mineral oil-induced tumor MOPC-21 with γ1 specificity (a gift from Dr. Howard Grey). The M'yG 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'yG, bovine serum albumin (BSA) (Pentex Biochemical, Kankakee, Ill.), or ovalbumin (OA) (Miles Lab., Inc., Elkart, Ind.).

Immunization.—BALB/c mice were immunized intraperitoneally with 400 μg of NIP-M'yG in complete Freund's adjuvant (CFA) (Difco Laboratories, Inc., Detroit, Mich.).

Tolerization.—Tolerization was accomplished by treating (ACA X CBA) or LAF1 mice with 1 mg of soluble NIP-M'yG 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'yG (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 μg of streptomycin/ml. 3 days after culturing the cells in triplicate at 37°C in a humidified CO2 incubator (5% CO2 and 95% air), 1 μCi of tritiated thymidine (T-3H) (Schwartz/Mann, Div. Becton, Dickinson, & Co., Orangeburg, N. Y.) (6 Ci/m 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 X CBA) mice anti-NIP response was measured as micrograms of antigen bound per 1 ml of undiluted serum at 50% binding using 10⁻⁶ M BSA-¹²⁵I. The results for LAF1 mice are per cent antigen precipitated at 1:3 serum dilution using 10⁻⁶ M EACA-N¹²⁵I.

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 γ1 and γ2a 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'yG.—The immunogenicity of NIP-M'yG 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

| Antigen in vitro | µg/Culture | Exp. 1     | Exp. 2     | Exp. 3     |
|------------------|------------|-----------|-----------|-----------|
|                  | cpm/culture ± SE |          |          |          |
| 0                | 1421 ± 399 | 1001 ± 221 | 613 ± 94  |           |
| NIP-MγG          | 100        | 5575 ± 1346 | 14,170 ± 392 | 15,822 ± 2365 |
| NIP-MγG          | 500        | 22,558 ± 899 | 18,968 ± 1269 |          |
| MγG              | 100        | 1759 ± 126  | 961 ± 216  |           |
| MγG              | 500        | 1584 ± 42   | 1140 ± 225 |           |
| NIP-EACA         | 10         | 672 ± 34    |           |           |
| NIP-EACA         | 130        | 470 ± 95    |           |           |

BALB/c female mice were immunized i.p. with NIP-MγG (CFA). 2-4 wk later, 4 X 10⁶ spleen cells were cultured with antigens for 3 days and pulsed with 1 µCi T-³H for 5 hr. Results are expressed as counts per minute per culture. (Mean of triplicates ± standard error.)

Fig. 1. Uptake of thymidine-³H in vitro by cells from mice primed with NIP-MγG (CFA). 4 X 10⁶ spleen cells from primed mice were cultured for 3 days with varying concentrations of NIP-MγG or MγG. Thymidine-³H was added for last 5 hr of culture. Results are expressed as counts per minute (CPM) per culture tube and graphed as mean ± 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.

| NIP-M\gamma G (\mu g/Culture) | NIP-EACA (\mu g/Culture) | Exp. 1 | Exp. 2 |
|-------------------------------|--------------------------|-------|-------|
|                               |                          | cpm/Culture | Per cent inhibition | cpm/Culture | Per cent inhibition |
| 0                             | 0                        | 613 \pm 94 | 0 %              | 304 \pm 33 | 0 %              |
| 500                           | 0                        | 15,822 \pm 2365 | 0 %             | 4148 \pm 475 | 0 %              |
| 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 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-MγG 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**

| Mouse strain (No. of mice) | Pretreatment | Challenge antigen (CFA) | Time after challenge | Mean anti-NIP ± SE | Mean anti-BSA ± SE |
|---------------------------|--------------|-------------------------|---------------------|-------------------|-------------------|
| ACA × CBA                 | Pretreatment | Challenge antigen (CFA) | Time after challenge | Mean anti-NIP ± SE | Mean anti-BSA ± SE |
| Control (6)               | None         | NIP-BSA                 | 15                  | 1.56 ± 0.397      | 19.03 ± 3.78      |
| Tolerant (8)              | NIP-MγG      | NIP-BSA                 | 15                  | 0.03 ± 0.034      | 20.16 ± 3.26      |
| Control (6)               | None         | NIP-BSA                 | 22                  | 11.10 ± 2.34      | 0.95 ± 0.14       |
| Tolerant (8)              | NIP-MγG      | NIP-BSA                 | 22                  | 0.07 ± 0.023      | 0.95 ± 0.08       |
| Control (6)               | None         | NIP-BSA                 | 32                  | 16.08 ± 2.079     | 1.72 ± 0.17       |
| Tolerant (8)              | NIP-MγG      | NIP-BSA                 | 32                  | 0.71 ± 0.175      | 1.79 ± 0.16       |
| LAF1                      | Pretreatment | Challenge antigen (CFA) | Time after challenge | Mean anti-NIP ± SE | Mean anti-BSA ± SE |
| Control                   | None         | NIP-OA                  | 15                  | 36 ± 5.48         |                   |
| Tolerant                  | NIP-MγG      | NIP-OA                  | 15                  | 2.1 ± 0.8         |                   |
| Control                   | None         | NIP-OA                  | 21                  | 76. ± 4.5         |                   |
| Tolerant                  | NIP-MγG      | NIP-OA                  | 21                  | 25.3 ± 2.73       |                   |

Pretreated mice were injected 3 X weekly for 4 wk with soluble NIP-MγG. 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 × CBA mice, anti-NIP response measured as μg bound/ml serum at 50% binding, using 10⁻⁶ M EACA-N¹²⁵I and anti-BSA response measured as μg bound/ml serum at 50% binding using 10⁻⁶ M BSA-¹²⁵I. For LAF1 mice, results are per cent antigen precipitated at 1:3 serum dilution using 10⁻⁶ M EACA-N¹²⁵I.

Further experiments were done testing hapten-specific tolerance induced by soluble NIP-MγG by challenging in vivo with the same antigen in a form known to be immunogenic, namely NIP-MγG in CFA, and then stimulating the cells in vitro using NIP-MγG. Untreated LAF1 mice challenged in vivo with NIP-MγG (CFA) had spleen cells capable of responding to NIP-MγG in vitro with a dose-dependent increase in T²H uptake as in Table I and Fig. 1, but mice pretreated with soluble NIP-MγG did not show this stimulation of thymidine uptake (Table IV). In neither case did carrier alone stimulate T²H uptake. This lack of stimulation further substantiates an in vitro hapten-specific response.
TABLE IV
Effect of Pretreatment of Mice with Soluble NIP-MyG on In Vitro Challenge of Cells Immunized with NIP-MyG (CFA)

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

LAF1 mice were untreated (control) or treated 3 X weekly for 4 wk with 1 mg of soluble NIP-MyG (tolerant). After a 10 day rest, all mice were given i.p. 0.4 mg of NIP-MyG (CFA). Spleen cells were cultured 17 and 23 days later as in Tables I and II and results expressed as cpm ± 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-MyG) mixed with CFA elicits hapten-specific immunity as measured by NIP-specific PFC, specific binding of N125IP 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 MyG 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-MyG.

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-MyG 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-MyG 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-MγG is not highly immunogenic in vivo. Recent experiments have shown that 200 μg of NIP-MγG can be immunogenic in vivo. 500 μg of NIP-MγG 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-MγG gave a fivefold response when the stimulatory antigen was NIP-MγG (500 μg) and only a twofold response when the stimulatory antigen was NIP-OA (500 μg). 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 MγG (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 MγG. Although this is not possible at present, it is clear that NIP-MγG (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 MγG. This implies that helper cell activity in vivo arises from T cell recognition of the NIP-MγG complex (16). A similar situation applies to the in vitro stimulation of cells primed to NIP-MγG (CFA) in that antigen-driven DNA synthesis occurs with the complex, NIP-MγG, but not with MγG alone, either in its native form or as MγG “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, 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, MyG, 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-MyG to stimulate cells from tolerant mice in vitro (Table IV) has additional implications, we believe. Since mice immunized to NIP-MyG have cells capable of recognizing NIP-MyG (but not MyG 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-MyG have B cell tolerance (to NIP) as well as T cell tolerance (to NIP-MyG).

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 (MyG) 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 NIP, 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-MyG 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-MyG 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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