HAPTEN-SPECIFIC T CELL RESPONSES
TO 4-HYDROXY-3-NITROPHENYL ACETYL
VII. Idiotype-specific Suppression
of Plaque-forming Cell Responses*

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The network theory of immune responsiveness, as originally proposed by Jerne (1), suggests that recognition of unique determinants on immunoglobulins by other antibodies results in self-regulation of antibody production. To explain the apparent idiotype specific restrictions of T-T and T-B cell collaboration the network theory has been expanded to encompass the interaction of lymphoid cells as well as antibodies (2-14). Thus, interaction of target cells with T-helper (2-5) or T suppressor (5-14) cells may depend on recognition of unique idiotypic determinants on the surface of the target cell. Furthermore, these interactions may be mediated by regulatory molecules that either bear the idiotype themselves (10, 15-18) or are idiotype specific (10).

In most studies of idiotype-specific suppression of a B cell response, suppressor cells have been induced by administration of anti-idiotypic antiserum (7, 10, 19), idiotype alone (4, 20), or idiotype conjugated to syngeneic cells (9). The operation of such an idiotypic suppressor mechanism during the normal course of a humoral immune response would be more directly demonstrated if antigen-induced suppressor cells, acting in an idiotype-specific manner, could be elicited by antigen. We have, therefore, used a model of immune suppression involving hapten-coupled syngeneic cells to study suppressor T lymphocytes acting in an idiotype-specific fashion to depress plaque-forming-cell (PFC)1 responses.

Previously, we have reported that the intravenous injection of spleen cells covalently coupled with the 4-hydroxy-3-nitrophenyl acetyl (NP) hapten induces T cell-mediated suppression of a hapten-specific delayed-type hypersensitivity (DTH) response in mice of the IgGb allotype (12-14). In the present work, we have extended these findings to a B cell system. Thus, spleen cells from mice treated 7 d previously with NP-conjugated syngeneic spleen cells are capable of selectively suppressing the expression of idiotype-positive B cells either when transferred to normal recipients in vivo or

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1 Abbreviations used in this paper: C, complement; DTH, delayed-type hypersensitivity; GLA, poly-(L-Glu-L-Lys-L-Phe); GI~, poly-(c-Glu-L-Lys-L-Ala); KLH, keyhole limpet hemocyanin; MEM, minimum essential medium; NIP, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl; NMS, normal mouse serum; NP, (4-hydroxy-3-nitrophenyl)acetyl hapten; PBS, phosphate-buffered saline; PFC, plaque-forming cell(s); SRBC, sheep erythrocytes.

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when subcultured for a 4-d period and then added to NP-primed cultures in vitro. Furthermore, the suppression is T cell dependent because treatment with anti-Thy-1.2 antiserum + complement C ablates the transfer of nonresponsiveness both in vivo and in vitro. Purified T cells are capable of transferring this idiotype-specific suppression. The data support the network hypothesis of T-B cell interaction via idiotypic recognition because antigen-induced T suppressor cells selectively affect the responsiveness of idiotype-bearing B cell clones.

Materials and Methods

**Mice.** C57BL/6 male, CBA/J male, and BALB/cJ female mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c nu/nu mice were a gift of Dr. L. Perry, Department of Pathology, Harvard Medical School, Boston, Mass. All experimental animals were between 8 and 12 wk of age at the beginning of immunization. Animals were age-matched in each experiment.

**Antigens.** NP-poly-(L-Glu/Lys-Ala) (GLA), NP-poly-(L-Ola/L-Lys/L-Phe) (GLP), NP-keyhole limpet hemocyanin (KLH), and NP-Ficoll were conjugated by the reaction of NP-O-succinimide (Biosearch, San Rafael, Calif) with GLA (prepared by E. Blout of Harvard Medical School), GLP (lot GLP1-71-199; Miles-Yeda Ltd., Rehovot, Israel), KLH (Sigma Chemical Co., St. Louis, Mo.), or AECMTS-Ficoll (Biosearch) as described previously (21). The NP-GLA and NP-GLP had an average of 7 and 6 hapten groups per carrier molecule respectively. The NP-KLH and NP-Ficoll had an average of 30 and 17 NP groups/100,000 mol wt, respectively. Dextran (B1355S linkages) was kindly provided by Dr. R. Riblet (The Fox Chase Institute for Cancer Research, Philadelphia, Pa.).

**Immunization.** Mice were immunized with 150 µg NP-KLH i.p. or 70 µg i.p. NP-GLA in a 0.2 ml mixture that contained 25% pertussis vaccine (Michigan Department of Public Health, Lansing, Mich.), with 25 µg NP-Ficoll, or with 50 µg dextran in 0.2 ml saline.

**Haptenated Cell Preparation.** NP-coupled spleen cells were made as described previously (14). Briefly, single-cell suspensions were treated with Tris-NH4Cl to lyse erythrocytes. After two washes, the cells were resuspended in phosphate-buffered saline (PBS), pH 7.6, and reacted with 2.5 µM NP-O-succinimide in dimethylsulfoxide for 3 min. The reaction was stopped with minimum essential medium (MEM) containing 1.2 mg/ml glycylglycine. Cells were washed extensively in MEM before injection of 2 × 10^7 to 3 × 10^7 NP-coupled cells i.v. into syngeneic recipients.

**Anti-Thy-1.2 Treatment.** 2 × 10^8 to 3 × 10^9 spleen cells from NP-coupled-cell-treated mice were pelleted and incubated in 1.0 ml of a 1:5 dilution of AKR anti-C3H thymocyte serum or in normal mouse serum (NMS) as control. After 30 min at 20°C, the cells were washed and resuspended in 1.0 ml rabbit C3H diluted 1:5 in MEM that containing 1% DNase. After a 30-min incubation at 37°C, cells were washed in MEM and 2–3 × 10^7 viable cells were transferred to normal recipients. In vitro studies, the treatment was carried out under sterile conditions using 0.3 ml AKR anti-C3H thymocyte serum diluted 1:5 and 0.3 ml mouse complement present for every 2 × 10^7 cells recovered from 4-d subcultures.

**Ant-NP Antiserum.** Anti-idiotype antiserum specific for the NP^b idiotype was prepared as described previously (22). Briefly, guinea pigs were injected biweekly every 2 wk with 200 µg of affinity-purified C57BL/6 anti-NP antibodies, obtained from a pool of primary sera from mice immunized with NP-bovine gammaglobulin. 2 wk after the third injection, animals were bled and the antisera extensively adsorbed with immunoadsorbsents conjugated with MOPC 104E (µL), and (C57BL/6 × DBA/2) normal mouse gammaglobulin. The specificity of this reagent has been determined by an inhibition of idiotype-binding assay (22). 30 µl of normal mouse sera, MOPC 104E, 30 µl C3H anti-NP sera, and 1.25 × 10^-4 M dinitrophenyl caproate did not inhibit idiotype binding. However 1.0 µl of primary B6 anti-NP sera, 4.25 × 10^-8 M NP-caproate or 2.0 × 10^-6 M (4-hydroxy-5-iodo-3-nitrophenyl)acetil (NIP)-caproate could efficiently inhibit idiotype binding. The gammaglobulin fraction of this anti-idiotype antiserum was prepared by 40% (NH_4)SO_4 fractionation, followed by extensive dialysis against PBS. Normal guinea pig gammaglobulin was similarly prepared.

**Transfer of Suppression.** For in vivo studies spleen cells from mice treated 7 d previously with
control or NP-coupled spleen cells were teased into a single cell suspension and $3 \times 10^7$ cells were injected i.v. into normal syngeneic recipients. Recipients were then immunized with 25 μg NP-Ficoll in saline or 70 μg NP-GLA in 25% pertussis vaccine. Direct (IgM) splenic NP-specific PFC responses were assayed 5 d after immunization with NP-Ficoll and indirect (IgG) splenic NP-specific PFC responses were assayed 10 d after immunization with NP-GLA. For in vitro studies, spleen cells from control mice or mice treated 7 d previously with NP-coupled spleen cells were teased and washed under sterile conditions. These cells were added to Linbro culture wells (Linbro Chemical Co., Hamden, Conn.) at a concentration of $7.5 \times 10^6$ cells/ml per well in Mishell-Dutton culture medium that contained 10% fetal calf serum (lot 95045; Microbiological Associates, Walkersville, Md.) (23). Cultures were incubated at 37°C in a 10% CO₂ atmosphere in rocking culture boxes and fed daily with 0.1 ml supplementary medium for a 4-d subculture period. Cells were then pooled, washed, and $5 \times 10^5$ viable normal or suppressor cells were added to $7.5 \times 10^6$ NP-primed cells.

Purification of T Cells. The method of Mage et al. (24) was used to purify splenic T cells. Purified rabbit anti-mouse immunoglobulin (5 ml of 1 mg/ml solution) was added to 100-× 15-mm polystyrene petri plates (1005; Falcon Labware, Div., Becton, Dickinson & Co., Oxnard, Calif.) and incubated for 1 h at room temperature. Unbound antibody was washed off and $8 \times 10^7$ spleen cells were added for a 1-h incubation period at 20°C. The nonadherent T cell population was recovered by gentle swirling. Less than 2.0% of these cells stained with a fluorescent rabbit anti-mouse immunoglobulin antiserum.

In Vitro Cultures. C57BL/6 mice were immunized with 150 μg NP-KLH i.p. with 25% pertussis vaccine. 4 wk later, their spleens were removed and teased into a single-cell suspension under sterile conditions. Cells were washed and resuspended in Mishell-Dutton media that contained 10% fetal calf serum. $7.5 \times 10^6$ was added to each well of Linbro culture plates viable NP-primed cells. Cultures were challenged with 100 ng NP-GLA or NP-Ficoll in 20 μl. A primry sheep erythrocyte (SRBC) response was obtained by addition of $2 \times 10^6$ washed SRBC to culture wells that contained $7.5 \times 10^6$ normal, unprimed spleen cells. All cultures were incubated in rocking boxes at 37°C in a 10% CO₂ atmosphere and were fed daily with 0.1 ml supplementary medium. 5 d after challenge, duplicate wells were pooled and assayed for total NP- or SRBC-specific PFC responses on replicate slides.

PFC Assay. NP- or SRBC-specific PFC were assayed by the Dresser and Greaves (25) modification of the plaque assay of Jerne et al. (26). NP-conjugated SRBC were prepared as follows. 2 ml of washed and packed SRBC was resuspended in 8 ml of borate-buffered saline, pH 8.8. NP-O-succinimide (20 mg in 2.0 ml dimethylformamide) was added dropwise. After a 10-min incubation period, the reaction was stopped by addition of cold PBS that contained 1.2 mg/ml glycylglycine. The cells were washed extensively in PBS before use. Dextran SRBC were prepared by coupling palmitoyl-derivatized dextran (a gift of Dr. R. Riblet) with washed SRBC as described previously (27). A goat anti-mouse IgM (lot 41073; Gateway Immunosera Co., Kankakee, Ill.) was used at a 1:20 dilution to inhibit IgM PFC for assay of indirect PFC. NP<sup>b</sup>-bearing PFC were inhibited from forming plaques by the addition of 3.0-4.0 μl anti-idiotypic reagent to each slide at the beginning of the assay. The control response was determined by addition of 3.0-4.0 μl normal guinea pig gammaglobulin. The percent inhibition of PFC was calculated according to the formula:

$$\text{percent inhibition} = \left(1 - \frac{\text{experimental PFC}}{\text{control PFC}}\right) \times 100\%.$$  

Slides were incubated at 45°C for 1 h. A 1:20 dilution of lyophilized guinea pig C (Pel-Freeze Biologicals Inc., Rogers, Ark.) was then added for an additional 1-h incubation period. Indirect or total PFC were developed with a 1:250 dilution of rabbit anti-mouse anti-IgG antiserum.

Results

Specificity of Guinea Pig Anti-NP<sup>b</sup> Antiserum. Mice of the Igh-1<sup>b</sup> allotype produce antibody that bears the NP<sup>b</sup> idiotype after immunization with NP-coupled compounds (28, 29). To determine if idiotype-secreting B cells can be specifically inhibited with anti-idiotypic antibody in a plaque assay system, C57BL/6 mice were immunized...
with NP-GLA or NP-Ficoll. 5 d after immunization with NP-Ficoll and 10 d after immunization with NP-GLA, the ability of anti-NP<sup>b</sup> antibodies to inhibit direct (NP-Ficoll) or indirect (NP-GLA) PFC responses was assayed. The data in Table I indicate that ~50% of the primary NP-specific B cells induced with NP-GLA and NP-Ficoll can be inhibited from forming plaques with anti-idiotypic antiserum. 3 μl of anti-idiotypic seemed to give plateau levels of inhibition, because increasing the amount of anti-idiotype to 10 μl did not increase the percentage of inhibition (Table I). The data suggest that a significant fraction of NP-specific B cells in C57BL/6 mice bear the NP<sup>b</sup> idiotype. To show the antigen specificity of the anti-idiotype serum, spleen cells from mice immunized with SRBC were assayed in the presence of anti-NP<sup>b</sup> idiotype. Under the conditions employed, no inhibition of SRBC-specific PFC was detected (Table I). Because mice that do not bear the IgH-1<sup>b</sup> allotype do not express NP<sup>b</sup>-bearing NP-specific antibodies, it would be predicted that PFC from NP-primed BALB/c (Ig<sup>b</sup>) or CBA/J (Ig<sup>j</sup>) mice would not be inhibitable with anti-idiotypic antiserum. The results (Table I) support this prediction because the addition of 3.0 μl anti-NP<sup>b</sup> antiserum to spleen cells from BALB/c or CBA/J mice primed with NP-Ficoll did not inhibit plaque formation. Because the antibody response to NP compounds has been shown to consist predominantly of light (λ) -chain bearing molecules in mice of the IgH-1<sup>b</sup> allotype (29), the apparent antigen or strain specificity of the putative anti-NP<sup>b</sup> antiserum could be a result of anti-λ-chain reactivity. To exclude this possibility, BALB/c mice were immunized with 50 μg dextran B1355S. The immune response of BALB/c mice to this antigen consists primarily of λ-chain-bearing antibodies (30). 5 d later, the ability of the anti-NP<sup>b</sup> antiserum to inhibit

### Table I

| Strain | Antigen (mice/group) | Percent inhibition with anti-NP<sup>b</sup> ± SE | Specific anti-NP PFC/spleen ×±log SE
|--------|----------------------|-----------------------------|----------------------------------|
|        |                      | 3.0 μl 10 μl                 |                                  |
| C57BL/6| NP-GLA (4)           | 57 ± 6  —                    | 17,600 × 1.2                     |
| C57BL/6| NP-Ficoll (9)        | 50 ± 6  49 ± 3               | 14,500 × 1.1                     |
| C57BL/6| SRBC (3)             | —3 ± 13  —                   | 39,300 × 1.5                     |
| CBA    | NP-Ficoll (3)        | —5 ± 5  —                    | 7,600 × 1.7                      |
| BALB/c | NP-Ficoll (12)       | 7 ± 8  —                      | 34,400 × 1.3                     |
| BALB/c | Dextran (3)          | 7 ± 9  —                      | 36,800 × 1.7                     |
| BALB/c | NP-GLA (3) (nu/nu)   | —  —                           | 33,600 × 1.7                     |

* C57BL/6, CBA, BALB/c, or BALB/c (nu/nu) mice were immunized with 70 μg NP-GLA, in pertussis vaccine, 25 μg NP-Ficoll, 0.2 ml of 10% SRBC or 50 μg dextran (B1355S). NP- or dextran-specific IgM PFC were assayed 5 d after immunization with NP-Ficoll or dextran. NP- or SRBC-specific IgG PFC were assayed 10 d after immunization with NP-GLA or SRBC.

The percent idiotype-positive response is expressed as the arithmetic mean ± SE of the percent inhibition of plaque formation in the presence of 3.0 or 10 μl anti-NP<sup>b</sup> antiserum relative to the response in the presence of normal guinea pig IgG.

The magnitude of the response is expressed as the geometric mean ×± log SE.
dextran-specific PFC formation was tested. The inability of anti-NP\textsuperscript{b} antiserum to affect this response (Table I) further demonstrated its idiotype specificity. Thus, this guinea pig antiserum detects an idiotypic determinant present on NP-specific PFC from C57BL/6 mice that corresponds to the NP\textsuperscript{b} idiotype described in previous serological studies (28, 29).

To evaluate the requirement for the presence of a thymus or conventional thymus-derived lymphocytes for the in vivo NP-Ficoll response, athymic BALB/c nu/nu mice were immunized with 25 \( \mu \)g NP-Ficoll. After 5 d, the PFC responses were assayed. As shown in Table I, the responses of BALB/c nu/nu mice were of comparable magnitude to those of conventional BALB/c mice. In contrast, BALB/c nu/nu mice produce fewer than 2,000 NP-specific PFC/spleen to the antigen NP-GLA (data not shown).

T Cell-mediated, Idiotype-specific Suppression of NP-GLA and NP-Ficoll Responses In Vivo. We have previously reported that the intravenous injection of NP-modified spleen cells induces T lymphocytes that are capable of suppressing the DTH response to NP (12–14). To determine if this treatment can induce suppressor cells that can specifically affect PFC responses, C57BL/6 mice were given an injection of \( 3 \times 10^7 \) control or NP-conjugated spleen cells i.v. 7 d later \( 3 \times 10^7 \) spleen cells from these donors were transferred to normal syngeneic recipients. Recipients and remaining donor groups were then immunized and their splenic IgM (NP-Ficoll) or IgG (NP-GLA) PFC responses assayed 5 or 10 d later for the magnitude of the response and the percent N\textsuperscript{b}-bearing B cells. It can be seen (Table II) that control mice produced between 54 and 45% idiotype-positive PFC after immunization with NP-GLA or NP-Ficoll as determined by plaque inhibition with anti-NP\textsuperscript{b} antiserum. However, plaques from mice injected intravenously with NP-spleen cells were not inhibitable with anti-idiotype under identical assay conditions. The suppression of idiotype-positive B cells could be transferred to normal recipients with \( 3 \times 10^7 \) spleen cells from suppressed donors. Although the magnitude of the response may decrease concurrently, as demonstrated by mice immunized with NP-GLA, a significant decrease in the number of PFC is not required to manifest suppression of idiotype-positive clones. Thus, although the percent idiotype-positive B cells dropped from 50 to 6% in NP-Ficoll-immunized recipients of suppressor cells, the magnitude of the response was only marginally affected. It may be concluded then that idiotype-specific suppressor cells induced with hapten-modified syngeneic cells specifically suppress the N\textsuperscript{b} idiotype-positive clones while leaving the responsiveness of N\textsuperscript{b}-idiotype-negative clones unaffected. The specificity of this suppressor mechanism is further demonstrated by the inability of treatment with NP-coupled spleen cells to reduce a SRBC PFC response (Table II).

To determine if T cells are required to transfer idiotype-specific suppression, spleen cells from mice injected 7 d previously with NP-coupled syngeneic cells were treated with NMS or anti-Thy-1.2 serum + C before transfer to normal recipients. Recipients were immunized either with NP-GLA or NP-Ficoll as indicated in Table III. The data again confirm that although ~40% of the PFC response was inhibited with anti-idiotypic antiserum, the PFC response of mice that had received NMS-treated suppressor cells was not inhibitable with anti-NP\textsuperscript{b} antiserum. Treatment of cells with anti-Thy-1.2 serum + C completely ablated the ability of these cells to transfer idiotype-specific suppression to normal recipients. Thy-1.2-positive cells, therefore, appear necessary to transfer idiotype-specific suppression. Surprisingly, after treatment
**Table II**

*Idiotype-specific Suppression of B Cell Responses In Vivo*

| Antigen   | Tolerogen (donor mice/group) | Percent inhibition with anti-NP* antiserum$\ddagger$ | Specific PFC/spleen $\times$ log SE$§$ |
|-----------|-------------------------------|----------------------------------------------------------|-----------------------------------------|
|           | Spleen cells transferred (recipient mice/group) | Anti-NP | Anti-SRBC |
| NP-GLA    | Control (7)                   | --       | 54 ± 6   | 9,800 $\times$ 1.3 | -- |
|           | NP-spleen (7)                 | --       | 1 ± 12   | 3,800 $\times$ 1.3 | -- |
|           | Control                      | 3 x 10⁷ (11) | 36 ± 8   | 15,700 $\times$ 1.2 | -- |
|           | NP-spleen                    | 3 x 10⁷ (12) | -- 16 ± 11 | 5,400 $\times$ 1.2 | -- |
| NP-Ficoll | Control (4)                  | --       | 45 ± 11  | 7,300 $\times$ 1.3 | -- |
|           | NP-spleen (4)                | --       | -- 18 ± 12 | 4,200 $\times$ 1.2 | -- |
|           | Control                      | 3 x 10⁷ (9) | 50 ± 10  | 11,100 $\times$ 1.1 | -- |
|           | NP-spleen                    | 3 x 10⁷ (8) | 6 ± 5   | 8,700 $\times$ 1.2 | -- |
| SRBC      | Control (7)                  | --       | --       | -- 12,600 $\times$ 1.2 | -- |
|           | NP-spleen (7)                | --       | --       | -- 15,200 $\times$ 1.2 | -- |

$*$ 7 d after the injection of 3 x 10⁷ syngeneic control or NP-coupled spleen cells i.v., 3 x 10⁷ spleen cells from C57BL/6 mice were transferred to normal syngeneic recipients. Donor and recipient mice were then immunized with 70 μg NP-GLA in pertussis vaccine, 25 μg NP-Ficoll, or SRBC 10 (NP-GLA and SRBC) or 7 (NP-Ficoll) d later, their spleens were assayed for magnitude and percent idiotype-positive IgG or IgM PFC responses, respectively.

$\ddagger$ The percent idiotype-positive response is expressed as the arithmetic mean ± SE of the percent inhibition of the PFC response in the presence of 4.0 μl anti-NP* antiserum, relative to the response in the presence of normal guinea pig IgG.

$§$ The magnitude of the response is expressed as the geometric mean $\times$ log SE.

$\dagger$ Significant decrease in the response relative to controls, P < 0.007.

$\ddagger$ Significant decrease in the response relative to controls, P < 0.05.

with anti-Thy-1.2 antiserum + C, the magnitude of the NP-specific PFC response did not return to control levels, even though the percentage of idiotype-bearing PFC corresponded to control values. These data add support to the contention that nonresponsiveness is mediated by idiotype-specific suppressor T cells.

To determine if T cells are sufficient for transfer of idiotype-specific suppression, spleen cells from mice treated 7 d previously with NP-conjugated cells were passed over rabbit anti-mouse immunoglobulin-coated plates before transfer to normal recipients. The ability of these purified T cells to transfer significant idiotype suppression (Table III) indicates that T cells are both required and sufficient to mediate immune suppression.

**T Cell-mediated, Idiotype-specific Suppression of the PFC Response In Vitro.** An in vitro secondary assay system using NP-KLH-primed spleen cells challenged with NP-Ficoll or NP-GLA was used to confirm the activity of idiotype-specific suppressor T cells on B cell responses. To induce suppressor cells, C57BL/6 mice were injected with 3 x 10⁷ NP-coupled spleen cells i.v. 7 d later, spleen cells from control or NP-spleen treated mice were cultured in Linbro wells. After a 4-d subculture period, 5 x 10⁵ control or suppressor cells were added to hapten-primed spleen cell cultures and the cultures...
Table III

| Antigen   | Tolerogen (mice/group) | Treatment of transferred cells | Percent inhibition with anti-NP b antiserum ± SE§ | NP-specific PFC/spleen × log SE§ |
|-----------|------------------------|---------------------------------|-------------------------------------------------|----------------------------------|
| NP-GLA    | Control (5)            | NMS + C                          | 39 ± 15                                          | 23,800 × 1.1                     |
|           | NP-spleen (4)          | NMS + C                          | -16 ± 28                                        | 7,200 × 1.1¶                     |
|           | Anti-Thy-1.2 + C       |                                  | 51 ± 5                                          | 11,900 × 1.1¶                    |
| NP-Ficoll | Control (5)            | NMS + C                          | 43 ± 9                                          | 13,600 × 1.1                     |
|           | NP-spleen (4)          | NMS + C                          | 9 ± 8||                                          | 9,000 × 1.2                      |
|           | Anti-Thy-1.2 + C       |                                  | 46 ± 4                                          | 7,100 × 1.1¶                     |
| NP-Ficoll | Control (4)            | Control                          | 62 ± 5                                          | 15,400 × 1.1                     |
|           | NP-spleen (4)          | Purified T cells                 | 19 ± 14||                                        | 5,800 × 1.3¶                    |

* 7 d after the injection of 3 × 10^7 syngeneic control or NP-coupled spleen cells i.v., spleen cells from C57BL/6 mice were either treated with NMS + C or anti-Thy-1.2 + C, or were passed over rabbit anti-mouse immunoglobulin-coated plates to purify T cells. 2 × 10^7 viable cells were then transferred to normal syngeneic recipients. 10 d after immunization with NP-GLA and 7 d after immunization with NP-Ficoll, recipient mice were assayed for splenic IgG and IgM PFC responses, respectively.

† The percent idiotype-positive response is expressed as the arithmetic mean ± SE of the percent inhibition of the PFC response in the presence of 4.0 µl anti-NP b antiserum relative to the response in the presence of normal guinea pig IgG.

§ The magnitude of the response is expressed as the geometric mean ×/± log SE.

|| Significant decrease in the response relative to controls, P < 0.04.

¶ Significant decrease in the response relative to controls, P < 0.01.

were challenged with NP-GLA or NP-Ficoll. The results presented in Table IV clearly show that cells obtained from idiotype suppressed donors were capable of significantly suppressing the magnitude of an in vitro response to NP-Ficoll and to NP-GLA. This suppression was antigen specific because the magnitude of a primary SRBC response remained unaltered. In addition, anti-Thy-1.2 + C treatment of these cells significantly depleted their ability to suppress these responses. The data obtained for idiotype-bearing clones may be similarly interpreted. Although the control response to NP-Ficoll or to NP-GLA consisted of 86–100% idiotype-bearing PFC, the response of cultures to which 5 × 10^5 suppressor cells had been added was composed exclusively of idiotype-negative populations. Anti-Thy-1.2 + C treatment of suppressor cells allowed the expression of idiotype-positive PFC. The suppression appeared specific in these in vitro studies because the SRBC response was not inhibited. Furthermore, the specificity of the anti-idiotypic was demonstrated, as this reagent did not significantly decrease the number of SRBC-specific PFC.

Discussion

To assay for idiotype-specific suppression of a B cell response, a guinea pig antiserum raised against anti-NP antibodies purified from C57BL/6 mice was used to inhibit NP-specific plaque formation. This antiserum was judged idiotype specific by virtue of its ability to inhibit NP-specific plaque formation up to 57% in vivo and 100% in vitro with microliter quantities without affecting SRBC-specific PFC from C57BL/6 mice. In addition, it appeared that this anti-idiotypic antiserum recognized
TABLE IV

| Antigen  | Donor treatment | Treatment of cells added to cultures | Percent inhibition with anti-NP<sup>b</sup> antiserum ± SE<sup>¶</sup> | Specific PFC/culture X log SE<sup>§</sup> |
|----------|----------------|--------------------------------------|---------------------------------------------------------------|----------------------------------------|
| NP-Ficoll | Control        | —                                    | 86 ± 4                                                         | 3,090 X ± 1.2                          |
|          |                | NP-spleen                            | 7 ± 41                                                         | 740 X ± 1.8                            |
|          |                | NP-spleen                            | 100 ± 0                                                        | 1,870 X ± 1.7                          |
| NP-GLØ   | Control        | —                                    | 91 ± 6                                                         | 1,770 X ± 1.4                          |
|          |                | NP-spleen                            | 0                                                              | 40 X ± 3.6                             |
|          |                | NP-spleen                            | 64 ± 36                                                        | 1,600 X ± 2.0                          |
| SRBC     | Control        | —                                    | 3                                                              | 920                                    |
|          | NP-spleen      | —                                    | 910                                                            |                                        |

* C57BL/6 mice received an injection of 3 x 10<sup>7</sup> control or NP spleens i.v. 7 d later, spleens were teased, and cultured at 7.5 x 10<sup>6</sup> cells/ml in Linbro culture wells. After a 4-d subculture period, cells were washed, treated as indicated, and added to duplicate wells that contained 7.5 x 10<sup>6</sup> responder spleen cells from mice immunized 4 wk previously with NP-KLH. The cultures were then challenged with 100 µg NP-Ficoll or NP-GLØ. For an SRBC-specific response, 2 x 10<sup>6</sup> washed SRBC were added to 7.5 x 10<sup>6</sup> normal spleen cell responders. 5 d after antigenic challenge, duplicate wells were pooled and assayed for total NP- or SRBC-specific PFC responses.

† Results are expressed as the arithmetic mean ± SE of the percent inhibition of the PFC response in the presence of 4.0 µl anti-NP<sup>b</sup> antiserum relative to the response in the presence of 4.0 µl guinea pig IgG. The idiotype-positive responses of suppressed cultures challenged with NP-GLØ and control cultures challenged with SRBC were obtained in single experiments. All other data were pooled from two or three experiments. Only cultures containing >600 specific PFC were assayed for inhibition with anti-idiotype antiserum.

§ Background PFC were subtracted from the PFC response in cultures that contained antigen to obtain specific PFC. Data are expressed as the geometric mean X log SE of three or more experiments.

| Anti-NP | Anti-SRBC |
|---------|-----------|
|         |           |

the NP<sup>b</sup> idiotype as described in serologic studies (28, 29) because it inhibited anti-NP PFC only from mice possessing the Igh-1<sup>b</sup> allotype (Table I). Although the mechanism of plaque inhibition is not certain, it is possible that binding of idiotype-bearing surface receptors by anti-idiotype prevents secretion of antibody. This mechanism has been proposed in other systems (11, 31). Alternatively, inhibition of plaque formation may be manifest by direct binding of anti-idiotype to antibody as it is secreted from B cells.

In this paper the ability of T suppressor cells induced by the intravenous administration of NP-modified syngeneic cells to reduce an idiotypic B cell response was studied in both an in vivo and an in vitro system. In previous studies, it has been shown that the intravenous injection of NP-modified syngeneic cells induces T cell populations capable of suppressing a DTH response to the NP haptenic determinant in C57BL/6 mice (12–14). At least two interacting subpopulations have been identified. The induction-phase suppressors, thus termed because of their activity when transferred before immunization with NP, bind antigen, bear the NP<sup>b</sup> idiotype, and are capable of suppressing an NIP response in mice of the Igh-1<sup>b</sup> allotype. The specific specificity of these suppressors appears analogous to the specificity patterns of the heteroclitic humoral anti-NP response reported by other groups and is consistent with
the interpretation that this suppressor cell population bears an NP\textsuperscript{b} idiotypic receptor (28, 29, 32, 33). It also appears that these induction-phase suppressor cells induce a second population of cells in the presence of antigen, called effector-phase suppressors, that acts on the effector limb of the DTH response and neither binds antigen nor bears the NP\textsuperscript{b} idiotype. Effector-phase suppressors have an anti-idiotypic receptor and are restricted by Igh-V and H-2 region genes (13, 14).

Our experiments did not address the question of which population(s) of suppressor T cells mediate the suppression of the PFC response. In contrast to the in vivo studies, spleen cells from mice treated 7 d previously with NP-coupled spleen cells do not cause significant levels of suppression when added directly to in vitro cultures (data not shown). The requirement for a 4-d subculture period may reflect the induction of a second population of T cells involved in a suppressor pathway similar to that induced in the NP-specific DTH system. Subculturing is also required to demonstrate suppression in other systems (34). Perhaps this procedure increases the activity or numbers of effector cells. Preliminary data indicating that subcultured spleen cells from NP-spleen treated mice can suppress an effector phase NP response in vitro (data not shown) is consistent with the concept that effector cells have been induced during this subculture period. Recruitment of effector cells in vivo may be envisioned to occur in recipients of cells from NP-spleen-treated donors. Furthermore, because suppression of the PFC response is idiotype specific, it appears that the effector-phase suppressor population has an anti-idiotypic receptor. Experiments are in progress to determine which suppressor T cell subset is directly responsible for idiotype-specific suppression and to distinguish idiotype-specific suppressor cells from idiotype-specific cytolytic T lymphocytes.

The cellular target of the suppressor cells is most likely an NP\textsuperscript{b}-idiotype-bearing B cell. Because NP-induced suppressor cells are unable to nonspecifically reduce SRBC responses in vivo or in vitro, it may be concluded that the suppression is NP specific. Furthermore, the presence of GLA-specific T cell help in vivo suggests that any decrease in the NP-specific response will be a result of suppression at the B cell level. This conclusion is supported by the results obtained after immunization or challenge with NP-Ficoll both in vivo and in vitro. Thus, as Ficoll is a relatively T-independent carrier (35, 36 and Table I), suppression of any fraction of the NP-Ficoll response probably reflects a decrease in the activity of the NP-specific B cell population. It appears, then, that T suppressor cells generated after treatment with NP-derivatized spleen cells specifically recognize idiotype-bearing B cells. However, we cannot exclude the possibility that the target of the suppressor cell is a regulatory T cell which specifically helps NP\textsuperscript{b}-bearing B cell clones. Such idiotype-bearing T helper cells have been described elsewhere (2-4). Alternatively, an idiotype-bearing suppressor cell may interfere with T helper cells that bear anti-idiotypic receptors.

In the vivo studies, spleen cells from NP-spleen-treated mice were capable of suppressing idiotype-positive B cells essentially 100% after immunization with NP-GLA. Reduction of the magnitude of the response to \(\sim 50\%\) of control levels may then reflect the functional elimination of all of the NP-specific cells that bear the NP\textsuperscript{b} idiotype. Therefore, suppression appears to affect a specific idiotypic population and does not alter the responsiveness of NP\textsuperscript{b}-negative clones. Interestingly, whereas the idiotypic B cell response to NP-Ficoll is eliminated almost completely by the transfer of T suppressor cells in vivo, the magnitude of the response is not consistently affected.
Because control mice still express 50% idiotype-positive B cells, the marginal decrease in the magnitude is probably not a result of a lower percentage of idiotype-bearing cells but rather may represent a reconstitution of the NP response by idiotype-negative clones. These results may be contrasted with those obtained in the NP-specific DTH system in which induction phase suppressor T cells eliminate essentially all of the response induced by priming with NP-BGG and challenge with either NP-BSA or NIP-BSA. The effectiveness of inducer suppressor cells in the DTH system may be a result of the predominantly idiotypic nature of the NP-specific DTH response. Idiotype suppressor cells are thereby capable of affecting the entire NP-specific response.

The in vivo data demonstrate that T suppressor cells can selectively eliminate a major subpopulation of B cells carrying the predominant idiotype. A similar argument can be made for the data generated in vitro. Thus, the greater degree of suppression of the magnitude of response to NP-GLα (98%) or to NP-Ficoll (76%) may be attributed to the increased percentage of idiotype-positive clones (91 and 86%, respectively) in control responses. This increase in idiotype-bearing cells may either be a consequence of the culture conditions employed or may be a characteristic of the response after priming and challenge with NP on different carriers. The latter hypothesis may be contrasted with previous studies which indicate that in hyperimmune mice the concentration of NPb idiotype is reduced (29). However, in earlier studies, the assay used to determine idiotype content was highly affinity dependent and therefore may not have accurately reflected the number of NPb-idiotype-bearing molecules (29). The ability of suppressor cells to decrease the T dependent, NP-specific response by 98% after challenge with NP-GLα may also reflect the suppression of NP-specific helper T cells. These hapten-specific helper cells may be generated in NP-KLH-primed mice and their activity observed under the present culture conditions. In support of this contention is the fact that the NP-GLα responses may be obtained in these experiments with cells from mice generally incapable of providing GLα-specific T cell help (37).

Because in most investigations of immune suppression the idiotypic specificity is not evaluated, it is possible that the antigen-specific suppression reported may represent modulation of responsiveness by a population of idiotype-specific suppressor cells instead of suppressor cells capable of recognizing unique antigenic determinants. When an antigen capable of eliciting a relatively heterogeneous response is used to induce suppression, a series of idiotype-specific suppressor cells may be generated. It should be noted in this regard that the ability to demonstrate suppressive activity by transfer of nonresponsiveness to normal or reconstituted recipients may then depend on the presence of idiotypic determinants common to both the donors and recipients of suppressor cells. Evidence for such Igh-restricted suppressor cells has been generated in some systems (5-9). The possibility that such T-T or T-B cell interactions occur under normal physiologic conditions is more strongly supported by the demonstration that naturally occurring (38) or antigen-induced (11) suppressor cells are idiotype specific. The data presented in this report and that of Alevy and Bellone (11) extend these findings to well-defined hapten systems in which B cell responsiveness is suppressed. Because in the present studies, suppressor cells are generated with hapten coupled onto syngeneic cells, the results may more accurately represent physiologic suppressor mechanisms responsible for hyporesponsiveness to antigens presented in the context of self components. Thus, further study of the idiotype-restricted interac-
tions of lymphocytes in the NP-modified-spleen-cell system may elucidate naturally occurring mechanisms of self-tolerance.

Summary

The ability of suppressor cells induced by the intravenous administration of 4-hydroxy-3-nitrophenyl acetyl (NP)-modified syngeneic cells to reduce an idiotype B cell response was studied in both an in vivo and an in vitro system. Idiotype-positive B cells were assayed by the ability of guinea pig anti-idiotypic antiserum to specifically inhibit idiotype-positive plaque formation. It was found that up to 57% of the PFC response in vivo and 100% of the PFC response in vitro was inhibitable with anti-idiotypic antiserum. The expression of these idiotype-positive B cells could be suppressed by the transfer of spleen cells from mice treated 7 d previously with NP-coupled syngeneic cells. T cells are both required and sufficient for the transfer of idiotype-specific suppression. The induction of these idiotype-specific T suppressor cells directly with antigen suggests that recognition of unique determinants on cell surfaces is important for regulation of lymphoid cell interactions. The role of idiotype-specific suppressor cells in the network of lymphoid interactions is discussed.

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