HAPTON-SPECIFIC T CELL RESPONSES TO 4-HYDROXY-3-NITROPHENYL ACETYL

V. Role of Idiotypes in the Suppressor Pathway*

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Among the many complex lymphocyte interactions, the mechanisms of immune suppression are among the most intensively investigated. Several laboratories have shown that T cell-mediated suppression involves the interaction of at least two distinct subsets of T lymphocytes (1-5). We recently described the characteristics of two T cell subpopulations mediating suppression of delayed-type hypersensitivity (DTH)1 to the haptenic determinant 4-hydroxy-3-nitrophenyl (NP) (6, 7). When mice were injected with NP-derivatized syngeneic spleen cells, suppressive activity could be transferred to syngeneic recipients either before immunization with the NP compound, termed induction-phase suppression, or immediately before challenge for a DTH response, termed effector-phase suppression. Whereas the fine specificity of the induction-phase suppressor of Igh-lb-bearing mice was heteroclitic (cross-reactive with 4-hydroxy,5-iodo-3-nitrophenyl acetyl hapten [NIP]), just as the serum antibody, the fine specificity of the effector suppressors was not heteroclitic. Treatment of suppressor cell populations with anti-idiotype plus complement could abrogate induction-phase suppressor transfer without interfering with effector-phase suppressor transfer. Thus, it was concluded that these two modes of suppression were essentially assays of distinct suppressor T cell subsets. It was also shown that effector-suppressor transfer is restricted by Igh-V and -I region genes (7). To provide a more complete understanding of the mechanisms of immune suppression and to determine the significance of these genetic restrictions on the immune suppression pathway, the specificity of these two cell populations was investigated using direct antigen-binding methods. We have established that the NP idiotype-positive induction-phase suppressor T cells (Ts') are in fact antigen binding, whereas the NP*-negative effector-phase suppressor T cell population (Ts") is anti-idiotype. Furthermore, we provide evidence that the Ts' cell population induces the Ts" population.

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1 Abbreviations used in this paper: ABA, azobenzene arsonate; APC, antigen-presenting cell; BGG, bovine gamma globulin; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DTH, delayed-type hypersensitivity; FCS, fetal calf serum; MEM, Eagle's minimum essential medium; MHC, major histocompatibility complex; NIP, 4-hydroxy,5-iodo-3-nitrophenylacetyl; NP, 4-hydroxy-3-nitrophenyl; PBS, phosphate-buffered saline; RAMIg, rabbit anti-mouse immunoglobulin; Ts, suppressor T cells; Ts', induction-phase suppressor T cells; V_h, variable portion of the Ig heavy chain.
ROLE OF IDIOTYPES IN THE SUPPRESSOR PATHWAY

Materials and Methods

Mice. C57BL/6 mice were either purchased from The Jackson Laboratory, Bar Harbor, Maine, or were bred in the animal facilities of Harvard Medical School. Mice were used at 2–10 mo of age, and were maintained on laboratory chow and acidified, chlorinated water, ad lib.

Antigens. Bovine gamma globulin (BGG) was purchased from Sigma Chemical Co., St. Louis, Mo. NP-O-succinimide and NIP-O-succinimide were purchased from Biosearch, San Rafael, Calif. The preparation of NP-conjugated proteins has been previously described (8). The molar conjugation ratio of haptenic groups used in this work was NP17BGG, and NPT4-bovine serum albumin (BSA).

Haptenated Cell Preparation. Single cell suspensions of spleen cells were prepared in Eagle’s minimum essential medium (MEM) that contained 0.5% heparin. The supension was pelleted and treated with Tris-NH₄Cl to lyse erythrocytes. After two washes, the spleen cells were resuspended in phosphate-buffered saline (PBS), pH 7.6, at 10⁶ cells/ml. 25 μl of a 24-mg/ml solution of NP-O-succinimide in dimethylsulfoxide was added per milliliter of cells. After allowing the reaction to proceed for 3 min at room temperature, 10–30 ml cold PBS, pH 7.4, containing 1.2 mg/ml glycyl-glycine was added to stop the reaction. The cells were extensively washed in MEM containing 0.5% heparin before use.

Immunization. Experimental animals were primed with NP-BGG, in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.) containing 2 mg/ml Mycobacterium butyricum, so that the final concentration of antigen was 500 μg/ml in the CFA emulsion. 3 d before antigen priming, animals were pretreated with 20 mg/kg cyclophosphamide (Mead Johnson & Co., Evansville, Ind.) in 0.2 ml PBS injected intraperitoneally. A total of 0.2 ml antigen emulsion was injected subcutaneously divided between two sites on the dorsal flanks.

DTH Response. 6 d after immunization, mice were challenged for the DTH response by injecting 25 μl of 1 mg/ml of NP-BSA in PBS into the left footpad using a 27-gauge needle. Footpad swelling was measured 24 h after challenge using an engineer’s micrometer (Schlesingers For Tools Ltd., Brooklyn, N.Y.). Swelling was determined as the difference, in units of 10⁻⁴, between the left footpad thickness and the right footpad thickness. All animals were measured by two independent observers, whose measurements were then averaged. The responses thus elicited have been previously demonstrated to be a classical T cell-mediated DTH reaction by the following criteria: (a) kinetically the reaction reached a peak at 20–36 h after challenge; (b) T cells were required to transfer this reactivity to naïve recipients; and (c) major histocompatibility complex (MHC) identity at the I-A subregion was necessary for transfer of reactivity (8).

Reagents. Idiotypic antibodies were prepared using affinity-purified B6 anti-NP antibodies obtained from primary sera of a pool of C57BL/6 mice immunized with 100 μg NP-BGG in alum with 0.1 ml pertussis vaccine (U.S. Department of Public Health, Ann Arbor, Mich.). The antibodies were purified by precipitation with 45% saturated ammonium sulfate followed by affinity purification on a NP-BSA-coupled Sepharose 4B immunoadsorbent. Specific antibody was eluted from the column by washing the column with a 0.03 M NIP-caproate (Biosearch) in PBS solution. The eluate was extensively dialyzed in PBS. The eluate from this column contained λ₁-light chain and μ-, γ₁-, and small amounts of γ₂-heavy chains as detected by radioimmunodiffusion (9). Furthermore, isoelectric focusing of a sample of the radioiodinated purified immunoglobulin in a pH 3–10 range polyacrylamide gel revealed a remarkable restricted spectrotpe. The pool of idiotypic antibody was shown to be able to significantly inhibit heterologous idioype-anti-idioype interactions.

Normal C57BL/6 Ig was prepared using CFA-induced ascites according to Tung et al. (10). The ascites were collected, precipitated with 45% ammonium sulfate, extensively dialyzed, and stored at 1 mg/ml in PBS.

Cell Fractionation. T cells were purified according to Mage et al. (11). 5 ml of a 1 mg/ml solution of affinity purified rabbit antimouse immunoglobulin (RAMIg) were added to 100-× 15-mm polystyrene Petri plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and incubated for 1 h at room temperature. Unbound antibody was removed, and the plates were then washed extensively with PBS; then PBS containing 5% fetal calf serum (FCS). Spleen cells, previously treated for 3 min at 37°C with Tris-NH₄Cl to lyse erythrocytes, were
resuspended at a concentration of \(1.2 \times 10^7\) cells/ml. 6 ml of cells was added to the antibody-coated plates and incubated for 1 h at 4°C. The nonadherent T cell population was recovered by gentle swirling.

Specific suppressor cells were separated according to a modification of the method of Taniguchi and Miller (12). NP-BSA-coated plates were prepared by overnight incubation of 5.0 ml NP-BSA at a concentration of 1 mg/ml on 100- × 15-mm polystyrene Petri dishes at 4°C. The nonbinding NP-BSA was removed by washing as indicated above. NPb or normal C57BL/6 Ig-coated dishes were prepared according to the method of Abbas et al. (13). Briefly, 3.6–4.0 ml of anti-NP or normal Ig (0.5–1.0 mg/ml in PBS) was incubated on 100- × 15-mm polystyrene Petri dishes for 1–2 h at room temperature. After removal of immunoglobulin solutions, the plates were washed as above.

60–65 × 10^6 T cells were added to each plate in a total vol of 5 ml PBS 5% FCS. These cells were incubated for 1 h at 20°C. Nonadherent cells were then removed with two cycles of swirling and washing with warm (20°C) PBS with 5% FCS. Then, 5 ml of chilled PBS 5% FCS was added to all plates, which were then placed at 4°C for a further 30 min. The adherent cells were suspended by vigorous pipetting. After an additional chilled PBS 5% FCS wash with vigorous pipetting, the adherent cells were pooled, spun at 200 g for 10 min, and resuspended twice with chilled Hanks' balanced salt solution (HBSS) and brought to appropriate volume before transfer.

Results

Specificity of Induction and Effector Phase Suppression. To induce NP-specific suppressor cells, a suspension of erythrocyte-free spleen cells was derivatized with NP-O-succinimide. 2 × 10^7 NP-derivatized syngeneic spleen cells were injected intravenously into C57BL/6 mice. After 7 d, these mice were sacrificed, and their spleen cells (suppressor cells) were fractionated as described below. After fractionation, the indicated numbers of cells were transferred to mice at either the induction or effector phase of the DTH response. To analyze induction phase immune suppression, groups of 4–5 C57BL/6 mice were treated with 20 mg/kg cyclophosphamide. 3 d later (day 0) these animals received either normal spleen cells or fractionated suppressor T cells. After cell transfer, the animals were immediately immunized with 100 μg NP-BGG in CFA. 6 d after immunization the animals were challenged by injection of 25 μl NP-BSA in PBS into the left footpad. The delayed hypersensitivity response was measured as the increment of footpad swelling 24 h after challenge of the left foot as compared with the uninjected right foot. This protocol assays afferent or induction phase suppressive activity, because the suppressor cell populations are transferred before priming. In the second protocol, suppressor cell or normal cell populations were transferred to cyclophosphamide-pretreated, NP-BGG-primed mice on the day of antigen challenge (day 6). This protocol assays effector phase suppression. The data represent the average of at least two separate experiments.

The suppressor spleen cell population was passed over RAMIg-coated plates to enrich for T cells. T cell populations prepared with this reagent contain <5% surface Ig-positive cells by immunofluorescence. The activity of the unfractionated T cell population was titrated for suppressor activity. The T cell population was then adhered to NP-BSA-coated Petri dishes or to specifically purified C57BL/6 primary anti-NP antibody (idiotype)-coated Petri dishes. After adherence to antigen or idiotype plates, the nonadherent T cell populations were recovered. These are referred to as NP-nonadherent and idiotype-nonadherent cell populations, respectively. In the various experiments done, the percentage of nonadherent cells recovered was 90–97%
of the T cells applied to the dishes. The adherent cell populations were then recovered by temperature shift, as described in Materials and Methods. The recovered adherent cell populations represented 0.7–3% of the unfractionated T cell population. All cell populations were titrated for suppressive activity at both the induction (day 0) and effector (day 6) phases.

Antigen-induced NP suppressor T cell populations were adhered to antigen (NP-BSA)-coated petri dishes. Figs. 1 and 2 summarize experiments on the ability of these cells to act as induction phase and effector phase suppressors, respectively. Adherence to the antigen-coated plate depleted induction-phase suppressive activity from the T cell population; conversely, the cells that adhered to the antigen plate were highly enriched for induction phase suppressor activity (Fig. 1). When the same cell populations were assayed for effector suppressor activity, no detectable depletion or enrichment was noted in either cell population (Fig. 2).

Because the NP-effector phase suppressors are H-2 restricted (7), a formal possibility for their inability to bind antigen could be attributed to the general inability of H-2-
restricted cells to adhere to antigen except in the context of a cell surface MHC product (14). It is also possible that the appropriate antigen for binding of the effector suppressor cells is not NP itself, but that the effector suppressor cell bears an anti-idiotypic receptor. Such idiotype-specific suppressor T cells (Ts) have been described in other systems (15–18). Our previous demonstration of Igh-V restriction of the effector suppressors (7), taken together with reports by others on the existence of idiotype-specific Ts led us to ask if effector suppressor T cells would adhere to idiotype-coated petri dishes. Adherence to an idiotype-coated plate did not enrich induction phase (day 0) suppressive activity in either the idiotype nonadherent and idiotype adherent suppressor cell populations (Fig. 1). Whereas the idiotype nonadherent population did lose some of its induction phase suppressive activity, the idiotype-adherent population was not detectably enriched for this activity. This dichotomy may be attributable to nonspecific losses. When the same cell populations of idiotype-adherent and idiotype-nonadherent suppressor T cells were assayed for effector-phase (day 6) suppressive activity (Fig. 2), markedly different results were noted. Effector-phase suppressive activity was depleted in the idiotype-nonadherent population, and was markedly enriched in the idiotype-adherent population. Thus, induction-phase suppressive activity can be enriched by specific binding to antigen-coated plates, whereas effector-phase suppressive activity can be enriched by binding to idiotype, but not antigen.

Relationship between Induction and Effector-Phase Suppressor T Cell Populations. To investigate the possible causal relationship between the Ts\textsuperscript{i} and Ts\textsuperscript{e} populations, groups of C57BL/6 mice were pretreated with 20 mg/kg cyclophosphamide. The animals then received either 2 × 10\textsuperscript{7} NP-derivatized spleen cells, 5 × 10\textsuperscript{5} NP-adherent induction phase suppressors (Ts\textsuperscript{i}), or a mixture of both cell populations. The population of antigen-adherent Ts\textsuperscript{i} was not directly able to cause effector-phase suppression (Table I). 5 d after receipt of these indicated cell populations, 4 × 10\textsuperscript{7} spleen cells were transferred from each group of donors to groups of C57BL/6 mice that were previously primed for NP-specific DTH responses. Immediately after cell transfer, the recipients were challenged for NP-specific responses, to assay for the presence of effector-phase suppression. The results are shown in Table I. Cyclophosphamide-

| Group | Cyclophosphamide | Cells injected | Cells transferred into NP-BGG-primed recipients | DTH response |
|-------|------------------|----------------|-----------------------------------------------|--------------|
| A     | +                | --             | Group A                                       | 42.3 ± 1.6   |
| B     | +                | 2 × 10\textsuperscript{7} NP-spleen | Group B                                       | 37.8 ± 1.6   |
| C     | +                | 5 × 10\textsuperscript{5} NP adherent Ts | Group C                                       | 40.2 ± 2.8   |
| D     | +                | 5 × 10\textsuperscript{5} NP adherent Ts + 2 × 10\textsuperscript{4} NP-spleen | Group D                                       | 14.2 ± 3.3*  |
|       |                  | --             | Normal spleen                                 | 39.4 ± 3.3   |
|       |                  | --             | 5 × 10\textsuperscript{5} NP-adherent Ts      | 48.0 ± 4.7   |
|       |                  | --             | 1.5 × 10\textsuperscript{5} NP-nonadherent Ts | 11.8 ± 2.8*  |

*See text for protocol.

* P < 0.01.
pretreated groups of mice receiving haptenated spleen cells intravenously were not able to transfer effector suppression. Similarly, animals receiving NP-adherent induction phase suppressors did not manifest an ability to transfer effector-phase suppression. However, when cyclophosphamide-pretreated animals were given NP-adherent suppressor T cells together with NP-derivatized spleen cells, effector-phase suppression could easily be demonstrated. Thus, antigen-adherent induction-phase suppressors can, in the presence of NP-derivatized spleen cells, induce a cyclophosphamide-resistant population of cells to mature and manifest effector suppression.

Discussion

Our data demonstrate that two populations of suppressor T cells that arise as a consequence of immunization with hapten-conjugated syngeneic cells may be distinguished based upon their receptor-binding specificities. The induction-phase suppressor cell binds antigen, and the effector-phase suppressor cell binds to idiotypic antibody molecules. These findings directly demonstrate that introduction of antigen alone is sufficient to trigger regulatory T cells, which appear to be members of an idiotypic network. We have previously shown that the effector suppressor cell population active on NP-specific DTH is both variable portion of the Ig heavy chain (V<sub>H</sub>) and I-A restricted in its activity by both Igh-V and I region genes. It now appears that the V<sub>H</sub> restriction is a consequence of an anti-idiotype-bearing receptor on the effector suppressor cell. It is remarkable, however, that an I-restricted population of cells apparently was bound to an idiotype-coated plastic dish, i.e., not in the context of a cell-surface alloantigen. This may be attributed to several mechanisms. One possibility is that the population of T cells responsible for the I restriction is distinct from the population with anti-idiotypic receptors. The apparent ability to enrich the suppressive ability would then be a result of cotransfer, either specifically or nonspecifically, of such H-2-restricted cells. Another possibility is that the same cells are I restricted and have anti-idiotypic specificity. If this hypothesis is to be verified, whether one or two receptors are involved in the dual specificity should be determined. In such a model, one would postulate that the anti-idiotypic (portion of the) receptor has sufficiently high affinity to bind to high densities of idiotype on the surface of a solid surface. A third possibility is that the effector suppressor cell secretes a soluble factor which requires I region homology for its activity. Our data do not allow us to distinguish among these possibilities.

Our results are consistent with a large number of previous observations. Thus, both Waltenbaugh et al. (1) and Tada (2) found that antigen-specific soluble suppressor factor acted by triggering a second set of suppressor cells. Germain et al. (19) extended these observations, showing that this process requires both I-J<sup>+</sup> suppressor factor, shared idiotypic determinants with antibodies of the same specificity (as the Ts<sup>i</sup> shares NP<sup>i</sup> idiotype with anti-NP antibodies), and antigen. Sy et al. (3) have recently reported that antigen-induced azobenzene arsonate (ABA)-specific suppressor factor which shares cross-reactive idiotypic determinants with antiarsonate antibodies, induces Ts<sub>2</sub> which are anti-idiotypic, as demonstrated by direct binding. However, elicitation of this anti-idiotypic response was not demonstrated using antigen alone in the ABA system at the time when they are detected in the NP system nor was the Ts<sup>i</sup> vs. Ts<sup>e</sup> nature of the induced Ts determined. Several groups have reported idiotypic or anti-idiotypic T cell-derived suppressor factors or cells in mice specifically sup-
pressed with anti-idiotypic antibodies (3, 20-22). Finally, Harvey et al. (23) have also found idiotypic determinants on Ts active in a humoral response suppressor circuit, and Moorhead (24, 25) have documented the existence of H-2-restricted Ts for dinitrofluorobenzene contact sensitivity.

Although the idiotype-adherent T cell population is capable of mediating immune suppression of the efferent phase of the DTH response, administration of idiotype-adherent cells at the time of antigen priming fails to suppress the NP-induced NP or NIP-elicited DTH responses in C57BL/6 mice (Fig. 1; and J. Z. Weinberger. Unpublished observations.). The inability of these idiotypic-adherent cells to suppress these DTH responses in the afferent mode may be attributed to the failure of this cell population to survive or to function in the absence of Ts\(^i\) cells, at least under the conditions used in our experiments.

The data presented in this report provide a framework for integrating these various observations into a single antigen-induced suppressor pathway. A schematic model of the various cell populations and their postulated interactions is shown in Fig. 3. Many of the features of the network hypothesis (26) are strongly supported by the data presented. Haptenated spleen cells induce a population of induction phase I-J-bearing suppressor T cells, termed Ts\(_i\) in other systems which bear an idiotypic, antigen-binding receptor. No H-2 restriction has as yet been demonstrated in the triggering of this cell. The Ts\(_i\) population stimulates, in the presence of antigen, maturation of a second population of suppressor T cells, the effector suppressor or Ts\(_e\) population, which bears a receptor complementary to the induction-phase suppressor. The Ts\(_e\) receptor is, therefore, anti-idiotypic in its nature. The requirement for antigen in the induction of Ts\(_e\) populations has also been demonstrated in other systems (27). We have previously demonstrated that the DTH effector T cell population is itself under VH control. It is likely then that a cell in the effector pathway bears an idiotypic, antihapten receptor. Because the effector suppressor has an anti-idiotypic receptor, it could easily recognize specific clones of DTH effector T cells, and transmit a suppressive signal. One possibility might be that the effector suppressor I-A restriction is a consequence of an anti-I-A receptor which must interact with antigen-presenting cell I-A determinants, whereas the VH restriction is a consequence of an anti-idiotypic

![Fig. 3. Immunoregulation of NP-induced T cell response. A model for the cellular interactions occurring during immunosuppression. The interacting cell populations are indicated by large squares. ■ indicates the NP haptenic determinant. (T) indicates a receptor with NP specificity sharing idiotypic determinants with serum anti-NP antibody. (T) indicates a receptor with specificity for the anti-NP-specific idiotypic receptor. Cyclo, cyclophosphamide; Ag, antigen.](image)
role which must interact with the DTH effector T cell. Possibly, both these interactions are required to interfere effectively with antigen-presenting cell (APC) triggering of DTH-T cell activity. Whatever the actual target cell of the effector suppressor cell, the data clearly support idiotype-anti-idiotype interactions in an antigen-driven suppressor T cell pathway.

Summary

4-Hydroxy-3-nitrophenyl (NP) derivatized syngeneic spleen cells injected intravenously stimulate maturation of an antigen-binding, idiotype-bearing induction-phase suppressor cell population, as well as an idiotype-binding anti-idiotype-bearing effector-phase suppressor cell population. Both cell types are present simultaneously in the spleen cell population 7-d after their induction. Furthermore, the cell population with antigen-binding properties can, in the presence of NP-derivatized syngeneic cells, induce a population of effector suppressor cells. The precursors of the effector suppressor population are not sensitive to concentrations of cyclophosphamide which prevented the generation of induction phase suppressor cells. These data provide direct evidence in support of the theory of network regulation of immune suppression.

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