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

XII. Fine Specificity of Anti-Idiotypic Suppressor T Cells (Ts2)*

By DAVID H. SHERR, SHYR-TE JU, AND MARTIN E. DORF

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

It has been well documented (1–11) that the manifestation of immune suppression is the result of interactions among several T lymphocyte subsets. These cellular interactions often depend upon the recognition of unique idiotypic determinants present on the various lymphoid elements (4–11).

We have previously (6) characterized one such system in which murine suppressor T cells, which are induced by the intravenous administration of syngenic spleen cells covalently coupled with the 4-hydroxy-3-nitrophenyl acetyl (NP)[1] hapten, specifically affected the responses of NP^b idiotype-bearing B lymphocytes both in vivo and in vitro. The ability of these Lyt-1⁻, Lyt-2⁺, Igh-restricted suppressor T cells to bind idiotype and to function during the effector phase of an in vitro response (7) suggested that they corresponded to the effector-phase suppressor T cell (Ts^e or Ts_2) involved in the suppression of NP-specific delayed-type hypersensitivity and cutaneous sensitivity responses (5, 11). The recognition of idiotypic determinants by suppressor cells appeared then to be important for the regulation of both T and B cell responses.

It is well established (12–15) that many defined idiotypic systems are composed of a family of idiotypically related but nonidentical molecules. In the NP^b idiotypic system, for example, most monoclonal anti-NP antibodies exhibit only a fraction of serum NP^b idiotypic specificities (14). To determine whether T cell receptors recognize the same repertoire of NP^b idiotypic determinants as anti-idiotypic antiserum, the ability of effector-phase suppressor cells to bind monoclonal anti-NP antibodies bearing different levels of NP^b idiotypic determinants was studied. The results suggest that anti-idiotypic effector-phase suppressor T cells do not recognize the predominant serologically detected NP^b idiotypic determinants.

Materials and Methods

Mice. C57BL/6 and SJL male mice were purchased from The Jackson Laboratories, Bar Harbor, Maine. C3H.NB mice were bred in the Harvard Medical School Facilities.

Antigens. NP conjugated to keyhole limpet hemocyanin (KLH), bovine gamma globulin (BGG), and Ficoll were prepared as described previously (7). The NP-KLH and NP-Ficoll had

[1] *Abbreviations used in this paper:* ABA, azobenzenearsonate hapten; BGG, bovine gamma globulin; KLH, keyhole limpet hemocyanin; MEM, minimal essential medium; NP, (4-hydroxy-3-nitrophenyl) acetyl hapten; PFC, plaque-forming cell(s); RAMG, rabbit anti-mouse immunoglobulin.

an average of 30 and 17 NP groups per 100,000 mol wt, respectively. The molar conjugation ratio of NP-BGG was 12.

Immunization. Cells for in vitro responder cultures were obtained from C57BL/6 mice immunized intraperitoneally 4 wk previously with 150 μg NP-KLH in a 0.2-ml mixture containing 25% pertussis vaccine (Michigan Department of Public Health, Lansing, Mich.). To prepare purified anti-NP antibodies, C57BL/6 or C3H.NB mice were immunized with 100 μg NP-BGG in maalox/pertussis vaccine. Pooled sera collected 7–21 d after primary immunization were specifically purified on NP-bovine serum albumin conjugated affinity columns, as detailed elsewhere (5). Ouchterlony analysis suggested that the purified antibodies were primarily IgM, although weak precipitin lines were also observed with anti-IgG subclass antisera.

Anti-Idiotypic Antisera. Guinea pigs were hyperimmunized with 100 μg purified primary C57BL/6 anti-NP antibodies in complete Freund's adjuvant as detailed elsewhere (5). The anti-idiotypic antisera were extensively adsorbed with columns conjugated with gamma globulin fractions of MOPC 104E (μ, λ_1) and (C57BL/6 $\times$ DBA/2)F$_1$ ascitic fluid. The adsorbed antisera did not react with IgM, IgG, κ, or λ chains by Ouchterlony analysis.

Generation of Suppressor Cells. NP-coupled spleen cells were made as described previously (5). Cells were washed extensively in minimum essential medium (MEM) before injection of 3 $\times$ 10^7 NP-coupled cells i.v. into syngeneic recipients.

Suppressor Cell Induction. 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 16-mm culture wells (Flow Laboratories, Inc., Hamden, Conn.) at a concentration of 7.5 $\times$ 10^6 cells/ml/well in Mishell-Dutton culture medium containing 10% fetal calf serum (lot 98678; Microbiological Associates, Walkersville, Md.). Cultures were incubated at 37° in a 10% CO_2 atmosphere in rocking culture boxes and were fed daily with 0.1 ml supplementary medium for a 4-d subculture period. Cells prepared in this fashion have been shown to correspond to the second-order suppressor cells (Ts^e or Ts$_2$) previously characterized in the NP system (5–7).

Purification of T Cells and Fractionation of Idiotype-binding Cells. The method of Mage et al. (16) was used to purify splenic T cells from subculture. To separate idiotype-specific suppressor T cells, 3 ml of a 0.5 mg/ml solution of affinity-purified serum or monoclonal anti-NP antibodies were incubated on 60 $\times$ 15 mm polystyrene petri dishes for 1 h at room temperature. After removal of immunoglobulin solutions the plates were washed extensively. 5 $\times$ 10^6 to 10 $\times$ 10^6 purified suppressor T cells were added to each plate in a total volume of 2.5 ml MEM containing 5% fetal calf serum. Cells were incubated for 1 h at room temperature. Nonadherent cells were recovered, and the dishes were washed extensively with warm (20°C) media. After addition of 2.5 ml cold media, dishes were incubated on ice for 30 min. Adherent cells were resuspended with vigorous pipetting, washed, and counted. 10^5 to 5 $\times$ 10^5 viable fractionated cells were then added to responder cultures challenged 4 d previously with 100 ng NP-Ficoll; the next day cultures were assayed for plaque-forming cells (PFC).

Monoclonal NP-specific Antibodies. The N (μ, λ_2), 4C2 (μ, κ) and 6100.15 (μ, λ_1) hybridoma cell lines were established, as described previously (15). These anti-NP antibodies share Igh^b allotypic determinants. The monoclonal N and 4C2 antibodies were derived from SJL (Igh^b) splenocytes, and the 6100.15 hybrid was derived from C.B-17 (Igh^b) splenocytes.

Radioimmunoassays. Inhibition of idiotype-binding assays were carried out with suboptimal quantities of anti-idiotypic antiserum that bind ~40% of the ^{125}I-labeled specifically purified C57BL/6 serum anti-NP antibody ligand, as detailed elsewhere (15). The results were expressed as percent inhibition of idiotype binding.

PFC Assay. NP or sheep erythrocyte-specific PFC were assayed as reported previously (6, 7).

Results

Idiotype Specificity of Effector-Phase Suppressor T Cells. To confirm the idiotype specificity of effector-phase suppressor T cells, C57BL/6J mice were treated with an intravenous injection of control or NP-coupled syngeneic spleen cells. 7 d later their spleens were removed and subcultured for a 4-d period. Control or suppressor cells

were then enriched for T cells by fractionation over rabbit anti-mouse immunoglob-ulin (RAMG)-coated plates. Suppressor T cells were further fractionated on petri dishes coated either with affinity-purified C57BL/6J (Igh^b) or C3H.NB (Igh^j) anti-NP antibody. 1 × 10^5 to 5 × 10^5 adherent or nonadherent T cells were added to responder cultures challenged 4 d previously with NP-Ficoll. Cultures were assayed 1 d later for direct NP-specific PFC. The results presented in Table I indicate that effector-phase suppressor T cells were completely depleted by passage over NP^b idiotype-coated plates. Suppressor activity could be completely recovered from the adherent population. In contrast, the suppressive activity was not depleted by passage over C3H.NB anti-NP antibody (NP^j)-coated plates. These data confirm previous observations (7) and again demonstrate that all of the suppressive activity can be specifically removed on NP^b-coated dishes.

To further define their specificity, suppressor T cells were fractionated on petri dishes coated either with N, 4C2, or 6100.15 monoclonal anti-NP antibody. Each of these monoclonal antibodies is of the IgM class and was derived from mice of the Igh^b allotype. The data presented in Table II indicate that suppressor T cell activity could be detected in the N antibody adherent and, to a lesser extent, in the nonadherent fractions. This contrasts with the results shown in Table I, which demonstrated that these suppressor cells were completely depleted by passage over serum anti-NP antibodies. The results with N hybridoma imply that effector-phase suppressor T cells are a heterogeneous population of cells, some of which recognize determinants present on this monoclonal anti-NP antibody. In contrast, all of the suppressive activity could be found in the 4C2 or 6100.15 nonadherent fractions, indicating that effector-phase suppressor T cells (Ts2) do not recognize idiotypic determinants present on the 4C2 or 6100.15 anti-NP antibodies.

To establish that all of these anti-NP antibodies adhered to the plastic plates, the antibodies were adsorbed to plastic and then reacted with a radiolabeled anti-μ

TABLE I

*NP^b Idiotype Specificity of C57BL/6 Effector-Phase Suppressor T Cells**

Number of T cells added	Idiotype used for fractionation	Fraction	Percent suppression ± SE‡
5 × 10^5	None	—	82 ± 14
1-5 × 10^5	Serum NP^b	Nonadherent	−19 ± 9§
1-3 × 10^5	Serum NP^b	Adherent	79 ± 17
1-5 × 10^5	Serum NP^j	Nonadherent	67 ± 9
1 × 10^5	Serum NP^j	Adherent	0 ± 5§

* C57BL/6 mice received an intravenous injection of 3 × 10^7 control or NP-coupled syngeneic spleen cells. 7 d later, these spleens were cultured for a 4-d period. Control or suppressor cells were then enriched for T cells by passage over RAMG plates. Suppressor T cells were further fractionated on NP^j or NP^b-coated dishes. 1 × 10^5 to 5 × 10^5 control or fractionated suppressor T cells were then added to responder cultures challenged 4 d previously with 100 ng NP-Ficoll. 1 d later duplicate wells were pooled and assayed for direct NP-specific PFC responses. The data represent the pooled results obtained from three separate experiments.

‡ The percent suppression relative to controls is presented as the arithmetic mean ± SE. The geometric mean of control PFC responses was 2,720 $\stackrel{\times}{\div}$ 1.6 PFC/culture.

§ Significant decrease in suppression relative to the suppression in the unfractionated T suppressor cell group, $P < 0.03$.

TABLE II

Fine Specificity of Idiotype-specific Effector-Phase Suppresser T Cells*

Number of T cells added	Monoclonal antibody used for fractionation	Fraction	Percent suppression ± SE (number of experiments)‡
5×10^5	None	—	55 ± 10 (5)
5×10^5	N (Igh^b, IgM, λ_2)	Nonadherent	29 ± 9 (5)
$1-2 \times 10^5$	N	Adherent	49 ± 9 (5)
5×10^5	6100.15 (Igh^b, IgM, λ_1)	Nonadherent	51 ± 9 (4)
$1-2 \times 10^5$	6100.15	Adherent	−8 ± 8 (4)§
5×10^5	4C2 (Igh^b, IgM, κ)	Nonadherent	60 ± 17 (3)
$1-2 \times 10^5$	4C2	Adherent	−3 ± 4 (3)§

* C57BL/6 mice received an intravenous injection of 3×10^7 control or NP-coupled syngeneic spleen cells. 7 d later these spleens were cultured for a 4-d subculture period. Control or suppressor cells from these subcultures were then T cell-enriched by passage over RAMG-coated petri dishes before passage on dishes coated with N, 4C2, or 6100.15 monoclonal anti-NP antibodies. 1×10^5 to 5×10^5 viable control or fractionated T suppressor cells were then added to responder cultures challenged 4 d previously with 100 ng NP-Ficoll. 1 d thereafter, duplicate wells were pooled and assayed for direct NP-specific PFC responses in triplicate slides. The geometric mean of control PFC responses from five experiments was 1,500 $\overset{\times}{\div}$ 1.5.

‡ Percent suppression relative to controls ± SE. The number of experiments is indicated in parentheses.

§ Significant difference in suppression relative to unfractionated suppressor cells, $P < 0.006$.

antibody. The results indicated that the three monoclonal anti-NP antibodies had similar plastic adherence properties (data not shown).

Idiotypic Determinants on Monoclonal Anti-NP Antibodies. To determine whether the three monoclonal anti-NP antibodies used to fractionate suppressor T cells possessed determinants recognized by a guinea pig anti-idiotypic antiserum, inhibition of NP^b idiotype binding was determined by radioimmunoassay, as described previously (15). The data in Fig. 1 indicate that 10 μg 6100.15, N, and 4C2 monoclonal anti-NP antibodies inhibit the binding of guinea pig anti-NP^b idiotype to ^{125}I-labeled serum anti-NP antibodies 74%, 36%, and 5% respectively. As expected, 10 μg of heterologous C57BL/6 anti-NP antibodies used in the preparation of anti-idiotypic antiserum inhibited this reaction 95%. As specificity controls, 10 μg of C57BL/6 anti-azoben-zenearsonate hapten (ABA) or 30 μg MOPC 315 or MOPC 104E failed to inhibit binding (Fig. 1). From these data it appears that the determinants present on 6100.15 antibody share a majority of the NP^b idiotypic determinants, whereas determinants intrinsic to the N-hybridoma antibody represent only a minority of these NP^b determinants (36% inhibition). The 4C2 antibody apparently shares little or no NP^b idiotypic determinants present on the serum anti-NP ligand that are recognized by anti-idiotypic antiserum. This was expected because κ-bearing antibodies lack NP^b idiotype (17).

When equal amounts of 6100.15 and N antibodies were added as inhibitor, the results were identical to those obtained with 6100.15 alone, indicating that the NP^b idiotypic determinants present on N-hybridoma, as defined by this assay, are a subset of those present on 6100.15 antibody. Because the majority of the effector-phase suppressor T cells recognized determinants present on N but not 6100.15 antibody, it
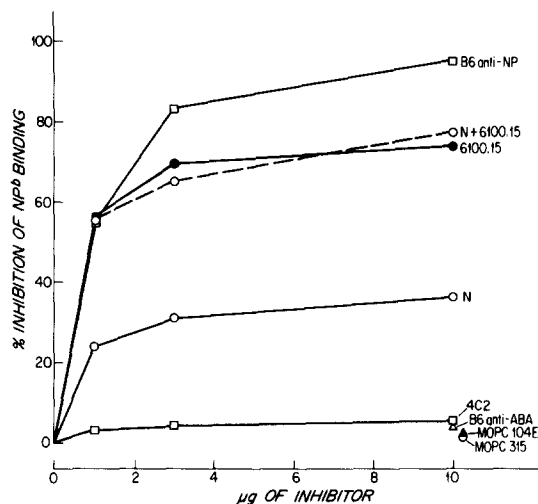
Fig. 1.   Affinity-purified C57BL/6 serum (□), 6100.15 monoclonal (●), N monoclonal (○), and 4C2 (□) monoclonal anti-NP antibodies and C57BL/6 serum anti-ABA antibodies (△) were tested for their ability to inhibit the binding of ^{125}I-labeled serum anti-NP antibodies to limiting amounts of anti-NP^b idiotypic antibodies. 30 μg of MOPC 104E (μ, λ) or MOPC 315 (α, λ_2) myeloma antibodies also failed to inhibit binding, verifying the specificity of this idiotypic system. The ability of a mixture of 6100.15 and N antibodies to inhibit binding was tested by addition of equal amounts of each antibody, as indicated on the abscissa (dashed line). Results are expressed as the percent inhibition of NP^b idiotype binding with 1–10 ng purified antibody.

may be concluded that the major set of serologically defined determinants are not recognized by anti-idiotypic suppressor Ts₂ cells.

Discussion

The ability of suppressor T cells to recognize idiotypic determinants present on monoclonal anti-NP antibodies was studied. Previously it had been shown (6, 7) that spleen cells from mice given an intravenous injection of NP-conjugated syngeneic spleen cells were capable of suppressing the NP-specific PFC response either when transferred in vivo to normal Igh-compatible recipients or when subcultured for a 4-d period and then added to in vitro cultures (6, 7). These suppressor T cells apparently correspond to second-order suppressor cells (Ts₂ or Ts^e) described in other systems (1, 5, 11) because they are Lyt-1$^-$, Lyt-2$^+$ T cells that function during the effector phase of the response (7). The ability of these lymphocytes to recognize idiotypic determinants present on serum anti-NP antibody from mice of the Igh^b but not the Igh^j allotype (Table I and reference 7) and their failure to suppress across an Igh allotype barrier (7) support the theory that these cells interact with their targets by recognition of idiotypic determinants linked to the Igh^b complex. Because the NP^b idiotype consists of a family of related but nonidentical anti-NP antibody molelcules (13, 14), it was of interest to determine whether Ts₂ suppressor cells recognize the same members of the NP^b family as those recognized by anti-idiotypic antibody. To test this hypothesis, effector-phase suppressor cells (Ts₂) were tested for their ability to bind monoclonal anti-NP antibody. Antibodies of the IgM class were chosen because it has been suggested that IgM molecules bear V-region determinants that closely resemble germ-line products (18). The data demonstrated that T suppressor cells do

not recognize the major NP^b idiotypic determinants because Ts$_2$ cells failed to recognize the determinants present on the 6100.15 antibody despite the presence of a major fraction of NP^b idiotypic determinants on this monoclonal antibody as measured by the inhibition of idiotype binding (Fig. 1).

That the suppressor cells interact with some determinants expressed on one anti-NP monoclonal antibody was demonstrated by the ability of most suppressor cells to adhere to dishes coated with the N-hybridoma antibody. This indicates that the Ts$_2$ cells are actually specific for immunoglobulin molecules and do not bind to a T cell-derived material that might have copurified with the serum anti-NP antibodies. However, not all of the suppressor cells adhered. Thus, it appears that these Ts$_2$ suppressor cells represent a heterogeneous population of idiotype-specific cells, only a fraction of which recognize determinants on N-hybridoma antibody. Interestingly, the determinants present on the N antibody that were recognized by T cells appear not to be the NP^b idiotypic specificities. This is demonstrated by the observation that the fraction of NP^b idiotypic specificities on N antibody were also present on 6100.15 hybridoma antibody, i.e., 6100.15 plus N antibodies inhibited binding no better than 6100.15 antibody alone. Yet the 6100.15 anti-NP antibody-coated plate did not remove suppressor cell activity. Thus, there is no apparent correlation between the idiotypic determinants recognized by Ts$_2$ cells and those recognized by anti-idiotypic antiserum under the conditions tested.

The suppressive activity remaining in the N monoclonal nonadherent fraction apparently was not the result of saturation of the antibody-coated plate because the anti-NP antibodies had similar plastic adherence properties and all suppressor cell activity could be depleted by passage over dishes coated with C57BL/6 anti-NP serum antibodies under identical conditions. The ability of dishes coated with the serum-derived anti-NP antibodies to bind all of the anti-idiotypic suppressor cells implies that anti-NP antiserum from C57BL (Igh^b) mice possess a sufficiently diverse repertoire of idiotypic determinants to bind essentially all members of a heterogenous anti-idiotypic suppressor T cell population, whereas the monoclonal N antibody detects only a subset of these cells.

A considerable amount of evidence has accumulated indicating that T cell receptors may express determinants that are idiotypically related to determinants on serum antibodies (19–21). However, our studies suggest that the repertoire of idiotypes present on cells that interact with effector-phase suppressor T cells (Ts$_2$) may differ from that expressed on serum antibodies. One explanation for this result is that the V genes used by T suppressor cells to produce NP-specific receptor molecules might be different from, or a minor fraction of, the V genes used by B cells to make immunoglobulin of the same specificity. Alternatively, it might be postulated that although the appropriate heavy and light chains are required to form idiotypic determinants on immunoglobulins (17, 22), light chains are not involved in T cell receptors and therefore contribute nothing to T cell idiotypic determinants (23, 24). Studies are currently underway to analyze the role of heavy and light chain determinants on T lymphocyte receptors.

Summary

The fine specificity of anti-idiotypic, effector-phase suppressor T cells (Ts$_2$) induced by the intravenous injection of syngeneic spleen cells covalently coupled with the 4-

hydroxy-3-nitrophenyl acetyl (NP) hapten was studied in an in vitro plaque-forming cell system. By comparing the ability of these suppressor cells to bind monoclonal anti-NP antibodies that express different levels of serologically detected NP^b idiotypic determinants, it was shown that anti-idiotypic suppressor T cells do not recognize the predominant NP^b idiotypic determinants that are defined by serologic analysis. The implications for the possible expression and/or recognition of different sets of idiotypic determinants on T and B cells are discussed.

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