ANALYSIS OF T CELL HYBRIDOMAS

III. Distinctions between Two Types of Hapten-specific Suppressor Factors that Affect Plaque-forming Cell Responses

BY DAVID H. SHERR,‡ MUTSUHIKO MINAMI, KENJI OKUDA, AND MARTIN E. DORF

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

The intravenous injection of 4-hydroxy-3-nitrophenyl acetyl hapten (NP)\(^1\)-modified syngeneic spleen cells results in profound suppression of contact sensitivity (CS), delayed-type hypersensitivity (DTH), and plaque-forming cell (PFC) responses (1–9). This hapten-specific suppression is mediated by a cascade of T cell interactions. The first cell in the pathway responsible for suppression of CS and PFC responses is present in spleens of mice treated 7 d previously with an intravenous injection of NP-coupled spleen cells (1). A hybridoma line derived from this population secretes a suppressor factor capable of inducing a second-order, effector-phase suppressor T cell population (\(T_{S2}\)) in vivo (2). The \(T_{S2}\) suppressor cell population interacts directly with an I-A\(^b\)-bearing, NP-specific T cell population (\(T_{S3}\)) induced in NP-primed mice (5, 9). The ultimate effect of this suppressor T cell pathway is the specific suppression of CS responses to NP (2–5) and preferential suppression of NP\(^b\) idiotype-bearing B cell clones both in vivo (6) and in vitro (7).

All of the studies performed in the PFC system have involved heterogeneous cell populations obtained either directly from spleens of treated mice or from cultures. The results indicate that a similar suppressor T cell pathway may affect both T cell-mediated CS and B cell-mediated PFC responses. The purpose of the present studies was to determine whether the same T cells within these mixed populations are capable of suppressing both CS and PFC responses. To this end, cloned T cell hybridomas corresponding to the \(T_{S1}\) and \(T_{S3}\) populations were prepared. We demonstrate that the hybridoma-derived factors previously shown to suppress in vivo CS responses also suppress the in vitro PFC responses, despite the difference in target populations. In addition, the characteristics of these hybridomas and the biologically active factor that they secrete are compared. The data indicate that, whereas both hybridoma-

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Abbreviations used in this paper: AECM, N-(2-aminoethyl) carbamylmethylacetyl; BA, Brucella abortus; BSA, bovine serum albumin; C, complement; CS, contact sensitivity; DTH, delayed-type hypersensitivity; DTT, dithiothreitol; GL\(\phi\), poly-(L-Glu\(^3\).L-Lys\(^3\).L-Phe\(^3\)); KLH, keyhole limpet hemocyanin; MEM, minimal essential medium; NGPS, normal guinea pig serum; NMS, normal mouse serum; NP, (4-hydroxy-3-nitrophenyl) acetyl hapten; NP\(^b\), IgM-linked idiotypic determinants on anti-NP antibody; O-Su, O-succinimide; PBS, phosphate-buffered saline; PFC, plaque-forming cell(s); RAMG, rabbit anti-mouse immunoglobulin; SRBC, sheep erythrocytes; TNP, trinitrophenyl hapten; TsF, monoclonal suppressor factor; \(T_{S1}\), first-order NP-specific T cells; \(T_{S2}\), second-order NP\(^b\)-specific T cells; \(T_{S3}\), third-order NP-specific T cells.
derived factors have NP-binding receptors and express I-Jβ and NPβ-related determinants, they can be distinguished on the basis of kinetics of suppression, ability to induce effector-phase suppressor cells, and ability to replace Ts8 populations. In addition, the factor secreted by the Ts8 hybridoma probably interacts directly with B lymphocyte targets and has two chains, only one of which binds NP.

Materials and Methods

Mice. Male C57BL/6 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All animals were between 8 and 12 wk of age at the time of immunization. Animals were age matched in each experiment.

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Dept. of Health, Education, and Welfare Publication (NIH) 78-23, revised 1978).

Antigen. NP-bovine serum albumin (BSA), NP-keyhole limpet hemocyanin (KLH) and NP-Ficoll were conjugated by the reaction of NP-O-succinimide (NP-O-Su) (Biosearch Inc., San Rafael, CA) with BSA, KLH (Sigma Chemical Co., St. Louis, MO), or N-(2-aminoethyl) carbamylmethylicacetyl (AECM)-Ficoll (Biosearch) as described (6). The NP-BSA, NP-KLH, and NP-Ficoll had an average of 12, 30, and 17 NP groups per 100,000 daltons, respectively. NP-Brucella abortus (BA) was prepared by the reaction of 1 mg NP-O-Su in dimethyl formamide with 1 ml of stock BA (Ring Test antigen, U. S. Department of Agriculture, Ames, IA) in Na2CO3 buffer, pH 8.0. Trinitrophenyl hapten (TNP)-BA was prepared according to the method of Mond et al. (10).

Immunization. Responder cells for in vitro cultures or for generation of Ts8 populations were obtained from mice immunized intraperitoneally 4 wk previously with 50 µg of NP-KLH in a 0.2-ml mixture containing 25% pertussis vaccine (Michigan Department of Public Health, Lansing, MI).

Suppressor T Cell Populations. Ts2 suppressor cell populations were obtained by the 4-d subculture of spleen cells from mice treated 7 d previously with NP-coupled syngeneic spleen cells as described (7). Ts8 suppressor cell populations were obtained from spleen cells of mice immunized 4 wk previously with NP-KLH (9). T cell-enriched populations were obtained by passage over rabbit anti-mouse immunoglobulin (RAMG)-coated petri dishes according to the procedure of Mage et al. (11). T cells purified on RAMG-coated dishes were <2% immunoglobulin positive as determined by fluorescence analysis.

Suppressor T Cell Hybridomas and Suppressor Factors. The generation of the B6-Tsl-29 hybridoma has been described (2). Briefly, spleen cells from mice treated 7 d previously with an intravenous injection of NP-coupled syngeneic spleen cells were hybridized with BW5147 tumor cells. Supernatants from cloned hybridoma cells that carried I-Jβ- and NPβ-related idiotypic cell surface markers were tested for biological activity as described (2, 3).

The production of the B6-Ts8-8 hybridoma by fusion of BW5147 with T cells from C57BL/6 mice that received an intravenous injection of NP-coupled syngeneic spleen cells is detailed elsewhere (4). The hybridoma cells were screened by a cytotoxicity test with anti-I-Jβ alloantiserum. The B6-Ts8-8 cell line was cloned and factors derived from this line have been characterized in the CS system (4). Several hybridomas corresponding to Ts8 suppressor cells were simultaneously obtained from this fusion and are described elsewhere (3).

All hybridomas were cultured in RPMI 1640 containing 8% fetal calf serum and 0.01 M Hepes buffer. The supernatants used in the present experiments as the source for suppressor factor were collected from cultures at a density of ~7.5 × 10⁶ cells/ml. Pools of supernatants were frozen at −70°C in aliquots.

Responder Cultures. Spleen cells from C57BL/6 mice immunized 4 wk previously with NP-KLH or TNP-KLH were teased into a single-cell suspension under sterile conditions. Cells were washed and resuspended in Mishell-Dutton medium containing 10% fetal calf serum. 7.5 × 10⁶ viable primed cells were added to each well of a 16-mm culture plate. Cultures were challenged with 200 ng of NP-Ficoll, a 1:20 dilution of NP-BA, or 1:80 dilution of TNP-BA in
40 μl minimal essential medium (MEM). Sheep red blood cell (SRBC)-specific PFC responses were induced by the addition of 2 × 10^6 SRBC to cultures of 7.5 × 10^6 normal C57BL/6 spleen cells. Cultures were incubated in rocking boxes at 37°C in a 10% CO2 atmosphere and fed daily with 0.1 ml of supplementary medium. 5 d after challenge, duplicate wells were pooled, washed, and assayed for direct NP- or SRBC-specific PFC responses on triplicate slides in a modified Jerne plaque assay (12).

**Anti-I-J and Anti-Thy-1.2 Antiserum Treatments.** 2 × 10^6 spleen cells from C57BL/6 mice immunized 4 wk previously with NP-KLH were washed in MEM, pelleted, and treated with 0.3 ml of 5R anti-3R (anti-I-J^b) or AKR anti-CBA thymocyte (anti-Thy-1.2) antiserum diluted 1:5 in MEM for 30 min at room temperature. Cells were then washed, pelleted, and treated for 30 min at 37°C with 0.3 ml of rabbit complement (C) diluted 1:5 in MEM containing 0.01 mg/ml DNase. Cells were washed, resuspended in Mishell-Dutton medium, and 7.5 × 10^6 viable cells were added to each well. The anti-I-J^b antiserum used in these experiments is capable of preventing the induction of transferable suppressor cells in vivo (13) and of lysing at least two distinct subpopulations of suppressor cells, i.e., Ts2 and Ts3 cells (3, 9). The anti-Thy-1.2 antiserum consistently ablates in vitro responsiveness to the T-dependent antigens NP-poly-L-Gly56-L-Lys35-L-Phe 9 (GL~) and NP-ovalbumin (data not shown).

5R anti-3R and 3R anti-5R antisera were used to prepare immunoadsorbent columns. The anti-I-J^b antiserum-containing columns had biological activity, as demonstrated by their ability to adsorb Ts2 and Ts3 factor from B6 and CKB mice (3, 4).

**NP^b Idiotype.** The preparation of affinity-purified C57BL/6 anti-NP antibody has been detailed elsewhere (8).

**Anti-NP^b Idiotypic Antiserum.** Guinea pigs were hyperimmunized with 100 μg of purified C57BL/6 anti-NP antibodies in complete Freund’s adjuvant as detailed elsewhere (8). The anti-idiotype antisera were extensively adsorbed with columns conjugated with γ-globulin fractions of MOPC-104E (μ, λ) and (C57BL/6 × DBA/2)F1 ascitic fluid. The adsorbed antisera did not react with IgM, IgG, κ, or λ chains by Ouchterlony analysis.

Columns. All columns were prepared by the reaction of protein with CNBr-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) as described (4). The coupling efficiency was routinely >90%.

**Reduction of Suppressor Factor.** The B6-Ts3-8-derived hybridoma factor was reduced according to the method of Taniguchi et al. (14). Briefly described, B6-Ts3-8 factor was reduced with 5 mM dithiothreitol (DTT) at room temperature for 45 min. The reduced factor was then passed over immunoadsorbent columns under reducing conditions (i.e., under N2) and eluted with 0.2 M glycine-HCl (pH 3.0) as described (15). Filtrates, eluates, or mixtures of the column fractions were then dialyzed extensively against PBS.

**Statistics.** Results were analyzed with a two-tailed Student’s t test.

**Results**

**NP-specific Suppression of PFC Responses In Vitro with B6-Ts1-29 and B6-Ts8-8 Hybridomas.** It has been demonstrated that hybridoma factor derived from first-order suppressor T cells (Ts) can induce effector-phase suppressor cells in vivo (2). The activity and specificity of the B6-Ts1-29-derived hybridoma factor to suppress in vitro PFC responses was compared with another NP-specific hybridoma-derived suppressor factor, B6-Ts8-8, both of which were previously characterized for suppression of T cell-mediated CS responses (2, 4). Cells or supernatants from BW5147 thymoma, B6-Ts1-29, or B6-Ts8-8 hybridoma cell cultures were added to NP-KLH-primed or normal responder cells challenged with NP-Ficoll or SRBC (Table I). For these experiments, the Ts1 factor (TsF1) was given during the induction phase (day 0), whereas the Ts8 factor (TsF3) was added in the terminal phase (day 4) of the 5-d culture period. 5 days after antigen challenge, cultures were assayed for NP- or SRBC-specific PFC responses. The results presented in Table I indicate that both B6-Ts1-29 and B6-Ts8-
### Table I

**NP-Specific Suppression of In Vitro PFC Responses with Ts₁ or Ts₃ Hybridoma Cells or Supernatants**

| Experimental series | Cells or supernatant added | NP-specific PFC/culture \( \log \text{SE} \) | SRBC-specific PFC/culture \( \log \text{SE} \) |
|---------------------|---------------------------|---------------------------------|---------------------------------|
| 1                   | BW5147 supernatant        | 1,300 × 1.2                     | 730 × 1.4                       |
|                     | B6-Ts₁-29 supernatant     | 420 × 1.2§                      | 840 × 1.3                       |
| 2                   | BW5147 cells              | 1,710 × 1.3                     | 1,380 × 1.5                     |
|                     | B6-Ts₃-8 cells            | 400 × 1.5§                      | 1,400 × 1.6                     |
| 3                   | BW5147 supernatant        | 2,100 × 1.4                     | 2,930 × 1.7                     |
|                     | B6-Ts₃-8 supernatant      | 600 × 1.5§                      | 3,100 × 1.7                     |

*2.5 × 10⁶ viable BW5147 thymoma or Ts₃-8 hybridoma cells or 40 μl of BW5147, B6-Ts₁-29, or B6-Ts₃-8 culture supernatants were added to 7.5 × 10⁶ NP-KLH-primed or unprimed responder cells the day of (B6-Ts₁-29) or 4 d after (B6-Ts₃-8) challenge with 200 ng of NP-Ficoll or 2 × 10⁶ SRBC. Cultures were assayed for NP- or SRBC-specific direct PFC responses 5 d after antigenic challenge.

Data are expressed as the geometric mean of the PFC response \( \log \text{SE} \). Results were obtained from three to nine experiments for each series.

§ Significant level of suppression relative to BW 5147 controls, \( P < 0.05 \).

### Table II

**B6-Ts₁-29 and B6-Ts₃-8 Factors Bind NP**

| Immunoabsorbent column | Column fractions | Percent suppression of anti-NP PFC ± SE\( \dagger \) |
|------------------------|------------------|-----------------------------------------------|
|                        |                  | B6-Ts₁-29 | B6-Ts₃-8 |
| None                   | —                | 86 ± 8    | 45 ± 8    |
| NP-BSA                 | Filtrate         | −21 ± 30  | −7 ± 14   |
| NP-BSA                 | Eluate           | 71 ± 13   | 52 ± 10   |
| BSA                    | Filtrate         | 78 ± 11   | NT§       |
| BSA                    | Eluate           | 27 ± 9    | NT        |
| NP₉                    | Filtrate         | NT        | 40 ± 15   |
| NP₉                    | Eluate           | NT        | 8 ± 5     |

* Supernatants from cultures of B6-Ts₁-29 or B6-Ts₃-8 hybridoma cells were passed over immunoabsorbent columns as indicated. 40 μl of column filtrates and eluates or supernatants from BW5147 thymoma cultures were added to 7.5 × 10⁶ NP-KLH-primed responder cells challenged on the same day (B6-Ts₁-29) or 4 d previously (B6-Ts₃-8) with NP-Ficoll. Cultures were assayed 5 d after antigenic challenge for direct NP-specific PFC responses.

\( \dagger \) Data are presented as the arithmetic means of percent suppression from two (B6-Ts₃-8) or three to six (B6-Ts₁-29) experiments ± SE. The geometric means of the control responses in separate experimental series for B6-Ts₁-29 and B6-Ts₃-8 were 2,260 × 1.4 and 7,300 × 1.3, respectively.

§ Not tested.
8 supernatants significantly reduced the NP-specific PFC responses. These suppressive activities were NP specific, since the response to SRBC was unaffected (Table I).

**TsF1 and TsF2 Both Bind NP.** To determine the binding specificity of the two hybridoma products, supernatants from cultures of B6-Ts1-29 or B6-Ts3b-8 hybridoma cells were passed over immunoadsorbent columns as indicated in Table II. Column filtrates or acid eluates were tested for their ability to suppress when added either in the induction (B6-Ts1-29) or effector (B6-Ts3b-8) phase of the PFC response. The results indicate that both hybridoma factors specifically bound to NP-BSA. B6-Ts1-29 factor did not significantly bind BSA-coupled immunoadsorbent columns. The binding specificity of the B6-Ts3b-8 hybridoma factor to bind antigenic and not idiotypic determinants suggests that the cell from which it is derived corresponds to the third-order, effector-phase, NP-specific Ts3 population (4, 9) and not the second-order, NPb-specific, Ts2 population (7, 8). As a control for the NPb immunoadsorbent columns, hybridoma factor derived from second-order Ts2 cells (B6-Ts2-28) was specifically absorbed and eluted from the NPb idiotype column (data not shown).

**TsF1 and TsF2 Express I-Jb- and NPb-Related Determinants.** To further characterize the B6-Ts1-29 and B6-Ts3b-8 factors, supernatants were passed over rabbit anti-mouse immunoglobulin, guinea pig anti-NPb idiotype, anti-I-Jb or anti-I-Jk antiserum-coupled immunoadsorbent columns as indicated in Table III. Column filtrates and acid eluates were tested for their ability to suppress in vitro PFC responses in the induction (B6-Ts1-29) or effector (B6-Ts3b-8) phase. Data presented in Table III demonstrate that the B6-Ts1-29- and B6-Ts3b-8-derived factors, which are capable of suppressing PFC responses, bear both I-Jb- and NPb-related idiotypic determinants; that is, suppressive activity measured in the PFC system was detected in the eluates from I-Jb.

### Table III

**Characterization of B6-Ts1-29 and B6-Ts3b-8 Factors**

| Immunoadsorbent column   | Column fraction | Percent suppression of anti-NP response ± SE § | B6-Ts1-29 | B6-Ts3b-8 |
|--------------------------|-----------------|-----------------------------------------------|-----------|-----------|
| Anti-mouse immunoglobulin| Filtrate        | NT§                                           | 55 ± 19   |           |
|                          | Eluate          | NT -6 ± 1                                     |           |           |
| Anti-NPb                 | Filtrate        | 11 ± 9                                        | -28 ± 32  |           |
|                          | Eluate          | 88 ± 12                                       | 33 ± 9    |           |
| Anti-I-Jb                 | Filtrate        | 4 ± 9                                         | 4 ± 6     |           |
|                          | Eluate          | 81 ± 19                                       | 51 ± 11   |           |
| Anti-I-Jk                 | Filtrate        | NT                                            | 37 ± 9    |           |
|                          | Eluate          | NT                                            | 8 ± 19    |           |

* Supernatants from cultures of BW5147 thymoma, B6-Ts1-29 hybridoma, or B6-Ts3b-8 hybridoma cells were passed over immunoadsorbent columns. 40 μl of the filtrate or the acid eluate was added to NP-KLH-primed responder cells on the day of (B6-Ts1-29) or 4 d after (B6-Ts3b-8) challenge with NP-Ficol. All cultures were assayed 5 d after challenge for direct PFC responses.

§ Data are expressed as the arithmetic mean of percent suppression of PFC responses from two (B6-Ts3b-8) or three (B6-Ts1-29) experiments ± SE. Control responses of separate experimental series ranged from 1,650 to 9,200 PFC.

§ Not tested.
and NP\textsuperscript{b} idiotype columns. Specificity controls included the demonstration that C57BL/6 (Igh\textsuperscript{b})-derived TsF\textsubscript{b} factor does not express immunoglobulin or I-J\textsubscript{b}-encoded determinants (Table III).

**Kinetics of Suppression with Hybridoma Supernatants.** Because the antigen-binding specificity of factor secreted by the B6-Tsl\textsubscript{29} hybridoma was the same as that secreted by the B6-Ts\textsubscript{8-8} hybridoma and because they both expressed I-J\textsubscript{b}-encoded and NP\textsuperscript{b} idiotypic determinants, it was important to differentiate between the two hybridomas or between their factors. To compare the kinetics of suppressive activity, B6-Tsl\textsubscript{29} or B6-Ts\textsubscript{8-8} supernatants were added to NP-KLH-primed responder cultures, either at the time of antigenic challenge (induction phase) or 3–4 d after antigenic challenge (effector phase). Cultures were assayed 5 d after the addition of antigen. The data presented in Table IV indicate that whereas B6-Tsl\textsubscript{29} and B6-Ts\textsubscript{8-8} suppressant suppressed the response when added in the induction phase, only B6-Ts\textsubscript{8-8} suppressant suppressed when added in the effector phase. The suppression observed after the addition of B6-Tsl\textsubscript{29} or B6-Ts\textsubscript{8-8} supernatants in the induction phase was NP specific inasmuch as the response to SRBC was not altered. The hybridomas differ, then, with regard to their ability to specifically suppress responses when added in the effector phase. The difference in kinetics of suppression apparently is not due to a higher concentration of B6-Ts\textsubscript{8-8} factor, because B6-Tsl\textsubscript{29} factor was effective at concentrations 10–100 times lower than B6-Ts\textsubscript{8-8} factor when both factors were added in the induction phase (data not shown).

**Induction of Effector-Phase Suppressor Cells with TsF\textsubscript{1} but not TsF\textsubscript{b}.** The administration of B6-Tsl\textsubscript{29} factor in vivo induces Ts\textsubscript{2} suppressor cells, which can affect contact sensitivity and PFC responses (2). To compare the abilities of B6-Tsl\textsubscript{29} and B6-Ts\textsubscript{8-8} hybridoma factors to induce effector-phase suppressor cells in vitro, normal spleen

**Table IV**

| Supernatant | Percent suppression of anti-NP response§ | Percent suppression of anti-SRBC response
|-------------|------------------------------------------|------------------------------------------|
|             | Induction phase | Effector phase | Induction phase |
| B6-Tsl\textsubscript{29} | 70 ± 21§ | -11 ± 16 | -30 ± 3 |
| B6-Ts\textsubscript{8-8} | 69 ± 13§ | 59 ± 14§ | -17 ± 4 |

* 40 µl of supernatants from BW5147 thymoma, B6-Tsl\textsubscript{29} hybridoma, or B6-Ts\textsubscript{8-8} hybridoma cell cultures were added to normal or NP-KLH-primed responder cells at the time of challenge with NP-Ficoll or SRBC (induction phase) or 3–4 d thereafter (effector phase). All cultures were assayed 5 d after antigen challenge for direct NP- or SRBC-specific PFC responses.

§ Data are expressed as the arithmetic means of percent suppression relative to BW 5147 controls ± SE. Data pooled from two to four experiments are presented. The geometric mean ± SE of the responses of NP-KLH-primed cultures to which BW 5147 supernatant was added in the induction or effector phases was 3,960 ± 1.6 and 4,830 ± 1.1, respectively. The control response to SRBC was 1,850 ± 2.4.

§§ Significant level of suppression relative to response of cultures to which B6-Tsl\textsubscript{29} supernatant was added in the effector phase, \( P < 0.04 \).
TABLE V

Comparison of the Ability of B6-Ts2-29 and B6-Ts2-8 Factors to Induce Suppressor Cells In Vivo*

| Factor added | Percent suppression ± SE‡ |
|--------------|---------------------------|
|              | NP-specific PFC | SRBC-specific PFC |
| B6-Ts2-29    | 75 ± 10§ (7)     | -32 ± 10 (2)      |
| B6-Ts2-8     | -13 ± 24 (0)     | NT§§             |

*7.5 × 10^6 normal spleen cells from C57BL/6 mice were cultured in the absence of antigen for 4 d. On day 0, 10–40 μl of supernatant from cultures of BW5147 thymoma, B6-Ts2-29 cells, or B6-Ts2-8 cells were added. 10 μl of these supernatants were added on days 1, 2, and 3. On day 4, cultured cells were washed extensively and 5 × 10^6 viable cells were added to NP-KLH-primed or normal responder cells challenged 4 d previously with NP-Ficoll or SRBC, respectively. 1 d later, all cultures were assayed for NP- or SRBC-specific PFC responses. The geometric mean ± log SE of the NP and SRBC control responses was 1,240 ± 1.2 and 1,360 ± 3.2, respectively.

‡ The data are presented as the arithmetic mean ± SE of the percent suppression, relative to BW 5147 controls.

§ Significant level of suppression relative to B6-Ts2-8 supernatant-treated cultures, P < 0.007.

§§ Not tested.

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...cells were cultured for 4 d in the presence of supernatants from BW5147 thymoma, B6-Ts2-29, or B6-Ts2-8 hybridoma cultures. Cultured cells then were extensively washed and added to normal or NP-KLH-primed responder cells challenged 4 d previously with NP-Ficoll or SRBC. Cultures were assayed 1 d later for PFC responses. The data presented in Table V extend previous in vivo experiments on the ability of B6-Ts2-29 supernatant to induce NP-specific, Ts2 suppressor cells (2) by demonstrating a similar inductive capacity in an in vitro induction system. In contrast, under identical conditions, supernatants from B6-Ts2-8 hybridoma cultures failed to induce active suppressor cells. Thus, the hybridoma-derived factors differ in their ability to induce effector-phase suppressor cells in vitro.

Suppression of Anti-I-J^β+ C-treated Responder Cells with TsF3 but not TsF1 Hybridoma Supernatant. Because addition of B6-Ts2-29 factor may result in the activation of effector-phase suppressor cells (Ts2 and/or Ts3 populations), it would be expected that the elimination of these I-J-bearing populations or their precursors with anti-I-J antiserum plus C would ablate the ability of B6-Ts2-29 supernatant but not that of B6-Ts2-8 supernatant to suppress the NP-specific PFC response. To test this hypothesis, supernatants from B6-Ts2-29 or B6-Ts2-8 cultures were added to untreated or anti-I-J^β antiserum plus C-treated responder cells at the time of antigenic challenge. To ensure that treatment of responder populations with anti-I-J^β antiserum plus C eliminated I-J^β-bearing cells, each experiment included groups in which Ts2 and/or Ts3 populations were added to anti-I-J^β-treated responder cells as described previously (9). As indicated elsewhere (5, 9, 16), the failure of active Ts2 populations to suppress was an indication of the functional elimination of the I-J-bearing Ts3 population (data not shown). The data presented in Table VI show that, although supernatants from B6-Ts2-29 or B6-Ts2-8 hybridoma cultures may specifically suppress anti-NP responses when added in the induction phase to untreated responder cultures, as has been demonstrated in a separate series of experiments (Table IV), only B6-Ts2-8
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### Table VI

**Comparison of the Ability of B6-Ts2-29 and B6-Ts3-8 Supernatants to Suppress Anti-I-Jb Plus C-treated Responder Cells**

| TsF added       | Day added | Treatment of responder cells | Percent suppression of anti-NP response ± SE‡ |
|-----------------|-----------|------------------------------|---------------------------------------------|
| B6-Ts2-29       | 0         | —                            | 66 ± 9§                                     |
| B6-Ts3-8        | 0         | —                            | 68 ± 13§                                    |
| B6-Ts2-29       | 0         | Anti-I-Jb* plus C            | 4 ± 6                                       |
| B6-Ts3-8        | 0         | Anti-I-Jb* plus C            | 61 ± 6§                                     |
| B6-Ts3-8        | 4         | Anti-I-Jb* plus C            | 81 ± 9§                                     |

* 40 µl of supernatant from BW5147 thymoma, B6-Ts2-29 hybridoma, or B6-Ts3-8 hybridoma cultures were added to NP-KLH-primed responder cultures at the time of (day 0) or 4 d after (day 4) challenge with NP-Ficol. Where indicated, responder cultures had been treated with anti-I-Jb antiserum plus C before culture. All cultures were assayed 5 d after challenge for direct NP-specific PFC responses.

‡ Data are presented as the arithmetic mean of percent suppression relative to BW 5147 controls from three to six experiments ± SE. The geometric means of control responses for untreated and anti-I-J plus C-treated responder cultures to which BW 5147 supernatant was added were 3,220 ± 1.7 and 4,430 ± 1.3, respectively.

§ Significant level of suppression relative to the percent suppression of anti-I-J plus C-treated cultures to which B6-Ts2-29 supernatant was added, P < 0.02.

supernatants were capable of suppressing the response of anti-I-J antiserum plus C-treated responder cells when added in the induction or the effector phase. Thus, the B6-Ts2-29 and B6-Ts3-8 hybridoma factors are distinguishable on the basis of their ability to function in the absence of I-Jb-bearing target cells.

**B6-Ts3-8 Hybridoma Factor is a Two-Chain Molecule.** Inasmuch as B6-Ts3-8 factor bears products linked to the **Igh** (NPb-related idiotype) and the **H-2** (I-J) gene complexes, it may be hypothesized that two gene products may be required to form the functional Ts3 suppressor factor. Indeed, recent evidence has suggested that antigen-binding suppressor factors may be separated by reduction (14, 15, 17) or digestion (18) into two moieties. To examine the structural organization of B6-Ts3-8 factor, ascites fluid induced with B6-Ts3-8 cells was passed over NP-BSA immunoadsorbent columns. The acid eluate (purified B6-Ts3-8-derived factor) was then reduced with 5 mM DTT. Some of the reduced factor was dialyzed, and the remainder was passed over an NP-BSA column. The filtrate, acid eluate, or a combination of these two factors was dialyzed against PBS. The B6-Ts3-8 fractions were then tested for their ability to suppress when added in the effector phase. The data presented in Table VII substantiate previous finding (Table II) that B6-Ts3-8 factor binds to and can be eluted from NP-coupled immunoadsorbent columns. Reduction of the purified factor does not eliminate its ability to suppress NP-specific PFC response. However, separation of the reduced factor into NP-binding and nonbinding fractions reveals that neither fraction alone is capable of significantly suppressing the PFC response in comparison to reduced unfractionated Ts3. In some experiments we observed activity in the fraction containing the reduced filtrate. This was not, however, consistent. In contrast, significant levels of suppression can be consistently reconstituted when a combination of the filtrate and the eluate is added in the effector phase of the
response. The data indicate that the B6-Ts8-8 suppressor factor, like some other TsF molecules (14, 15, 17, 19) exists as a two-chain structure. Whereas one chain binds antigen, both chains are required to manifest suppression. The suppression of the reconstituted Ts8 is NP specific since the response to SRBC remains unaffected.

**B6-Ts8-8 Hybridoma Supernatant Suppresses T-Independent Responses.** Although the B6-Ts8-8 factor functions during the effector phase of the response, it is not known whether it interacts directly with the B cell target. To determine whether B6-Ts8-8 factor is capable of suppressing a B cell response in the absence of T cells, NP-primed responder cells were treated with anti-Thy-1.2 antiserum plus C before challenge with

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**Table VII**

**Immunochemical Characterization of B6-Ts8 Factor**

| Reduction | NP-BSA immunoadsorbent column fraction | Percent suppression ± SE* |
|-----------|----------------------------------------|---------------------------|
|           | eluate                                 | 100 ± 0§                  |
| +         | filtrate                               | 68 ± 13§                  |
| +         | eluate                                 | 31 ± 14                   |
| +         | filtrate plus eluate                   | 62 ± 13§                  |

* B6-Ts8-8-induced ascites was passed over NP-BSA immunoadsorbent columns. An aliquot of the acid eluate (purified B6-Ts8-8 factor) was then reduced with 5 mM DTT. An aliquot of the reduced factor was dialyzed against PBS while the remainder was passed over another NP-BSA immunoadsorbent column under reducing conditions. The filtrate, acid eluate, or combination of these fractions was dialyzed against PBS. 40 μl of these aliquots of purified, reduced, or reduced and column-fractionated B6-Ts8-8 factors were added to NP-KLH-primed or normal responder cells challenged 4 d previously with NP-Ficoll or SRBC, respectively. 1 d later, all cultures were assayed for direct NP- or SRBC-specific PFC responses.

§ Significant level of suppression relative to the percent suppression obtained with reduced, NP-BSA-binding fractions.

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**Table VIII**

**B6-Ts8-8 Factor Suppresses the NP-BA Response**

| Supernatant added | PFC/culture × log SE* |
|-------------------|-----------------------|
|                   | NP specific | TNP specific |
| BW5147            | 1650 |- 1.1 | 600 |- 1.3 |
| B6-Ts8-8          | 220 |- 1.6§ | 535 |- 1.0 |

* 40 μl of BW5147 thymoma or B6-Ts8-8 hybridoma culture supernatants were added to anti-Thy1.2 plus C-treated NP-KLH-primed responder cells or untreated TNP-KLH-primed responder cells 3 d after challenge with NP-BA or TNP-BA. All cultures were assayed for NP- or TNP-specific direct PFC responses 5 d after antigenic challenge. TsF1 was not included because this factor will not suppress when added to anti-Thy-1.2 plus C-treated cultures.

§ Significant level of suppression relative to BW 5147 controls, P < 0.03.
the class I thymus-independent antigen NP-BA (20). The antiserum used to lyse Thy-1.2-bearing T cells consistently eliminated the capacity of NP-primed cells to respond to the thymus-dependent antigens NP-GLδ and NP-ovalbumin in vitro (data not shown). When supernatants from B6-Ts3-8 cultures were added on day 3 of a 5-d culture period, significant suppression of the response to NP-BA was observed (Table VIII). The specificity of this suppression was demonstrated by the inability of B6-Ts3-8 factor to affect the response to TNP-BA. The data are consistent with the hypothesis that Thy-1.2-bearing cells are not required for suppression mediated by B6-Ts3-8 supernatants.

Discussion

The intravenous injection of NP-modified syngeneic spleen cells results in the specific suppression of both NP-specific CS and PFC responses (1-9). This suppression is mediated by at least three interacting T cell subpopulations. NP-specific first-order suppressor cells (Ts1) induce second-order suppressor cells (Ts2), which are specific for NPb-related idiotypic determinants. The Ts2 population activates an NP-primed, NP-specific third-order T cell population (Ts3) to effect immune suppression within 24 h of either the CS or the PFC assay. Taken together, the data suggest that a common T cell pathway may be responsible for suppression of both the T and B cell compartments.

The purpose of the present study was to identify soluble factors derived from cloned Ts hybridoma cell lines that are capable of suppressing T cell-mediated CS responses as well as directly suppressing B cell (PFC) responses. Despite the similarities between the in vitro PFC and the CS system, factors secreted by these T cell populations had been defined only in the CS system. Because mixed populations of T cells were used to suppress PFC responses in previous studies (5, 7, 9), it was not known whether the same cells or cellular products could regulate both T cell-mediated CS and B cell-mediated PFC responses.

B6-Ts1-29 suppressor factor is derived from a hybridoma isolated from spleens of NP spleen-treated C57BL/6 mice (2). This factor has been well characterized in the CS system (2, 4). Factor present in supernatants of B6-Ts1-29 hybridoma cells (a) functions only when added in the induction phase of the CS response, (b) induces effector-phase suppressor cells when injected i.v., (c) binds NP, (d) bears NPb-related and I-Jb-encoded determinants, and (e) can function across H-2 and Igh disparities (2). Similar results, presented in the current report, were obtained in a PFC assay system. Thus, the cloned B6-Ts1-29 hybridoma-derived factor (a) is capable of suppressing the B cell-mediated PFC response only when added in the induction phase (day 0) of the Mishell-Dutton culture, (b) induces effector-phase suppressor cells in vitro, (c) binds NP, and (d) bears NPb idiotype-related and I-Jb-encoded determinants.

In a similar fashion, a factor present in supernatants of B6-Ts3-8 hybridoma cultures has now been characterized in both the CS and in vitro PFC systems. In both systems the factor binds NP but not NPb idiotypic determinants and bears I-Jδ-encoded and NPb idiotypic-related determinants. B6-Ts3-8 supernatants have been shown to suppress CS responses only in the effector phase of the response (4), whereas data presented in this report demonstrate that B6-Ts3-8 supernatant can affect in vitro PFC responses when added either in the induction or the effector phase. Similar
kinetics of TsF3 activity were reported by Waltenbaugh and Lei (26) using a GT-specific TsF. Although the reason for this disparity between the kinetics of TsF3 in CS and PFC responses is not known, it is possible that the in vivo half-life of the suppressor factor is relatively short. Nevertheless, the data indicate that monoclonal B6-Ts8-8 derived factor can affect both T- and B cell-mediated responses.

Given the immunochemical similarity of B6-Ts1-29 and B6-Ts8-8 factors it was necessary to provide evidence that the two hybridomas represent different T cell populations. The B6-Ts8-8 factor is effective when added to responder populations either at the time of antigen challenge or within 18–24 h of the PFC assay. In contrast, in the PFC, as in the CS, system (2), the B6-Ts1-29 factor appears to work only when added in the induction phase. This difference presumably reflects the requirement for the Ts1-derived factor to recruit Ts2 cells to mediate suppression. This interpretation is consistent with data indicating that only the Ts1 factor is capable of inducing second-order, effector-phase suppressor cells in normal spleen cell cultures (Table V). The ability of hapten-binding Ts1-derived factor to recruit suppressor cells from normal populations is reminiscent of other suppressor cell systems (21–25). Unlike some factors described in other systems (21–22), the B6-Ts1-29-derived factor does not require antigen to induce second-order Ts cell populations. The induction of effector-phase suppressor cells with B6-Ts1-29 factor is further reflected by the inability of B6-Ts1-29 supernatants to suppress responder cultures depleted of cells expressing I-J-encoded determinants. The inhibition of effector cell induction under these conditions may be due to elimination of Ts2 or Ts8 cell populations, because both of these cell types express I-J subregion-encoded determinants (5, 9).

Both the B6-Ts1-29 and the B6-Ts8-8 suppressor factors bind NP and express I-Jβ- and NPβ-related idiotypic determinants. Whether the I-J determinants expressed on the two factors are identical is not known. In this regard, it has recently been shown that the I-J subregion may code for more than one product, which are expressed to varying degrees on different populations of T cells (26). Similarly, it remains to be determined whether these two distinct cell types within the NP-specific suppressor cell pathway express the same or different NPβ-related idiotypic determinants. It may be argued that the anti-NPβ idiotype antiserum used to adsorb TsF may contain specificities for several components of the NPβ-related idiotypic family, only some of which may be expressed on any monoclonal suppressor factor. In this regard, we have recently demonstrated that, although anti-NPβ idiotype antiserum may recognize NPβ-related determinants on both NP-specific T and B lymphocytes, the NPβ idiotype-related determinants expressed on Ts do not appear to represent the predominant NPβ idiotypic determinants expressed on NP-specific antibody-producing cells (8).

Unlike the donors for previously described Ts8 populations, the donors of spleen cells for fusion with BW5147 thymoma cells were not immunized subcutaneously or intraperitoneally with NP-KLH or NP-O-Su. Rather, these donors were injected intravenously with NP-coupled syngeneic spleen cells. Spleen cells from mice treated 7 d previously with NP-coupled syngeneic spleen cells contain both Ts1 and Ts2 suppressor cell populations, which can be identified in the CS and/or PFC systems (1–8). We have not previously observed Ts8 cell generation following intravenous sensitization, although Ts8 cells can be generated after subcutaneous administration of NP-coupled cells (16). Thus, the identification of a Ts8 hybridoma cell line derived from mice given NP-coupled spleen cells intravenously indicates that all three
suppressor T cell populations may exist simultaneously. However, the frequency of Ts3 cells in these mice appears to be very low, since only one Ts3 hybridoma has been identified in three fusions and since Ts3 cells obtained from NP spleen-treated mice require an exogenous source of Ts3 cells to effect significant suppression (9).

The ability of supernatants from B6-Ts3-8 hybridoma cultures to suppress the response of populations depleted of I-J-bearing cells is consistent with the hypothesis that this factor represents the effector molecule in the suppressor pathway. The ability of B6-Ts3-8 supernatant to suppress the T independent response to NP-BA suggests that this factor acts directly on B lymphocytes. The data do not, however, exclude the possibility that an acceptor cell is required for binding of the suppressor factor and manifestation of its suppressor function (27). If an acceptor cell is required for suppression mediated by Ts3 factor then it expresses neither I-J nor Thy-1.2 determinants, since the B6-Ts3-8 suppressor factor functions in anti-I-J antisera plus C-treated responder cell cultures challenged with NP-Ficoll (Table VI) or anti-Thy-1.2 antisera plus C-treated cultures responding to the class I T-independent antigen NP-BA (Table VIII). If the Ts3 factor acts directly on B lymphocytes then preferential suppression of NPb idiotype B cell clones may be attributed to the preferential inactivation of high affinity NPb-bearing B cells by complexes of Ts3 factor and NP antigen. This hypothesis is currently under investigation.

It is conceivable that some antigen-binding factors may specifically inhibit in vitro generated responses by sequestering antigen and thereby making it unavailable to the responding cell populations. We can exclude the possibility that B6-Ts3-29-derived factor functions by depleting antigen, because the addition of this factor to naive spleen cells in the absence of antigen results in the generation of a population of second-order T suppressor cells. Furthermore, the addition of TsF1, to Ts3-depleted cultures fails to mediate suppression. It is also unlikely that B6-Ts3-8 functions by depleting antigen, because the addition of this factor to cultures in the effector phase of the response results in significant suppression. This conclusion is further supported by the observation that the isolated antigen-binding chains of the reduced factor failed to effect suppression.

Reduction of B6-Ts3-8 factor with DTT resulted in two moieties that could be separated on NP-BSA immunoabsorbent columns (Table VII). Although neither fraction alone significantly suppressed immune responsiveness, a combination of them reconstituted suppression. These data are taken to reflect a dimer structure of this Ts3-derived factor. One chain contains the receptor for NP, but both chains are required to deliver the suppressor signal. Inasmuch as the two chains were not alkylated before reconstitution, it seems likely that interchain disulfide bonds were reformed and that the active form of the factor was regenerated by reconstitution. The two-polypeptide-chain concept may explain the observation that products linked to the Igh complex on the 12th chromosome (NPb idiotype) and products encoded on the 17th chromosome (I-Jb) are expressed on the same molecule or molecular complex (4). These data are consistent with those previously reported for KLH- (14), SRBC- (18), and GT- (17)2 specific effector suppressor factors. Recent data from our laboratory also demonstrates that the TsF3 molecules, which suppress CS responses, also

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2 Lei, H.-Y., S.-T. Ju, M. E. Dorf, and C. Waltenbaugh. 1983. Regulation of immune responses by I-J gene products. III. GT-specific suppressor factor is composed of separate I-J- and idiotype-bearing chains. J. Immunol. In press.
consist of two chains, one with \(I-J\) encoded determinants and the other containing the NP-binding moieties (15). Thus, the TsF\(_3\) molecules that suppress T cell function have the same serological and molecular properties as the TsF\(_3\) molecules that act on B cells.

This report establishes the ability of two distinct cloned hybridoma products representing TsF\(_1\) and TsF\(_3\), respectively, to suppress PFC responses in a manner as yet indistinguishable from that observed in the CS system. Thus, suppression of both T cell-mediated CS and B cell-mediated PFC responses is effected with molecules which, except for kinetic differences, have similar properties.

**Summary**

The ability of two cloned T cell hybridomas and their products to specifically suppress the in vitro plaque-forming cell (PFC) response to the 4-hydroxy-3-nitrophenyl acetyl hapten (NP) was studied. Supernatant from one hybridoma (Ts\(_1\)) was shown to suppress in the induction but not the effector phase of the immune response. Supernatant from the Ts\(_3\) hybridoma was capable of inducing second-order (Ts\(_2\)) effector-phase suppressor cells in vitro but did not suppress the response of anti-I-J plus C-treated responder cells. In contrast, supernatant from a second hybridoma (Ts\(_3\)) was capable of suppressing PFC responses when added either in the induction or the effector phase of the response. Ts\(_3\) supernatant was unable to induce effector-phase suppressor cells but was capable of suppressing the response of anti-I-J plus C-treated responder cells. In addition, specific suppressor factors isolated from supernatants of the Ts\(_3\) and Ts\(_8\) hybridomas were shown to bind to NP, bear NP\(^b\) idiotypic and I\(-J\)-encoded but not immunoglobulin-constant region determinants. The factor secreted by the Ts\(_3\) hybridoma appears to act directly on B cell targets. Mild reduction of this factor results in two separable moieties, only one of which binds NP. Reconstitution experiments suggest that both chains are required for function. The collective data indicate that these hybridomas represent cells from first- and third-order suppressor T cell populations described previously in contact sensitivity and in vitro PFC systems. The implications of the ability of these hybridoma products to affect both T and B cell-mediated immune responses are discussed.

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