MECHANISM RESPONSIBLE FOR THE INDUCTION OF I-J RESTRICTIONS ON Tsa SUPPRESSOR CELLS*

BY MUTSUHIKO MINAMI, NAOKI HONJI, AND MARTIN E. DORF

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

The finding that the interactions of T lymphocytes are genetically restricted by genes within the major histocompatibility complex has been firmly established in many systems (1-5). In mice, helper, proliferating, and delayed-type hypersensitivity T cells are restricted by genes in the I-A, and for selected antigens the I-E, subregion of the H-2 complex (1, 6-11). In contrast, cytolytic T lymphocytes and contact sensitivity effector T cells are generally restricted by genes in the K or D regions of the major histocompatibility complex (3-5, 10, 12). In many suppressor T cell systems, the T cell interactions are controlled by still another series of genes within the H-2 complex, i.e., the I-J or I-C subregions (13-18). Thus, it appears that most products of the major histocompatibility complex (MHC) can serve as restricting elements for immune responses. The commitment of T cells to various MHC products is most frequently attributed to mechanisms involving associative recognition (19, 20). This theory of genetic restriction assumes that antigen is presented to T cells in the context of specific MHC gene products.

To date, most of the data on the induction of MHC restrictions are derived from studies of helper, proliferating, and cytolytic T cells. In this report, we investigate the mechanisms responsible for the induction of I-J restrictions in a particular subset of suppressor T cells. Three distinct suppressor T cell subpopulations have been identified in the 4-hydroxy-3-nitrophenyl acetyl (NP) system (21-23). These were termed Tsl, Ts2 and Tsa cells, respectively. Tsl cells or their soluble factors do not display any H-2 restriction (14, 24, 25). In contrast, Ts2 and Tsa cells and their factors are genetically restricted by genes in the I-J subregion (14, 16, 17, 22). This report focuses on the induction of Tsa cells. Tsa cells are present in conventionally primed mice, but they only exert their suppressive activity after activation by Ts2 cells or factors derived from Ts2 cells (17, 21, 23). In this report, we evaluate methods of generating NP-specific Tsa cells. The data suggest that I-J determinants may serve as the antigen-presenting structures for the induction of Tsa cells in a manner analogous to that proposed for I-A molecules in the induction of helper T cells. Furthermore, we demonstrate that two distinct populations of Tsa cells restricted by either parental H-2 haplotype can be generated in H-2 heterozygous F1 mice. These combined obser-

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1 Abbreviations used in this paper: B6, C57BL/6 mice; CY, cyclophosphamide; DMSO, dimethyl sulfoxide; DNFB, 2,4-dinitrofluorobenzene; DTH, delayed-type hypersensitivity; EDTA, ethylenediaminetetraacetic acid; HBSS, Hanks' balanced salt solution; MEM, minimal essential media; MHC, major histocompatibility complex; NP, 4-hydroxy-3-nitrophenyl acetyl; NP-O-Su, NP-O-succinimide; PBS, phosphate-buffered saline; Th, helper T cells; Ts2, Ts3, second- or third-order suppressor T cells; TsF2, TsFa, Ts2- or Tsa-derived suppressor factors.
vations resemble data previously obtained with helper T cells and imply a general scheme for the induction of antigen-specific T cell populations.

Materials and Methods

Mice. All mice were either purchased from The Jackson Laboratory, Bar Harbor, ME, or were bred in the animal facilities at Harvard Medical School, Boston, MA. Mice were used at 3–12 mo of age and were maintained on laboratory chow and acidified, chlorinated water ad lib.

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 (DHEW publication [NIH] 78-23, revised 1978).

Antigens. NP-O-Succinimide (NP-O-Su) was purchased from Biosearch Co., San Rafael, CA. Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific Co., Pittsburgh, PA. 2,4-dinitro-1-fluorobenzene (DNFB) was obtained from Eastman Kodak Co., Rochester, NY.

Cell Preparation. Spleen cell suspensions were made in Hanks’ balanced salt solution (HBSS), and the erythrocytes were lysed with Tris ammonium chloride. The spleen cells were washed and then used for further separation or for NP-conjugation.

Macrophage-enriched glass-adherent spleen cells were purified by a 4-h adherence to glass petri dishes, followed by removal with EDTA, as previously described (26). Macrophage-depleted T and B spleen lymphocytes were prepared by preparing 1–2 × 10^6 splenic leukocytes over a 25-ml column of Sephadex G-10 in a 35-ml syringe barrel and collecting the first 15 ml of eluate, as previously described (26). Cells prepared by glass adherence and by filtration through Sephadex G-10 have been extensively characterized in previous reports (26, 27). Briefly, 4-h glass-adherent cells contained 40–70% phagocytic cells; the nonphagocytic cells were comprised of equal numbers of Thy-1+, sIg+, and Thy-1-, sIg- cells. Unfractionated spleen cells were 4–8% phagocytic, and G-10-passed cells were 0.1–0.8% phagocytic, while retaining the same T cell to B cell ratios as the input cells. Phagocytosis was assessed by latex ingestion, as previously described (27).

Haptenated Cell Preparation. NP-coupled spleen cells were made as described previously (28). Briefly, 3–5 × 10^7 spleen cells or 2–6 × 10^6 spleen glass-adherent cells were resuspended in 4 ml phosphate-buffered saline solution (PBS), pH 7.7. 120 μl of 2.4% NP-O-Su in DMSO was reacted with the cells for 2.5 min at room temperature. The reaction was stopped with Eagle’s minimum essential medium (MEM) containing 1.2 mg/ml glycyglycine. After extensive washing in MEM, the NP-coupled cells were used for priming.

Assay for DTH Induction and Ts3 Induction after Immunization with NP-coupled Cells. To induce DTH and Ts3 cells, animals were primed subcutaneously with NP-coupled cells. 5 and 6 d after priming, each mouse received 0.5 ml HBSS containing 5 μl BW5147, B6-Ts2-28, or CKB-Ts2-59 derived ascitic fluids. These monoclonal TsF have been previously characterized (29). On day 6, mice were challenged in the left footpad with 0.025 ml PBS containing 30 μg NP-O-Su. Footpad swelling was measured 24 h after challenge. Swelling was determined as the difference, in units of 10^-3 cm, between the left and right footpad thickness.

Adoptive Transfer System for Assaying Ts3 Activity. Donor mice were immunized subcutaneously with 1 × 10^7 NP-coupled cells. 6 d after priming, the mice were killed, and inguinal and axillary lymph nodes were removed, teased into a single cell suspension, and used as a source of Ts3 donor cells.

To prepare Ts3-depleted recipient mice, animals were primed with 2 mg of NP-O-Su. 24 h later, they were treated with an intraperitoneal injection of 20 mg/kg cyclophosphamide (CY) in saline. On day 5, each recipient received 1 × 10^7 NP-primed Ts3 donor cells intravenously. Immediately after transfer and on day 6, 0.5 ml HBSS containing 5 μl control BW5147 tumor-derived or B6-Ts2-28-derived or CKB-Ts2-59-derived ascitic fluid was injected intravenously. On day 6, mice were challenged with NP-O-Su, and CS responses were measured 24 h later.

DNFB Contact Sensitivity Responses. Contact sensitivity was induced by two daily paintings on the shaved abdomen with 25 μl of 0.5% DNFB solution (Eastman Kodak Co., Rochester, NY) in acetone/olive oil (4:1). 6 d after the last painting, 20 μl of 0.2% DNFB in the same vehicle was applied to the left ear, and the ear swelling was measured as the difference between the left and right ear thicknesses.
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*Percent Suppression.* The percent suppression in the present study was calculated by the following formula: percent suppression = 100 × ([swelling of BW tumor supernatant-injected group − swelling of TsF-injected group]/[swelling of BW tumor supernatant-injected group − swelling of unprimed group]).

*Data Analysis.* Statistical analysis of the experimental data with respect to controls was calculated using the two-tailed Student’s t test.

**Results**

*Priming for DTH Responses with NP-coupled Cells.* In an initial series of experiments, NP-coupled syngeneic spleen cells were used for antigen priming. Optimal priming was achieved by subcutaneously injecting $1 \times 10^7$ NP-coupled cells. After 7 d, the mice were challenged in the left footpad with NP-O-Su, as previously described. The swelling responses were measured after 24 h. The magnitude of swelling varied among experiments but was generally in the range of 25-45 $\times 10^{-3}$ cm. To establish that these swelling responses were a measure of a cell-mediated DTH response, $4 \times 10^7$ lymph node cells from B10.MBR mice primed with NP-coupled syngeneic cells were adoptively transferred to H-2I-compatible but H-2K- and H-2D-incompatible B10.BR recipients. Lymphocytes from the primed B10.MBR donors could transfer significant levels of immunity to H-2I-compatible recipients (data not shown). In addition, the delayed (18-36 h) kinetics of the swelling response (data not shown) support the contention that these responses are a measure of DTH reactivity.

*Suppression of DTH Responses.* To determine whether these DTH responses could be suppressed by Tsz- and Tss-derived suppressor factors, (TsFz and TsFs), animals were primed with NP-coupled syngeneic cells. After 5 to 6 d, the mice were given 0.5 ml i.v. of media containing hybridoma-derived TsF. The data in Table I demonstrate that the DTH responses were suppressed after administration of monoclonal TsFz or TsFs factors. The fact that these mice could be suppressed with TsFz factor suggests

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**Table I**

*Suppression of DTH Responses with Monoclonal Suppressor Factors*  

| Strain | Priming with NP-coupled spleen cells | TsF source | Footpad swelling ± SE$\dagger$ |
|--------|-------------------------------------|------------|--------------------------------|
| C57BL/6| NP-B6                               | BW5147     | 42.2 ± 1.6                      |
|        |                                    | B6-Tsz-28  | 15.5 ± 1.2$§$                  |
|        |                                    | B6-Tss-2   | 16.0 ± 1.1$§$                  |
|        | None                                | None       | 7.8 ± 1.6                       |
| CKB    | NP-CKB                              | BW5147     | 43.3 ± 1.4                      |
|        |                                    | CKB-Tsz-59 | 20.0 ± 0.6$§$                  |
|        |                                    | CKB-Tss-3  | 18.5 ± 3.0$§$                  |
|        | None                                | None       | 7.3 ± 1.0                       |

$\dagger$ The data are expressed as the increment of specific footpad swelling ± SE in units of $10^{-3}$ cm.

*Groups consisting of four mice were primed with $1 \times 10^7$ NP-coupled syngeneic spleen cells. 5 and 6 d after priming, animals were injected intravenously with either monoclonal TsFz or TsFs suppressor factors that were derived from fusions of BW5147 tumor cells with C57BL/6 or CKB suppressor T cells (22, 29). The mice were challenged on day 6 with NP-O-Su, and the footpad swelling was measured 24 h later.

$§$ Significant suppression; $P < 0.01$. 
that \( T_3 \) cells, which are the target of \( T_3F_2 \), were present and were generated by priming with hapten-coupled cells.

We next performed a series of experiments to determine whether \( T_3 \) cells could be generated by priming with allogeneic hapten-coupled cells. Animals were immunized with \( 1 \times 10^7 \) NP-coupled allogeneic spleen cells and 5 to 6 d later were given i.v. injections of \( T_3F_2 \). Initially, the allogeneic combination used for these experiments differed at the entire H-2 complex. However, the swelling responses observed after such allogeneic immunizations were either absent or minimal (data not shown). Consequently, we used strain combinations that differed only at the I-J subregion, e.g., 3R and 5R (Table II). In such combinations, it was consistently possible to observe strong DTH responses. However, \( T_3F_2 \) was not able to suppress DTH responses induced by administration of I-J-incompatible NP-coupled cells. Thus, when 3R mice are primed with NP-coupled 5R cells, strong DTH responses are noted, and these responses can not be suppressed by administration of \( T_3F_2 \). In contrast, when the same NP-coupled 3R cells were used to prime 3R animals, the responses could be suppressed by administration of C57BL/6-derived \( T_3F_2 \) factor (Table II). In reciprocal

| Experiment number | Immunized strain | Cells used for priming | TsF source | Footpad swelling ± SE |
|-------------------|------------------|------------------------|------------|-----------------------|
| 1                 | 3R               | NP-3R                  | BW5147     | 36.3 ± 1.5            |
|                   |                  | NP-3R                  | B6-Ts2-28  | 20.3 ± 2.4*           |
|                   |                  | NP-3R                  | B6-TsR-2   | 16.8 ± 1.1*           |
|                   |                  | NP-5R                  | BW5147     | 26.3 ± 3.0            |
|                   |                  | NP-5R                  | B6-Ts2-28  | 28.0 ± 2.7            |
|                   |                  | NP-5R                  | B6-TsR-2   | 9.0 ± 1.5*            |
|                   |                  | None                   | None       | 8.4 ± 1.6             |
| 5R                |                  | NP-3R                  | BW5147     | 37.7 ± 1.7            |
|                   |                  | NP-3R                  | CKB-Ts2-59 | 34.0 ± 2.5            |
|                   |                  | NP-3R                  | CKB-TsR-3  | 26.5 ± 1.3*           |
|                   |                  | NP-5R                  | BW5147     | 44.8 ± 1.3            |
|                   |                  | NP-5R                  | CKB-Ts2-59 | 24.8 ± 1.9*           |
|                   |                  | NP-5R                  | CKB-TsR-3  | 20.3 ± 2.2*           |
|                   |                  | None                   | None       | 7.5 ± 0.6             |

* Refer to protocol for Table I. 3R or 5R recipients were primed with either syngeneic or I-J-incompatible NP-coupled 5R or 3R spleen cells.
experiments, we demonstrated that NP-coupled 5R cells primed syngeneic recipients and that the ensuing responses could be suppressed by CKB-derived TsF2. However, when the same NP-coupled 5R cells were used to prime I-J-disparate 3R recipients, TsF2 could no longer suppress the response. The inability of TsF2 to suppress DTH responses induced by priming with I-J-mismatched cells is not due to restrictions on T3 activation because neither C57BL/6 (I-Jb) nor CKB (I-Jk) TsF2 produced suppression (Table II, experiment 2). The controls for these experiments included groups that were injected with TsF3 (Table II, experiment 1). The ability of TsF3 to cause suppression in mice primed with either syngeneic or allogeneic cells demonstrates (a) that mice primed with I-J incompatible cells were not totally refractory to suppression and (b) that the specific defect in the suppressor pathway lies in the steps between TsF2 and TsF3, i.e., in the T3 population.

Requirement for I-J Homology for T3 Cell Induction. A series of adoptive transfer experiments were performed to determine whether priming with I-J-mismatched cells resulted in a failure to induce the T3 cells or in the inability of T3 cells to express their functional activity. We previously characterized an adoptive transfer system in which recipients are primed, treated with cyclophosphamide (CY) to prevent the generation of T3 cells, and are then given T3 cells or TsF2 along with a transfer containing the potential T3 cell source (17, 21). To generate T3 cells, C57BL/6 or B10.BR mice were primed with NP-coupled syngeneic or allogeneic cells that differed at various regions of the major histocompatibility complex. Lymph node cells from these potential T3 donors were then adoptively transferred into NP-O-Su-primed CY-treated syngeneic recipients along with control BW or monoclonal TsF2 factors. As shown in Table III, C57BL/6 lymph node cells obtained from T3 donors primed with syngeneic NP-coupled cells transferred suppression to syngeneic recipients that also received C57BL/6-derived TsF2. In reciprocal experiments, injections of H-2b-derived CKB-Ts2-59 factor and B10.BR T3 cells generated NP-specific suppression when given to B10.BR recipients. However, the CKB-Ts2-59 (I-Jb) factor would not activate C57BL/6 T3 cells, and the B6-Ts2-28 factor did not activate I-J-mismatched

| Strain of CY-treated recipients | NP-O-Su and DNFB priming | Spleen cells for priming of T3 | Source of TsF | Antigen challenge‡ |
|---------------------------------|--------------------------|-------------------------------|---------------|-------------------|
| C57BL/6                         | +                        | Normal B6                    | BW5147        | 30.4 ± 2.0 (7)    |
|                                | +                        | Normal B6                    | B6-Ts2-28     | 30.3 ± 1.4 (6)    |
|                                | +                        | NP-B6                        | BW5147        | 32.3 ± 2.3 (8)    |
|                                | +                        | NP-B6                        | B6-Ts2-28     | 12.9 ± 1.5§ (6)   |
|                                | +                        | CKB-Ts2-59                   | 31.0 ± 1.8    |
|                                | —                        | —                             | 7.0 ± 2.4     |

* C57BL/6 mice were doubly primed with NP-O-Su and DNFB, and 24 h later were given CY. After 5 d, they received adoptive transfers of 1 X 10⁷ T3 cells and TsF2 or BW5147 control factors. The T3 cells were generated in syngeneic C57BL/6 mice that were primed with NP-coupled C57BL/6 spleen cells; controls received normal syngeneic cells. After transfer of T3 cells, the mice were challenged with NP-O-Su (in the footpad) and DNFB (on the ear).

† The swelling responses were measured 24 h after challenge. The results of two experiments were pooled, and the number of mice tested is indicated in parentheses.

§ Significant levels of suppression; P < 0.001.
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B10.BR T\(\text{S}_3\) cells. This transfer protocol provides direct evidence that T\(\text{S}_3\) cells were generated by priming with hapten-coupled syngeneic cells and verifies the H-2 restrictions on T\(\text{S}_3\) cell activation (17).

In an additional series of experiments, NP-coupled 3R, 4R, and 5R allogeneic cells were also used to induce T\(\text{S}_3\) in either C57BL/6 or B10.BR hosts. When C57BL/6 recipients were given TsF\(_2\) and lymph node cells from C57BL/6 mice that had been previously primed with NP-coupled 3R or 4R cells, significant levels of suppression were observed (Table IV). In contrast, after priming of B10.BR mice with the same population of NP-coupled 3R or 4R cells, there was no generation of detectable T\(\text{S}_3\) activity (Table IV). However, priming of B10.BR hosts with NP-coupled 5R cells could generate functional T\(\text{S}_3\) cells, whereas priming of C57BL/6 mice with these NP-5R cells failed to induce a functional T\(\text{S}_3\) population. Analysis of the genetic disparities in these various combinations points out the critical role of the I-J region in controlling the ability to induce T\(\text{S}_3\) cells. Thus, T\(\text{S}_3\) cells were only generated in combinations in which the NP-coupled cells used for antigen priming carried an I-J allele in common with the host. This is most directly observed by comparing the results of priming with NP-coupled 3R and 5R cells. The 3R (I-J\(^b\)) and 5R (I-J\(^b\)) strains can be considered I-J congenic because they have different alleles at the I-J subregion but there are no known differences throughout the remainder of their genomes. NP-coupled 3R but

### Table IV

| CY-treated recipients | Transferred T\(\text{S}_3\) cells | Source of TsF | Footpad swelling ± SE:~ |
|-----------------------|---------------------------------|---------------|--------------------------|
| B6                    | B6                              | BW5147        | 36 ± 1                   |
|                       | NP-B6                           | B6-Ts\(_2\)-28 | 18 ± 3§                 |
|                       | NP-3R (K, IA, IB, IJ)           | BW5147        | 36 ± 2                   |
|                       | NP-3R                           | B6-Ts\(_2\)-28 | 15 ± 2§                 |
|                       | NP-4R (IB, IJ, IC, IE, S, D)    | BW5147        | 35 ± 1                   |
|                       | NP-4R                           | B6-Ts\(_2\)-28 | 22 ± 2§                 |
|                       | NP-5R (K, IA, IB)               | BW5147        | 34 ± 2                   |
|                       | NP-5R                           | B6-Ts\(_2\)-28 | 32 ± 1                   |
|                       | None                            | None          | 6 ± 3\]                  |
| B10.BR                | B10.BR                          | BW5147        | 41 ± 2                   |
|                       | NP-B10.BR                       | CKB-Ts\(_2\)-59 | 22 ± 1§                |
|                       | NP-B10.BR (IE)                  | BW5147        | 40 ± 1                   |
|                       | NP-3R                           | CKB-Ts\(_2\)-59 | 43 ± 4                |
|                       | NP-4R (K, IA)                   | BW5147        | 40 ± 3                   |
|                       | NP-4R                           | CKB-Ts\(_2\)-59 | 40 ± 4                |
|                       | NP-5R (IJ, IE)                  | BW5147        | 41 ± 2                   |
|                       | NP-5R                           | CKB-Ts\(_2\)-59 | 29 ± 1§                |
|                       | None                            | None          | 12 ± 2\]                 |

* C57BL/6 or B10.BR mice were used as donors for T\(\text{S}_3\) cells. The T\(\text{S}_3\) cells were induced by priming with NP-coupled syngeneic or allogeneic cells. The regions of H-2 homology are indicated in parentheses. The T\(\text{S}_3\) cells were transferred along with control (BW5147) or Ts2-derived suppressor factor to NP-O-Su-primed, CY-treated recipients.

§ Refer to legend of Table I.

\(\text{S}\) Significant suppression; \(P < 0.01.\)

\(\text{I}\) Background swelling responses in nonimmune mice.
not 5R cells induce Ts3 in I-J compatible C57BL/6 (I-Jb) mice, whereas NP-coupled 5R cells showed a reciprocal pattern inducing Ts3 in I-J-compatible B10.BR (I-Jk) but not in I-J-mismatched C57BL/6 mice. The ability of NP-5R cells to generate a Ts3 population in B10.BR hosts is notable because these two strains differ at the K, I-A, I-B, I-C, S and D regions and only share alleles at the I-J and I-E subregions of the major histocompatibility complex. Thus, homology at I-J (and I-E) appears to be sufficient for Ts3 induction, at least under these experimental conditions.

When priming with allogeneic cells, one must always consider the potential complications caused by allogeneic effects. To exclude these potential artifacts we have (a) demonstrated that the suppression generated by these Ts3 cells is antigen specific (data not shown); (b) used a syngeneic adoptive transfer system to assay Ts3 activity so that no allogeneic cells were actually present in the recipient mice; (c) suppression was not observed with control BW5147 supernatants, but only when a soluble mediator (TsF2) was added; and (d) various strain combinations displaying a variety of H-2 disparities were used.

**Ts3 Generation in F1 Mice.** The next series of experiments were aimed at evaluating the specificity of Ts3 cells derived from I-J heterozygous F1 mice. B6AF1 (I-Jb/I-Jk) mice were primed with NP-coupled C57BL/6 (I-Jb) or B10.BR (I-Jk) cells. After 5 d, lymph node cells from the F1 donors were adoptively transferred to CY-treated C57BL/6 or B10.BR recipients along with B6- or CKB-derived TsF2. Significant levels of suppression were consistently noted in the combinations in which these elements all shared gene products of the I-J subregion: i.e., (a) the cells used for Ts3 priming, (b) the TsF2, and (c) the recipients. Thus, the NP response was suppressed in C57BL/6 (I-Jb) mice after injection of both C57BL/6-derived TsF2 (B6-Ts2-28) and Ts3 cells from B6AF1 mice that were primed with NP-B6 cells (Table V). If CKB (I-Jk)-derived TsF2 (CKB-Ts2-59) was injected along with the same source of Ts3 cells, significant levels of suppression were no longer observed. In a reciprocal experiment, Ts3 cells derived from F1 donors primed with NP-coupled B10.BR (I-Jk) cells suppressed B10.BR recipients when injected along with CKB (I-Jk)-derived TsF2 (Table V). However, the same population of F1 Ts3 cells failed to induce significant levels of suppression when transferred to C57BL/6 recipients and reciprocally Ts3 induced with NP-B6 cells could not cause significant suppression when transferred to B10.BR recipients. The simplest interpretation of the above data is that I-J heterozygous animals can generate two distinct populations of Ts3 cells, depending on the manner of antigen priming. Thus, in I-Jb/I-Jk heterozygous mice, priming with antigen in the context of I-Jk generates a population of Ts3 cells that are genetically restricted to I-Jk for both activation and interaction, whereas priming with antigen in the context of I-Jb generates I-Jk-restricted Ts3 cells (17).

**Ts3 Induction Requires Antigen-presenting Adherent Cells.** The next series of experiments were aimed at identifying the nature of the cell population in the spleen that was responsible for the induction of Ts3 cells. Mice were primed with graded doses of NP-coupled adherent, nonadherent, or unfractionated syngeneic spleen cells. After 6 d, 1 × 10^7 lymph node cells were adoptively transferred to syngeneic recipients, and the appropriate groups were also injected with TsF2. The mice were challenged with NP-O-Su, and the CS responses were measured 24 h thereafter. Among the four experiments, the minimum number of NP-coupled cells required to induce the Ts3 varied by ~10-fold, but the overall patterns were consistent. The data from all four
**Table V**

| CY-treated recipients | Ts3 donor | Priming Ts3 donor | Source of TsF2 | Percent suppression ± SE$^\ddagger$ |
|-----------------------|-----------|-------------------|----------------|-------------------------------------|
| C57BL/6 B6AF1         | NP-C57BL/6| B6-Ts3-28         | B6-Ts3-28      | 67 ± 6§                             |
|                       |           |                   | CKB-Ts3-59     | 16 ± 16                             |
| B6-Ts3-28             | 7 ± 7     |
|                       |           |                   | CKB-Ts3-59     | 29 ± 14                             |
|                       |           |                   | (1)KB-Ts2-59   | 4 ± 1                               |
|                       |           |                   | B6-Ts2-28      | 18 ± 5                              |
|                       |           |                   | CKB-Ts2-59     | 4 ± 1                               |
|                       |           |                   | B6-Ts2-28      | 2 ± 5§                              |
|                       |           |                   | CKB-Ts2-59     | 42 ± 5§                             |

*Ts3 cells were generated in B6AF1 mice by priming with NP-coupled C57BL/6 or B10.CR spleen cells. After 5 d, the B6AF1 lymph node Ts3 cells were transferred to either C57BL/6 or B10.CR CY-treated recipients along with either BW5147-, B6-Ts3-28-, or CKB-Ts2-59-derived factors. The recipients were challenged after administration of these factors, and footpad swelling was measured 24 h thereafter.

$^\ddagger$ The results of three independent experiments were normalized and pooled using the response with BW5147 supernatants as the positive control and the nonimmune background response as the negative control.

§ Significant levels of suppression, $P < 0.01$.

Discussion

The purpose of these experiments was to analyze the mechanisms responsible for the induction of major histocompatibility complex restrictions on Ts cell interactions. In several independent systems, I-J (or I-C) restrictions have been observed on the interactions of suppressor T cells (13–18). In most systems, the Ts cells are stimulated by antigen priming. In the NP system, the ability of antigen-primed Ts3 cells or their factors to suppress contact sensitivity responses is also I-J restricted (17, 22). Furthermore, in H-2 heterozygous F1 mice, at least two distinct populations of Ts3 cells can be generated (Table V). The activity of each F1-derived Ts3 population is genetically restricted to a parental I-J haplotype (17). Based on the above information, we hypothesized that the mechanism responsible for the induction of MHC restrictions in antigen-primed Ts3 cells may mirror those previously described for the induction of MHC restrictions in populations of helper or proliferating T cells (17). To test this hypothesis, we first modified our method of inducing NP-specific Ts3 cells to permit priming with antigen-modified cells. The data demonstrate that this method of antigen priming induces hapten-specific Ts3 and DTH cells. The DTH effector cells have a characteristic delayed onset and can be adoptively transferred into H-2I region-compatible recipients. The DTH responses induced by priming with syngeneic experiments were normalized and pooled; the results are summarized in Table VI. NP-coupled adherent cells were most efficient at inducing Ts3 cells. Thus, priming with $10^3$ hapten-coupled adherent cells generated sufficient Ts3 to induce significant levels of suppression. Approximately $10^6$ NP-coupled unfractionated spleen cells were required to induce comparable levels of Ts3 activity. In contrast, priming with up to $10^7$ nonadherent cells failed to produce significant levels of Ts3 activity.
### Table VI

*Antigen-coupled Splenic Adherent Cells Are Required for Induction of Tsa Cells*

| Cells used for Tsa priming | Tsa2 | Percent suppression ± SE |
|---------------------------|------|-------------------------|
|                           | +    | ~3 ± 7                  |
| 10^7 NP-spleen            |     | ~4 ± 8                  |
| 10^6 NP-spleen            | +    | 48 ± 5§                 |
| 10^7 NP-spleen            | +    | 32 ± 11§                |
| 10^6 NP-adherent          | +    | 15 ± 7                  |
| 10^5 NP-adherent          |     | ~2 ± 10                 |
| 10^4 NP-adherent          | +    | 46 ± 9§                 |
| 10^3 NP-adherent          | +    | 47 ± 9§                 |
| 10^2 NP-adherent          | +    | 28 ± 10§                |
| 10^1 NP-adherent          | +    | 18 ± 20                 |
| 10^6 NP-nonadherent, G-10 |     | 17 ± 11                 |
| 10^5 NP-nonadherent, G-10 | +    | 9 ± 4                   |
| 10^4 NP-nonadherent, G-10 | +    | 13 ± 9                  |
| 10^3 NP-nonadherent, G-10 | +    | 4 ± 5                   |

* Tsa cells were generated by priming with either hapten-coupled syngeneic unfractionated spleen cells or NP-coupled nonadherent or adherent splenic cells. After 5 d, the lymph nodes containing the Tsa population were adoptively transferred to syngeneic recipients along with either TsF2 or splenic Tsa cells (prepared by injecting mice 6 d previously with NP-coupled spleen cells intravenously).

§ The results of four experiments were normalized and pooled. The results were compared with groups that received neither Tsa nor Ts2 cells (refer to legend of Table V).

§ Significant levels of suppression; P < 0.05.

NP-coupled cells can be suppressed by a variety of monoclonal TsF (Table I). In contrast, the DTH responses generated after priming with NP-coupled I-J-mismatched cells cannot be suppressed by TsF2 but remain sensitive to suppression by TsF3 (Table II). These results imply that priming with I-J-matched cells is required for the induction of Tsa cells.

To verify that the inability of TsF2 to suppress mice primed with NP-coupled I-J-mismatched cells was due to a functional absence of Tsa cells, a series of transfer experiments was performed. To directly assay Tsa activity, we injected TsF2 and lymph node cells derived from mice that were primed with either NP-coupled syngeneic or allogeneic cells into Tsa-depleted recipients (i.e., mice that were previously primed with NP-O-Su and then treated with CY to prevent the generation of Tsa cells). This transfer protocol permits analysis of Tsa generation independent of the generation of DTH effector cells. The data demonstrate that priming with NP-coupled I-J-compatible cells is required and sufficient to generate antigen-specific Tsa cells (Table III). Furthermore, the data verify our previous observations (17) that the interactions of Tsa populations are genetically restricted by genes in the MHC (Table IV). These points are supported by additional data derived from experiments in which H-2 heterozygous mice were primed with antigen-coupled parental cells (Table V). The data demonstrate that priming of B6AF1 mice with NP-coupled C57BL/6 (I-Jb) cells induced a population of Tsa cells that were only activated with C57BL/6-
derived TsF2 and not by CKB (I-Jk)-derived TsF2. Further, these Ts3 cells only caused suppression when transferred to H-2-matched C57BL/6 recipients. These results emphasized the requirement for H-2 restriction in the activation and interaction of Ts3 cells. Reciprocal experiments demonstrated that a second Ts3 population was generated by priming F1 mice with NP-coupled B10.BR (I-Jk) cells. In the latter situation, priming F1 mice with NP-B10.BR cells generated Ts3 cells that required I-Jk-derived TsF2 for activation, and these F1 Ts3 cells function most effectively in B10.BR recipients. Thus, antigen appears to functionally associate with I-J determinants on the immunizing cells. This complex then controls the specificity of the developing Ts3 population restricting both Ts3 activation and subsequent interaction.

The next issue of concern was the nature of the antigen-presenting cell required for the generation of Ts3 cells. Again, the adoptive transfer protocol was used to assess Ts3 generation. The data in Table VI indicate that 10⁷ NP-coupled nonadherent spleen cells could not induce Ts3 cells. In contrast, as few as 10⁶ NP-coupled splenic adherent cells could induce Ts3 activity. These data emphasize the role of a specialized adherent cell population in Ts3 generation. The conditions used to couple NP onto large numbers of unfraccionated or nonadherent spleen cells were the same as those used to couple 1/10 to 1/20 the number of splenic adherent cells. Thus, the apparent enhanced efficiency of Ts3 induction with NP-coupled splenic adherent cells (Table VI) may be attributable to higher hapten densities on the adherent population under these experimental conditions. Nonetheless, the inability of NP-coupled splenic non-adherent cells to generate Ts3 demonstrates the vital role of a specialized adherent population in the generation of Ts3 cells.

Taken together, the present data have numerous features in common with the situation noted in the generation of MHC-restricted helper (Th) or proliferating T cells. (a) For both Th and Ts3 generation, antigen must be presented in the context of MHC determinants on an adherent antigen-presenting cell (6-8, 30). (b) In H-2 heterozygous F1 mice, conventional antigen priming generates two distinct populations of helper or suppressor cells, each specific for antigen in the context of one of the parental H-2 haplotypes (31, 32). (c) Priming F1 animals with antigen in the context of only one set of parental H-2 determinants results in the generation of only one population of helper or suppressor cells (7, 8). (d) The activation of Th and Ts3 cells is genetically restricted by the H-2 haplotype of the parental cells used for priming (7, 8). (e) The subsequent interactions of activated Th and Ts3 cells with their target populations may also involve genetic restrictions identical to those required for activation (33).

One of the major differences noted between the helper and suppressor compartments are the MHC genes participating in the induction of these immune processes. Thus, genes in the I-A and I-E subregions control helper T cell induction, whereas genes in the I-J subregion control Ts3 induction. The functional role of I-A or I-E gene products in the presentation of antigen by macrophages or dendritic cells has been well documented (34, 36). We hypothesize that I-J-encoded structures on antigen-presenting cells can serve a similar presentation function. Receptors on functional Ts3 precursor cells must recognize antigen in the context of the appropriate I-J structure. Once the precursor population matures or differentiates, the terminal activation and interaction of Ts3 cells presumably requires triggering via anti-I-J and anti-idiotypic receptors(s).
Additional data supporting this hypothesis are provided in our previous report (17) and the accompanying report (37), which further documents the critical role of I-J gene products in the induction and activation of azobenzenearsenonate-induced T<sub>S</sub> cells. However, there are several issues concerning this hypothesis that remain unresolved. Among these is the comparison of the antigen-presenting cells required for helper and suppressor induction. Do these cells represent different cellular subsets? Because suppressor cells have been identified in some nonresponder strains (38–41), do antigen associations with I-A or I-J molecules direct these H-2-controlled responses?

We should caution that, although we assume that the cells that present antigen to T<sub>S</sub> precursors express cell surface I-J determinants, we have been unable to directly document this point. Technical obstacles are probably responsible for this failure. Nonetheless, other investigators have described I-J-bearing antigen-presenting cells (42). Interestingly, Niederhuber et al. (43) reported that this I-J-bearing antigen-presenting population also expressed I-A markers. Further experiments to characterize the antigen-presenting cells involved in T<sub>H</sub> and T<sub>S</sub> induction are required.

Although we do not wish to minimize the importance of the disparities in the induction of T helper and T suppressor cells, the majority of the data argue in favor of a common underlying mechanism for the induction of I-A and I-J genetic restrictions in their respective T cell populations.

**Summary**

The mechanisms responsible for the induction of I-J restrictions on third-order suppressor T cells (T<sub>S</sub>) were analyzed. The I-J phenotype of the antigen-coupled cells used for priming restricted the specificity of the T<sub>S</sub> population. Thus, T<sub>S</sub> cells were only generated after priming with antigen-coupled I-J homologous cells. Identity at the I-J (and I-E) subregions was sufficient for T<sub>S</sub> induction. Furthermore, priming of H-2 heterozygous mice with antigen-coupled parental cells generated T<sub>S</sub> that were restricted to the parental haplotype used for priming. The splenic cell population responsible for antigen presentation and induction of T<sub>S</sub> cells was fractionated. The cells involved in antigen presentation were found in the splenic adherent population and were absent in the fraction containing splenic nonadherent T and B cells. The subsequent activation and interaction of T<sub>S</sub> cells is also restricted by genes in the H-2 complex. The results are discussed in terms of a general mechanism responsible for the induction of restrictions in T helper and T<sub>S</sub> cells.

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