MECHANISM RESPONSIBLE FOR THE INDUCTION OF I-J RESTRICTIONS ON Ts_3 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)[1] 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 Ts_1, Ts_2 and Ts_3 cells, respectively. Ts_1 cells or their soluble factors do not display any H-2 restriction (14, 24, 25). In contrast, Ts_2 and Ts_3 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 Ts_3 cells. Ts_3 cells are present in conventionally primed mice, but they only exert their suppressive activity after activation by Ts_2 cells or factors derived from Ts_2 cells (17, 21, 23). In this report, we evaluate methods of generating NP-specific Ts_3 cells. The data suggest that I-J determinants may serve as the antigen-presenting structures for the induction of Ts_3 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 Ts_3 cells restricted by either parental H-2 haplotype can be generated in H-2 heterozygous F_1 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-dinitro fluorobenzene; DTH, delayed-type hypersensitivity; EDTA, ethylenediamine tetraacetic 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; T_h, helper T cells; Ts_2, Ts_3, second- or third-order suppressor T cells; TsF_2, TsF_3, Ts_2- or Ts_3-derived suppressor factors.

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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, clorinated 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 passing $1–2 \times 10^8$ 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, $3–5 \times 10^8$ spleen cells or $2–6 \times 10^6$ 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 glycylglycine. After extensive washing in MEM, the NP-coupled cells were used for priming.

Assay for DTH Induction and Ts_3 Induction after Immunization with NP-coupled Cells. To induce DTH and Ts_3 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 of BW5147, B6-Ts_2-28, or CKB-Ts_2-59 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 Ts_3 Activity. Donor mice were immunized subcutaneously with 1×10^7 NP-coupled cells. 6 d after priming, the mice were killed, and inguinal and axillar lymph nodes were removed, teased into a single cell suspension, and used as a source of Ts_3 donor cells.

To prepare Ts_3-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 Ts_3 donor cells intravenously. Immediately after transfer and on day 6, 0.5 ml HBSS containing 5 μl control BW5147 tumor-derived or B6-Ts_2-28-derived or CKB-Ts_2-59-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.

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 1×10^7 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, 4×10^7 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 Ts_2- and Ts_3-derived suppressor factors, (TsF_2 and TsF_3), 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 TsF_2 or TsF_3 factors. The fact that these mice could be suppressed with TsF_2 factor suggests

TABLE I

*Suppression of DTH Responses with Monoclonal Suppressor Factors**

Strain	Priming with NP-coupled spleen cells	TsF source	Footpad swelling ± SE‡
C57BL/6	NP-B6	BW5147	42.2 ± 1.6
		B6-Ts_2-28	15.5 ± 1.2§
		B6-Ts_3-2	16.0 ± 1.1§
	None	None	7.8 ± 1.6
CKB	NP-CKB	BW5147	43.3 ± 1.4
		CKB-Ts_2-59	20.0 ± 0.6§
		CKB-Ts_3-3	18.5 ± 3.5§
	None	None	7.3 ± 1.0

* Groups consisting of four mice were primed with 1×10^7 NP-coupled syngeneic spleen cells. 5 and 6 d after priming, animals wre injected intravenously with either monoclonal TsF_2 or TsF_3 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.

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

§ Significant suppression; $P < 0.01$.

that Ts_3 cells, which are the target of TsF_2, were present and were generated by priming with hapten-coupled cells.

We next performed a series of experiments to determine whether Ts_3 cells could be generated by priming with allogeneic hapten-coupled cells. Animals were immunized with 1×10^7 NP-coupled allogeneic spleen cells and 5 to 6 d later were given i.v. injections of TsF_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, eg., 3R and 5R (Table II). In such combinations, it was consistently possible to observe strong DTH responses. However, TsF_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 TsF_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 TsF_2 factor (Table II). In reciprocal

TABLE II

The I-J Genotype of the Cells Used for Antigen Priming Controls Ts_3 Expression

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-Ts_2-28	20.3 ± 2.4*
		NP-3R	B6-Ts_3-2	16.8 ± 1.1*
		NP-5R	BW5147	26.3 ± 3.0
		NP-5R	B6-Ts_2-28	28.0 ± 2.7
		NP-5R	B6-Ts_3-2	9.0 ± 1.5*
		None	None	8.4 ± 1.6
	5R	NP-3R	BW5147	37.7 ± 1.7
		NP-3R	CKB-Ts_2-59	34.0 ± 2.5
		NP-3R	CKB-Ts_3-3	26.5 ± 1.3*
		NP-5R	BW5147	44.8 ± 1.3
		NP-5R	CKB-Ts_2-59	24.8 ± 1.9*
		NP-5R	CKB-Ts_3-3	20.3 ± 2.2*
		None	None	7.5 ± 0.6
2	3R	NP-3R	BW5147	43.0 ± 3.2
		NP-3R	B6-Ts_2-28	19.5 ± 1.9*
		NP-5R	BW5147	40.3 ± 3.5
		NP-5R	B6-Ts_2-28	42.0 ± 3.0
		NP-5R	CKB-Ts_2-59	40.8 ± 3.0
		None	None	9.0 ± 1.0
	5R	NP-3R	BW5147	37.5 ± 3.1
		NP-3R	B6-Ts_2-28	37.0 ± 1.9
		NP-3R	CKB-Ts_2-59	38.0 ± 3.0
		NP-5R	BW5147	42.0 ± 3.2
		NP-5R	CKB-Ts_2-59	14.0 ± 1.7*
		None	None	9.3 ± 0.3

* 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 insuing responses could be suppressed by CKB-derived TsF_2. However, when the same NP-coupled 5R cells were used to prime I-J-disparate 3R recipients, TsF_2 could no longer suppress the response. The inability of TsF_2 to suppress DTH responses induced by priming with I-J-mismatched cells is not due to restrictions on Ts_3 activation because neither C57BL/6 (I-J^b) nor CKB (I-J^k) TsF_2 produced suppression (Table II, experiment 2). The controls for these experiments included groups that were injected with TsF_3 (Table II, experiment 1). The ability of TsF_3 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 TsF_2 and TsF_3, i.e., in the Ts_3 population.

Requirement for I-J Homology for Ts_3 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 Ts_3 cells or in the inability of Ts_3 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 Ts_3 cells, and are then given Ts_2 cells or TsF_2 along with a transfer containing the potential Ts_3 cell source (17, 21). To generate Ts_3 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 Ts_3 donors were then adoptively transferred into NP-O-Su-primed CY-treated syngeneic recipients along with control BW or monoclonal TsF_2 factors. As shown in Table III, C57BL/6 lymph node cells obtained from Ts_3 donors primed with syngeneic NP-coupled cells transferred suppression to syngeneic recipients that also received C57BL/6-derived TsF_2. In reciprocal experiments, injections of H-2^k-derived CKB-Ts_2-59 factor and B10.BR Ts_3 cells generated NP-specific suppression when given to B10.BR recipients. However, the CKB-Ts_2-59 (I-J^k) factor would not activate C57BL/6 Ts_3 cells, and the B6-Ts_2-28 factor did not activate I-J-mismatched

TABLE III

*Adoptive Transfer of Ts_3 Cells Induced by Priming with NP-coupled Syngeneic Cells**

Strain of CY-treated recipients	NP-O-Su and DNFB priming	Spleen cells for priming of Ts_3 donor	Source of TsF	Antigen challenge‡	
				NP-O-Su	DNFB
C57BL/6	+	Normal B6	BW5147	30.4 ± 2.0 (7)	11.3 ± 0.8 (7)
	+	Normal B6	B6-Ts_2-28	30.3 ± 1.4 (8)	12.3 ± 1.7 (8)
	+	NP-B6	BW5147	32.5 ± 2.3 (8)	11.5 ± 1.0 (8)
	+	NP-B6	B6-Ts_2-28	12.9 ± 1.5§ (8)	11.9 ± 0.5 (8)
	+	NP-B6	CKB-Ts_2-59	31.0 ± 1.8 (8)	12.3 ± 1.1 (8)
	—	—	—	7.0 ± 2.4 (6)	1.7 ± 0.8 (6)

* 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 1×10^7 Ts_3 cells and TsF_2 or BW5147 control factors. The Ts_3 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 Ts_3 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; $P < 0.001$.

B10.BR Ts_3 cells. This transfer protocol provides direct evidence that Ts_3 cells were generated by priming with hapten-coupled syngeneic cells and verifies the H-2 restrictions on Ts_3 cell activation (17).

In an additional series of experiments, NP-coupled 3R, 4R, and 5R allogeneic cells were also used to induce Ts_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 Ts_3 activity (Table IV). However, priming of B10.BR hosts with NP-coupled 5R cells could generate functional Ts_3 cells, whereas priming of C57BL/6 mice with these NP-5R cells failed to induce a functional Ts_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 Ts_3 cells. Thus, Ts_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 ($I\text{-}J^b$) and 5R ($I\text{-}J^k$) strains can be considered I-J congeneic 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

*Inability to Transfer Ts_3 Activity from Mice Primed with I-J Mismatched NP-coupled Cells**

CY-treated recipients	Transferred Ts_3 cells		Source of TsF	Footpad swelling ± SE‡
	Ts_3 donor	Priming of Ts_3 donor		
B6	B6	NP-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	NP-B10.BR	BW5147	41 ± 2
		NP-B10.BR	CKB-Ts_2-59	22 ± 1§
		NP-3R (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 Ts_3 cells. The Ts_3 cells were induced by priming with NP-coupled syngeneic or allogeneic cells. The regions of H-2 homology are indicated in parentheses. The Ts_3 cells were transferred along with control (BW5147) or Ts_2-derived suppressor factor to NP-O-Su-primed, CY-treated recipients.

‡ Refer to legend of Table I.

§ Significant suppression; $P < 0.01$.

‖ Background swelling responses in nonimmune mice.

not 5R cells induce Ts_3 in I-J compatible C57BL/6 (I-J^b) mice, whereas NP-coupled 5R cells showed a reciprocal pattern inducing Ts_3 in I-J-compatible B10.BR (I-J^k) but not in I-J-mismatched C57BL/6 mice. The ability of NP-5R cells to generate a Ts_3 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 Ts_3 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 Ts_3 cells is antigen specific (data not shown); (*b*) used a syngeneic adoptive transfer system to assay Ts_3 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 (TsF_2) was added; and (*d*) various strain combinations displaying a variety of H-2 disparities were used.

Ts_3 Generation in F_1 Mice. The next series of experiments were aimed at evaluating the specificity of Ts_3 cells derived from I-J heterozygous F_1 mice. $B6AF_1$ (I-J^b/I-J^k) mice were primed with NP-coupled C57BL/6 (I-J^b) or B10.BR (I-J^k) cells. After 5 d, lymph node cells from the F_1 donors were adoptively transferred to CY-treated C57BL/6 or B10.BR recipients along with B6- or CKB-derived TsF_2. Significant levels of suppression were consistantly noted in the combinations in which these elements all shared gene products of the I-J subregion: i.e., (*a*) the cells used for Ts_3 priming, (*b*) the TsF_2, and (*c*) the recipients. Thus, the NP response was suppressed in C57BL/6 (I-J^b) mice after injection of both C57BL/6-derived TsF_2 (B6-Ts_2-28) and Ts_3 cells from $B6AF_1$ mice that were primed with NP-B6 cells (Table V). If CKB (I-J^k)-derived TsF_2 (CKB-Ts_2-59) was injected along with the same source of Ts_3 cells, significant levels of suppression were no longer observed. In a reciprocal experiment, Ts_3 cells derived from F_1 donors primed with NP-coupled B10.BR (I-J^k) cells suppressed B10.BR recipients when injected along with CKB (I-J^k)-derived TsF_2 (Table V). However, the same population of F_1 Ts_3 cells failed to induce significant levels of suppression when transferred to C57BL/6 recipients and reciprocally Ts_3 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 Ts_3 cells, depending on the manner of antigen priming. Thus, in I-J^b/I-J^k heterozygous mice, priming with antigen in the context of I-J^b generates a population of Ts_3 cells that are genetically restricted to I-J^b for both activation and interaction, whereas priming with antigen in the context of I-J^k generates I-J^k-restricted Ts_3 cells (17).

Ts_3 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 Ts_3 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 TsF_2. 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 Ts_3 varied by ~10-fold, but the overall patterns were consistent. The data from all four

TABLE V
*Ts_3 Generation in F_1 Mice**

CY-treated recipients	Ts_3 donor	Priming Ts_3 donor	Source of TsF_2	Percent suppression ± SE‡
C57BL/6	$B6AF_1$	NP-C57BL/6	B6-Ts_2-28	67 ± 6§
			CKB-Ts_2-59	16 ± 16
		NP-B10.BR	B6-Ts_2-28	7 ± 7
			CKB-Ts_2-59	29 ± 14
B10.BR	$B6AF_1$	NP-C57BL/6	B6-Ts_2-28	18 ± 5
			CKB-Ts_2-59	4 ± 1
		NP-B10.BR	B6-Ts_2-28	2 ± 5
			CKB-Ts_2-59	42 ± 5§

* Ts_3 cells were generated in $B6AF_1$ mice by priming with NP-coupled C57BL/6 or B10.BR spleen cells. After 5 d, the $B6AF_1$ lymph node Ts_3 cells were transferred to either C57BL/6 or B10.BR CY-treated recipients along with either BW5147-, B6-Ts_2-28-, or CKB-Ts_2-59-derived factors. The recipients were challenged after administration of these factors, and footpad swelling was measured 24 h thereafter.

‡ 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, $P < 0.01$.

experiments were normalized and pooled; the results are summarized in Table VI. NP-coupled adherent cells were most efficient at inducing Ts_3 cells. Thus, priming with 10^3 hapten-coupled adherent cells generated sufficient Ts_3 to induce significant levels of suppression. Approximately 10^6 NP-coupled unfractionated spleen cells were required to induce comparable levels of Ts_3 activity. In contrast, priming with up to 10^7 nonadherent cells failed to produce significant levels of Ts_3 activity.

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 Ts_3 cells or their factors to suppress contact sensitivity responses is also I-J restricted (17, 22). Furthermore, in H-2 heterozygous F_1 mice, at least two distinct populations of Ts_3 cells can be generated (Table V). The activity of each F_1-derived Ts_3 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 Ts_3 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 Ts_3 cells to permit priming with antigen-modified cells. The data demonstrate that this method of antigen priming induces hapten-specific Ts_3 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

TABLE VI

*Antigen-coupled Splenic Adherent Cells Are Required for Induction of Ts_3 Cells**

Cells used for Ts_3 priming	Ts_2	Percent suppression ± SE‡
—	+	−3 ± 7
10^7 NP-spleen	−	−4 ± 8
10^7 NP-spleen	+	48 ± 5§
10^6 NP-spleen	+	32 ± 11§
10^5 NP-spleen	+	15 ± 7
10^5 NP-adherent	−	−2 ± 10
10^5 NP-adherent	+	46 ± 9§
10^4 NP-adherent	+	47 ± 9§
10^3 NP-adherent	+	28 ± 10§
10^2 NP-adherent	+	18 ± 20
10^7 NP-nonadherent, G-10	−	17 ± 11
10^7 NP-nonadherent, G-10	+	9 ± 4
10^6 NP-nonadherent, G-10	+	13 ± 9
10^5 NP-nonadherent, G-10	+	4 ± 5

* Ts_3 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 Ts_3 population were adoptively transferred to syngeneic recipients along with either TsF_2 or splenic Ts_2 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 Ts_3 nor Ts_2 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 TsF_2 but remain sensitive to suppression by TsF_3 (Table II). These results imply that priming with I-J-matched cells is required for the induction of Ts_3 cells.

To verify that the inability of TsF_2 to suppress mice primed with NP-coupled I-J-mismatched cells was due to a functional absence of Ts_3 cells, a series of transfer experiments was performed. To directly assay Ts_3 activity, we injected TsF_2 and lymph node cells derived from mice that were primed with either NP-coupled syngeneic or allogeneic cells into Ts_3-depleted recipients (i.e., mice that were previously primed with NP-O-Su and then treated with CY to prevent the generation of Ts_3 cells). This transfer protocol permits analysis of Ts_3 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 Ts_3 cells (Table III). Furthermore, the data verify our previous observations (17) that the interactions of Ts_3 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 $B6AF_1$ mice with NP-coupled C57BL/6 ($I\text{-}J^b$) cells induced a population of Ts_3 cells that were only activated with C57BL/6-

derived TsF_2 and not by CKB (I-J^k)-derived TsF_2. Further, these Ts_3 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 Ts_3 cells. Reciprocal experiments demonstrated that a second Ts_3 population was generated by priming F_1 mice with NP-coupled B10.BR (I-J^k) cells. In the latter situation, priming F_1 mice with NP-B10.BR cells generated Ts_3 cells that required I-J^k-derived TsF_2 for activation, and these F_1 Ts_3 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 Ts_3 population restricting both Ts_3 activation and subsequent interaction.

The next issue of concern was the nature of the antigen-presenting cell required for the generation of Ts_3 cells. Again, the adoptive transfer protocol was used to assess Ts_3 generation. The data in Table VI indicate that 10^7 NP-coupled nonadherent spleen cells could not induce Ts_3 cells. In contrast, as few as 10^3 NP-coupled splenic adherent cells could induce Ts_3 activity. These data emphasize the role of a specialized adherent cell population in Ts_3 generation. The conditions used to couple NP onto large numbers of unfractionated or nonadherent speen 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 Ts_3 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 nonadherent cells to generate Ts_3 demonstrates the vital role of a specialized adherent population in the generation of Ts_3 cells.

Taken together, the present data have numerous features in common with the situation noted in the generation of MHC-restricted helper (T_h) or proliferating T cells. (*a*) For both T_h and Ts_3 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 F_1 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 F_1 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 T_h and Ts_3 cells is genetically restricted by the H-2 haplotype of the parental cells used for priming (7, 8). (*e*) The subsequent interactions of activated T_h and Ts_3 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 Ts_3 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 Ts_3 precurser cells must recognize antigen in the context of the appropriate I-J structure. Once the precurser population matures or differentiates, the terminal activation and interaction of Ts_3 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 azobenzenearsonate-induced Ts_3 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 Ts_3 precursers 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_h and Ts_3 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 (Ts_3) were analyzed. The I-J phenotype of the antigen-coupled cells used for priming restricted the specificity of the Ts_3 population. Thus, Ts_3 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 Ts_3 induction. Furthermore, priming of H-2 heterozygous mice with antigen-coupled parental cells generated Ts_3 that were restricted to the parental haplotype used for priming. The splenic cell population responsible for antigen presentation and induction of Ts_3 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 Ts_3 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 Ts_3 cells.

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