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

VIII. Suppressor Cell Pathways in Cutaneous Sensitivity Responses*

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We have previously demonstrated that suppressor cells can abrogate 4-hydroxy-3-nitrophenyl acetyl (NP)[1]-specific delayed-type hypersensitivity (DTH) responses (1–3). We now examine the ability of the same population of suppressor cells to abrogate cutaneous sensitivity (CS) responses. Previous studies have noted several differences between the effector T cell populations which mediate DTH and CS (4–6). In particular, DTH responses can be transferred between strains of mice sharing only the H-2I region of the murine major histocompatibility complex, in contrast to CS responses, where homology at either H-2K, I, or D is sufficient for adoptive transfer of reactivity (4, 5). Further, it has been demonstrated that T cells mediating H-2I-restricted and H-2D-restricted immune responses may bear different idiotypic receptors (6, 7).

Recognizing the importance of idiotypic-anti-idiotypic interactions in the generation of antigen-specific immune suppression (3, 8), we now extend the comparison of DTH and CS responses by analyzing the mechanisms of suppression of CS reactions. Earlier investigations have implicated the involvement of two distinct T cells from the NP-tolerized lymphocyte population in the regulation of DTH immunity (1–3): induction-phase suppressor T cells (Ts^i or Ts_1), which can be generated by antigen alone; and effector-phase suppressors (Ts^e or Ts_2), which depend for their generation on both antigen and Ts^i or idiotype-positive factor derived from Ts^i. Recent evidence in another system (9) has further suggested the requirement of a third auxiliary T cell from the immune lymphocyte population for the suppression of 2,4-dinitro-1-fluorobenzene (DNFB) contact sensitivity by Ts^e. The current study unifies and extends these findings, demonstrating in a single system the development of all three suppressor cell types after in vivo antigen administration.

Materials and Methods

Mice. All mice were either purchased from The Jackson Laboratory, Bar Harbor, Maine, or bred in the animal facilities at Harvard Medical School, Boston, Mass. Mice were used at

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[1] *Abbreviations used in this paper*: BBS, borate-buffered saline; C, complement; CS, cutaneous sensitivity; DMSO, dimethyl sulfoxide; DNFB, 2,4-dinitrofluorobenzene; DTH, delayed-type hypersensitivity; Igh, immunoglobulin heavy chain-determining region; LN, lymph node; MEM, Eagle's minimum essential medium; NP, (4-hydroxy-3-nitrophenyl) acetyl; NP-*O*-Su, NP-*O*-succinimide; PBS, phosphate-buffered saline; T_{aux} or Ts_3, third-order "auxiliary" suppressor T cell; Ts^e or Ts_2, second-order "effector-phase" suppressor T cell; Ts^i or Ts_1, first-order 'induction-phase' suppressor T cell.

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1.5 to 12 mo of age, and were maintained on laboratory chow and acidified, chlorinated water ad lib.

Antigens. NP-*O*-succinimide (NP-*O*-Su) was purchased from Biosearch Co., San Rafael, Calif. DNFB was obtained from Sigma Chemical Co., St. Louis, Mo. Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific Co., Medford, Mass.

Immunization and Elicitation of CS Responses. Animals were shaved and primed subcutaneously with a saturated solution of NP-*O*-Su (7 g/100 ml) in DMSO. A total of 0.1 ml of antigen was divided equally between two sites on each ventral flank, followed by 0.1 ml of borate-buffered saline (BBS) at pH 8.6. 6 d after immunization or 1-2 h after adoptive transfer, mice were challenged for the CS response as described previously (5). In brief, 25 μl of NP-*O*-Su (0.1% solution in phosphate-buffered saline [PBS]) was injected into the left footpad. Footpad swelling was measured 24 h after challenge using an engineer's micrometer (Schlesingers for Tools, Brooklyn, N. Y.). Responses were determined as the difference, in units of 10^{-3} cm, between the left and right footpad thickness.

For specificity testing, animals were sensitized with two daily paintings of 25 μl 0.5% DNFB in a 4:1 mixture of acetone and olive oil, as previously described (9). 5 d after the last painting, 20 μl 0.2% DNFB in the same vehicle was applied to the dorsal surface of the ear, and the ear swelling response was measured as the difference between right and left ear thickness at 24 h.

Haptenated Cell Preparation. NP-coupled spleen cells were made as described previously (1). Briefly, single cell suspensions were treated with Tris-NH_3Cl to lyse erythrocytes. After two washes, the cells were resuspended in PBS, pH 7.7, and reacted with 2.5 mM NP-*O*-Su in DMSO for 4 min. The reaction was stopped with Eagle's minimum essential medium (MEM) containing 1.2 mg/ml glycylglycine. After extensive washing in MEM, 4×10^7 NP-coupled cells were injected into syngeneic recipients.

Antisera and Antibody Treatments. 6–7 $\times 10^8$ spleen cells from NP-coupled-cell-treated mice or NP-immune lymph node (LN) cells were pelleted and incubated in 1.0 ml of a 1:5 dilution of AKR anti-C3H thymocyte antiserum (anti-Thy-1.2), B10.A(3R) anti-B10.A(5R) (anti-I-J^k), B10.A(5R) anti-B10.A(3R) (anti-I-J^b), or as a control, normal mouse serum (NMS). After 30 min at 20°C, cells were washed and resuspended in 1.0 ml rabbit complement (C) diluted 1:5 in RPMI-1640 containing 1% DNase. After a 30-min incubation at 37°C, cells were washed in RPMI-1640 before transfer.

Adoptive Transfer of Suppression. 4×10^7 viable spleen cells from mice that had received NP-coupled spleen cells 6 d earlier were transferred intravenously into recipient mice either on day 0 (induction phase) or on day 6 (effector phase), 1 h before either antigen priming or challenge. Control groups received normal spleen cells. CS responses were elicited and measured as described above. Percent suppression is calculated as:

$$\% = \frac{(\text{control response} - \text{experimental response})}{(\text{control response} - \text{background})} \times 100.$$

Double Adoptive Transfer of CS Effector Cells and Ts^e. Mice were immunized as described above. 6 d after priming, the animals were killed, inguinal lymph nodes were removed, and a single cell suspension was prepared using a fine mesh screen. After washing, 4.0×10^7 viable cells were treated with antiserum plus C and mixed in a test tube with 4×10^7 NP-tolerized spleen cells or control normal spleen cells immediately before intravenous transfer into naive syngeneic recipients. In several groups of mice, 1×10^7 NP-immune LN cells were added to the mixture before transfer (see Table IV). Recipients and control mice were challenged with NP-*O*-Su 1 h after transfer, as describe above.

Data Analysis. Two-tailed Student's *t* tests were used to perform statistical analyses. Data are expressed as the mean incremental footpad or ear swelling ± SE in units of 10^{-3} cm.

Results

Phenotype of Splenocytes Responsible for Suppression of Syngeneic NP-specific CS Responses. Splenocytes from B10.BR congenic mice were depleted of erythrocytes and covalently coupled with NP-*O*-Su. 4×10^7 NP-derivatized spleen cells were

injected intravenously into 6–8-wk-old syngeneic recipients. 6 d later, 4×10^7 splenocytes from these mice (termed NP-tolerized spleen cells) or control spleen cells from normal mice were transferred intravenously into syngeneic mice at either the induction or effector phase of NP-specific CS responses. As shown in Table I, NP-tolerized spleen cells (Ts), but not control normal splenocytes, are capable of completely suppressing NP-CS responses at both the afferent and efferent limbs of the immune response.

T cells are required for the transfer of NP-nonresponsiveness as demonstrated by total abrogation of Ts activity by anti-Thy-1.2 antiserum plus C, as seen in Table I, experiment 1. Anti-I-J antiserum plus C was similarly able to lyse specifically both Ts populations for CS responses, as indicated by the results in experiments 2 and 3. The specificity of the anti-I-J^k alloantiserum was demonstrated by its failure to eliminate Ts cells obtained from R106 mice that carry the I-J^b allele.

Furthermore, treatment of Ts with anti-Lyt-2.2 hybridoma antibody plus C showed that the effector-phase suppressor cells are Lyt-2.2 positive (data not shown). However, this same treatment only partially abrogated the activity of induction-phase suppressors; thus, we cannot unequivocally determine the Lyt-2 phenotype of the Ts^i population (data not shown). In conclusion, both the induction- and effector-phase suppressor cells are I-J-bearing T cells, but they may differ in their susceptibility to anti-Lyt-2 treatment.

Specificity of NP-induced Suppressor Cells. We then proceeded to determine the antigen specificity of NP-tolerized spleen cells at the effector phase of CS responses.

TABLE I

*Phenotype of Splenocytes Suppressing Syngeneic NP-specific CS Responses**

Experiment	Strain	Treatment of transferred cells	Time of Ts administration‡	
			Induction phase	Effector phase
1	B10.BR	Control cells	26.5 ± 2.3 (6)§	26.4 ± 1.8 (7)§
		Ts + NMS + C	12.0 ± 1.8 (6)	13.2 ± 1.5 (6)
		Ts + anti-Thy-1.2 + C	25.8 ± 3.0 (4)‖	29.2 ± 1.7 (6)‖
2	B10.BR (I-J^k)	Control cells	26.5 ± 2.5 (6)§	27.6 ± 2.2 (8)§
		Ts + NMS + C	13.8 ± 1.8 (5)	15.5 ± 1.4 (4)
		Ts + anti-I-J^k + C	22.4 ± 1.8 (5)‖	25.8 ± 1.6 (5)‖
3	R106 (I-J^b)	Control cells	24.2 ± 1.3 (6)§	32.4 ± 2.7 (5)§
		Ts + NMS + C	11.0 ± 1.1 (4)	16.3 ± 2.3 (6)
		Ts + anti-I-J^k + C	8.8 ± 1.8 (5)	17.2 ± 1.6 (6)
		Ts + anti-I-J^b + C	26.8 ± 1.9 (4)‖	29.0 ± 2.3 (5)‖

* On day 0, mice were immunized with NP-*O*-Su (7 g/100 ml in DMSO) given subcutaneously on both flanks. 4×10^7 syngeneic NP-tolerized spleen cells were treated as indicated and transferred intravenously either on day 0 (induction phase) or on day 6 (effector phase) one h before NP-*O*-Su administration. Control groups received normal spleen cells. On day 6, mice were challenged in one footpad with NP-*O*-Su (0.1 g/100 ml). CS responses were measured 24 h later as the difference between the right and left footpad thickness.

‡ Responses expressed as the mean incremental footpad swelling in units of 10^{-3} cm ± 1 SE. Number of mice per group in parentheses.

§ $P < 0.007$ as compared with nonimmune mice challenged with NP-*O*-Su. Background footpad swellings measured 12.8 ± 0.9 U (5).

‖ $P < 0.01$ compared with immune mice receiving NP-tolerized spleen cells treated with NMS or medium plus C. Also, see footnote §.

Mice were immunized with NP-*O*-Su and/or DNFB, followed 6 d later by intravenous transfer of NP-tolerized spleen cells or control splenocytes immediately before challenge in the footpad with NP-*O*-Su or on the ear with DNFB. Responses were measured at 24 h as the incremental swelling between right and left (footpads or ear pinnae, respectively). The data are summarized in Table II. It is apparent that NP-tolerized spleen cells given intravenously are able to suppress NP-*O*-Su-induced, NP-*O*-Su-elicited CS responses to background levels, but have no significant effect on DNFB-primed, DNFB-elicited swelling. Further, when mice are sensitized with both NP-*O*-Su and DNFB, NP-tolerized spleen cells are still unable to suppress DNFB-specific immunity under the conditions used. Thus, NP-induced effector suppressor cells inhibit CS effector cells in an antigen-specific manner.

Genetic Restrictions of Effector-Phase Suppressor Cells. Next, we investigated the genetic restrictions on NP-induced effector-phase suppressor cell activity, using B10.BR mice as the Ts donors. The results of these studies are presented in Table III. It is clear that NP-tolerized Ts from B10.BR mice inhibit syngeneic NP-specific CS responses to within background levels. In contrast, B10.BR Ts have no discernible effect on CS reactions in B10 mice, which are congenic with B10.BR at the H-2 complex. This implies the presence of an H-2 restriction between the Ts^e donor and recipient.

Further, Ts^e from B10.BR mice are unable to suppress NP-specific CS responses in C3H mice, which share the $H\text{-}2^k$ haplotype with B10.BR but differ at background loci. In contrast, B10.BR Ts are able to inhibit NP-*O*-Su CS responses in CKB mice, which are congenic with C3H at the Igh region. These results, which implicate the involvement of allotype-linked genes in the activity of effector-phase Ts, have been confirmed in several experiments using different strain combinations. Thus, both H-2 and Igh-linked loci apparently have a role in the mechanism of action of effector-phase Ts for NP-specific CS responses.

Phenotype of Third Cell Population Involved in Suppressor Pathway. To extend our

TABLE II
*Specificity of NP-induced Suppressor Cells**

Cells transferred	Immunogen	Response‡	
		NP-*O*-Su (footpad)	DNFB (ear)
Control spleen cells	NP-*O*-Su	21.6 ± 1.1 (5)§	NT
NP-tolerized spleen cells	NP-*O*-Su	10.3 ± 1.3 (6)‖	NT
Control spleen cells	DNFB	NT¶	6.7 ± 1.4 (20)§
NP-tolerized spleen cells	DNFB	NT	7.0 ± 1.4 (20)§
Control spleen cells	NP-*O*-Su + DNFB	26.0 ± 2.8 (5)§	7.0 ± 0.8 (22)§
NP-tolerized spleen cells	NP-*O*-Su + DNFB	6.8 ± 1.5 (5)‖	5.8 ± 1.1 (22)§
None	None	7.8 ± 1.1 (5)	0.5 ± 0.2 (21)

* B10.HTT mice were sensitized either with NP-*O*-Su (7 g/100 ml DMSO) injected subcutaneously on both flanks on day 0, and/or with DNFB (0.5% in olive oil:acetone [1:4]) painted on the ventral surface on days 0 and 1. 6 d later, NP-tolerized spleen cells or control cells were transferred. 1 h after transfer, mice were challenged by the injection of one footpad with NP-*O*-Su and/or the painting of one ear with DNFB. Responses were measured at 24 h as the difference between right and left footpad or ear pinna thickness.

‡ Responses expressed as the mean incremental swelling ± 1 SE in units of 10^{-3} cm.

§ $P < 0.05$ compared with nonimmune mice challenged with the same agent.

‖ $P < 0.001$ compared with immune mice receiving control spleen cells.

¶ Not tested.

TABLE III
*Genetic Interactions of B10.BR Effector-Phase Suppressor Cells**

Recipient			Spleen cells transferred	Footpad response‡
Strain	H-2	Igh-1		
B10.BR	k	b	Control	23.8 ± 1.4 (5)§
			Ts	13.6 ± 1.4 (5)‖
B10	b	b	Control	24.6 ± 1.7 (7)§
			Ts	25.3 ± 2.4 (6)§
C3H	k	j	Control	33.3 ± 2.5 (4)§
			Ts	33.0 ± 4.8 (4)§
CKB	k	b	Control	31.0 ± 1.0 (5)§
			Ts	12.0 ± 2.9 (5)‖

* See legend to Table I for protocol.
‡ Responses expressed as the mean incremental footpad swelling in units of 10^{-3} cm ± SE. Number of mice per group in parentheses.
§ $P < 0.0001$ compared with nonimmune mice challenged with NP-*O*-Su, which gave background swellings of 10.9 ± 0.9 (17).
‖ $P < 0.0007$ compared with immune mice receiving control normal spleen cells.

TABLE IV
*Phenotype of Third Cell Involved in Suppressor Pathway**

Treatment of CS effector cells	Effector-phase suppressor cells	Treatment of Ts_3 cells	Footpad response‡	Percent suppression
NMS + C	Control cells	No cells	24.7 ± 1.4 (6)§	0
NMS + C	NP-tolerized cells	No cells	11.3 ± 1.4 (9)‖	85
Anti-I-J + C	Control cells	No cells	26.3 ± 1.5 (6)§	0
Anti-I-J + C	NP-tolerized cells	No cells	23.5 ± 2.0 (6)§	6
Anti-I-J + C	Control cells	NMS + C	8.4 ± 1.6 (5)‖	103
Anti-I-J + C	NP-tolerized cells	Anti-Thy-1 + C	22.8 ± 0.8 (4)§	20

* On day 0, donor mice were immunized subcutaneously with NP-*O*-Su. On day 6, the immune LN cells were harvested and treated with antiserum followed by complement, as indicated. 4×10^7 LN cells were mixed with 4×10^7 NP-tolerized spleen cells or control normal spleen cells, with or without additional immune LN cells. 1 h after intravenous transfer of cell mixtures into syngeneic recipients, mice were challenged in one footpad with NP-*O*-Su and responses were measured 24 h later.
‡ Responses expressed as the mean incremental footpad swelling in units of 10^{-3} cm ± SE. Number of mice per group in parentheses.
§ $P < 0.0001$ compared with control mice without cell transfer, receiving NP-*O*-Su challenge alone, which gave background swellings of 8.9 ± 1.2 (10).
‖ $P < 0.0002$ compared with mice receiving immune cells together with control spleen cells.

knowledge of suppressor cell interactions occurring at the effector phase of NP CS responses, the NP-*O*-Su immune LN cell population was treated with anti-I-J antiserum plus C before mixing and transfer, together with NP-tolerized Ts or with control normal cells into syngeneic recipients. It is apparent from the data in Table IV that NP-immune cells treated with either NMS or anti-I-J serum plus C, then transferred along with normal spleen cells, give rise to significant levels of footpad swelling. NP-tolerized cells were able to suppress almost completely the immune LN population that had been pretreated with NMS plus C, but had little, if any, suppressive effect on the same immune LN cell populations treated with anti-I-J serum plus C.

We initiated a series of reconstitution experiments to determine the phenotype of

the cell present in the immune cell population that was required for the activity of NP-effector suppressor cells. To a mixture of 4 × 10^7 anti-I-J-treated NP-immune cells and 4 × 10^7 NP-tolerized Ts were added 1 × 10^7 viable syngeneic NP-immune LN cells that had been treated beforehand with NMS plus C. As indicated in Table IV, this small number of NMS-treated immune cells was able to reconstitute the ability of Ts to inhibit the entire NP-specific CS response. The cell required to restore suppression was a T cell because treatment of the immune LN population with anti-Thy-1.2 plus C abrogated the ability to restore immune suppression. In this manner, we have established the requirement for a third I-J-bearing T cell, present in the immune cell population, for successful function of the suppressor cell circuit. This third cell population will be termed Ts_3.

Ts_3 Suppressor Cells Are Sensitive to Cyclophosphamide. To determine whether the Ts_3 suppressor cell population present in the immune animal is sensitive to cyclophosphamide, recipient mice were primed subcutaneously with NP-*O*-Su on day 0, followed 24 h later with either saline or 20 or 50 mg/kg cyclophosphamide injected intraperitoneally. On day 6, these mice received either 4 × 10^7 NP-Ts^e or control spleen cells from syngeneic donors. It is evident from the data in Table V that neither dose of cyclophosphamide significantly altered the level of the CS footpad swelling response in the groups receiving control splenocytes. However, treatment of immunized recipients with either 50 or 20 mg/kg cyclophosphamide abrogated the ability of syngeneic Ts^e to transfer suppression. Further, reconstitution of these cyclophosphamide-treated immune mice with 10^7 NP-*O*-Su-immune LN cells at the time of Ts^e transfer completely restored suppression. Finally, 10^7 LN cells from syngeneic mice treated

TABLE V

*Ts_3 Suppressor Cells Are Sensitive to Low-Dose Cyclophosphamide**

Cytoxan dose	Spleen cells transferred	Syngeneic Ts_3		Footpad response‡
		Cells added	Cyclophosphamide	
mg/kg				
None	Control	None	None	31.1 ± 1.5 (21)§
None	Ts^e	None	None	12.6 ± 0.8 (15)‖
50	Control	None	None	32.5 ± 1.6 (20)§
50	Ts^e	None	None	30.2 ± 1.4 (16)§
20	Control	None	None	30.9 ± 1.6 (7)§
20	Ts^e	None	None	29.0 ± 4.9 (3)§
50	Ts^e	10^7	None	13.0 ± 1.9 (4)‖
50	Ts^e	10^7	50 mg/kg	30.7 ± 5.2 (3)§

* Recipient mice were primed subcutaneously with NP-*O*-Su on day 0, followed 24 h later with an intraperitoneal injection of either saline or cyclophosphamide in saline at the indicated dosages. On day 6, these mice received 4 × 10^7 syngeneic NP-tolerized spleen cells or control normal spleen cells, with or without 10^7 additional LN cells from syngeneic mice primed with NP-*O*-Su on day 0 with or without 50 mg/kg cytoxan on day 1. Mice were challenged with NP-*O*-Su 1 h after transfer and responses were measured at 24 h.

‡ Responses expressed as the mean incremental footpad swelling in units of 10^{-3} cm ± 1 SE. Results from three experiments have been pooled. Number of mice per group in parentheses.

§ $P < 0.005$ compared with nonimmune mice without cell transfer, receiving NP-*O*-Su challenge alone, which gave background swellings of 9.8 ± 1.4 (16).

‖ $P < 0.002$ compared with immune mice receiving control spleen cells.

with 50 mg/kg cyclophosphamide 1 d after NP-*O*-Su priming were unable to reconstitute suppression.

Genetic Restriction of Ts^e-Ts_3 Interaction. To evaluate the genetic requirements for effective Ts^e-Ts_3 interaction, NP-immune recipient mice treated with 50 mg/kg cyclophosphamide on day 1 were subsequently given 4×10^7 syngeneic NP-Ts^e together with Ts_3 from various inbred strains of mice. As shown in Table VI, syngeneic Ts_3 from NP-*O*-Su-immune donors are able to reconstitute suppression in both B10.A(5R) and B10.BR mice. This reconstitution depends on specific antigen priming of the Ts_3 donors, because DNFB-immune LN cells are unable to restore suppression of NP-specific CS responses, as indicated for B10.BR recipients. Cyclophosphamide treatment of syngeneic Ts_3 donors abrogates the restorative capacity of NP-*O*-Su immune LN cells, as shown for 5R mice (Table VI).

The H-2 restriction between Ts^e-Ts_3 interactions was demonstrated by the inability of immune LN cells from B10.HTG mice, which are congenic with 5R recipients (differing at the H-2K, H-2I, and H-2D regions) to reconstitute suppression. This H-2 restriction was subsequently mapped to H-2I, as shown by the ability of Ts_3 from B10.MBR mice to restore suppression in B10.BR recipients. Although it is necessary for suppression, the H-2 homology requirement alone is not sufficient for effective Ts^e-Ts_3 interaction. It is clear that C3H Ts_3 (H-2^k, Igh-1^j) are unable to restore

TABLE VI
*Genetic Restriction of Ts^e-Ts_3 Interaction**

NP-*O*-Su-primed recipient	Syngeneic Ts^e	Source of Ts_3	Ts^e-Ts_3 homology		Percent suppression‡
			H-2	Igh	
5R	−	—	—	−	0
	+	—	—	−	25
	+	5R	K, I, S, D	+	95§
	+	5R (CY)‖	K, I, S, D	+	5
	+	B10.HTG	I-C, S	+	5
	+	B10.HTG (CY)‖	I-C, S	+	15
B10.BR	−	—	—	−	0
	+	—	—	−	19
	+	B10.BR	K, I, S, D	+	96§
	+	B10.BR (DNFB)¶	K, I, S, D	+	15
	+	B10.MBR	I, S	+	100§
	+	CKB	K, I, S, D	+	100§
	+	C3H	K, I, S, D	None	31
	+	C57BL/6	None	+	27
	+	(B6 × C3H)F_1	K, I, S, D	+	104§

* Recipient mice were primed subcutaneously with NP-*O*-Su on day 0, followed 24 h later with 50 mg/kg cyclophosphamide injected intraperitoneally. On day 6, these mice received 4×10^7 syngeneic NP-tolerized spleen cells (Ts^e) or control spleen cells, with or without 10^7 additional LN cells (Ts_3) from the donor strains indicated. These Ts_3 donors were immunized with NP-*O*-Su on day 0, except for two groups (see footnote ‡). Recipient mice were challenged with NP-*O*-Su 1 h after transfer, and responses were measured at 24 h.

‡ Percent suppression was calculated as in Materials and Methods. Actual footpad swelling responses, in units of 10^{-3} cm ± 1 SE (with 3–7 mice per group), were 32 ± 2 (5R) and 38 ± 2 (B10.BR). Nonimmune mice receiving NP-*O*-Su challenge alone gave background swelling of 12 ± 1 (5R) and 7 ± 3 (B10.BR).

§ $P < 0.0002$ compared with immune mice receiving control cells.

‖ Ts_3 donors received 50 mg/kg cyclophosphamide 24 h after NP-*O*-Su immunization.

¶ Ts_3 donor immunized with DNFB skin painting on day 0 and day 1 (instead of NP-*O*-Su injection).

suppression in B10.BR recipients (H-2^k, Igh-1^b). However, NP-immune LN cells from CKB mice (H-2^k, Igh-1^b), which are congenic with C3H at the Igh region, are fully able to restore suppression in B10.BR mice. Thus, the Ts^e-Ts_3 interaction is governed by genes closely linked to both the Igh and the H-2 gene complexes. The dual genetic requirement is further supported by the ability of Ts_3 from (C57BL/6 × C3H)F_1 mice to restore suppression to B10.BR recipients, although neither parental strain possesses this capability, due to lack of either H-2 (i.e., C57BL/6) or Igh (i.e., C3H) homology. In conclusion, the Ts^e-Ts_3 interaction depends on specific antigen priming of Ts_3 and is restricted by both H-2I and Igh complex-linked genes.

Discussion

Intravenous administration of hapten-coupled syngeneic cells induces tolerance in a variety of experimental systems (1, 8–12). In these models, tolerance is mediated by suppressor T lymphocytes. Similarly, in the present experiments, spleen cells from mice that had received NP-coupled syngeneic cells inhibited NP CS responses. As was previously demonstrated for NP-specific DTH responses (1, 3), the Ts can function at either the induction or effector phases of the CS response. The cells that transfer suppression are T lympocytes, because anti-Thy-1.2 antiserum plus C abrogated the inhibitory capacity of the suppressor cells in both the induction and effector phases (Table I). In addition, both populations bear I-J determinants, although we do not know whether these cells bear identical or distinct I-J determinants. Preliminary Lyt phenotyping indicated that at least the Ts^e population carries the Lyt-2 marker. Weinberger et al. (2) have demonstrated that the suppressor cells that function during the induction phase can be distinguished from effector-phase suppressors by their ability to bind antigen or idiotype.

Ts inhibit only NP-directed CS responses, and have no discernible effect on contact sensitivity to DNFB (Table II). Specificity was observed even when mice had been sensitized with both DNFB and NP-*O*-Su, and were challenged with both DNFB and NP-*O*-Su in the presence of NP-induced Ts. In the latter case, DNFB contact sensitivity responses were not inhibited, whereas NP CS reactions in the same mice were significantly depressed. Thus, Ts are triggered to exert suppression on NP–CS-reactive cells in an antigen-specific manner. These data do not rule out the possibility that Ts may act by elaborating nonspecific suppressor factors, which are known to operate both in vivo and in vitro (12–15), but which may only function across short distances and consequently would be unable to suppress immune responses at sites distant from the area of specific antigen challenge.

The strain specificity of the effector Ts was then investigated. It is apparent from the results presented in Table III that a dual requirement exists for both H-2 and Igh compatibility between Ts^e donor and antigen-primed recipient for successful transfer of effector-phase suppression. This conclusion is compatible with findings in other experimental models (2, 8, 15–17), and is important to our understanding of the cellular interactions involved, as will be described in more detail below.

A previous report (9) suggested the participation of an additional T cell in DNFB contact sensitivity suppressor circuits. Originally termed T_{aux}, this cell was detected in the lymphoid cells of the antigen-primed recipient. Thus, we sought to determine whether an analogous cell is required for the function of NP-specific suppressors for NP CS responses. As the results in Table IV indicate, the third cell population

involved in this suppressor network is an I-J-bearing T cell. Again, we have no evidence to suggest that this I-J is either identical to or distinct from I-J determinants on Ts^i and Ts^e populations, nor is it clear whether the I-J determinants are functionally involved in the suppressive mechanism. We have demonstrated that the Ts^e interaction is restricted by genes in the H-2I region (Table VI), which suggests a possible role for matching of structures encoded within the I subregions. In other models, suppressor cells have been found bearing I-J determinants (10, 18), as well as elaborating soluble suppressor factors bearing I-J determinants (8, 13, 15), which have been shown to mediate interactions between T cell subpopulations. Moreover, such suppressor cell interactions have been demonstrated to be I-J or I-C restricted in different systems (13, 14). Although we have not shown the operation of such factors in the NP CS model, this remains a viable explanation for the requirement for H-2I compatibility between the I-J-bearing Ts^e and Ts_3 suppressor cell populations. Moreover, our experiments clearly indicate that the Ts_3 population is sensitive to low doses of cyclophosphamide (Table V). This result appears to contrast with the data of Sy et al. (9), who claimed that the T_{aux} population is only sensitive to higher doses of cyclophosphamide. However, in that model, cyclophosphamide was given 2–3 d before immunization, whereas we injected cyclophosphamide 1 d after NP-*O*-Su primings. Considering the short half-life of cyclophosphamide (19), it is likely that immune cell populations in both models are exposed to the same range of drug concentrations during the maturation period after sensitization. Hence, we are probably describing a similar auxiliary suppressor population. More recently, transfer of fowl γ-globulin-specific suppression also has been shown to depend on low-dose cyclophosphamide-sensitive cells in immunized recipient mice (10), which suggests evidence for the involvement of an auxiliary suppressor population in yet another model.

Thus, at least in the NP system, the Ts^i and Ts_3 populations appear phenotypically similar in that both bear I-J determinants and their generation is sensitive to low doses of cyclophosphamide (3). In addition, the Igh restriction noted between the Ts^e and Ts_3 populations suggests that the Ts_3 population may bear idiotypic determinants similar, if not identical, to those previously demonstrated on the Ts^i population (1). If this is indeed the case, our findings support many elements of the network theory of immunoregulation (20). Experiments are in progress to further characterize these cell populations.

The present study unifies and extends the results of many previous investigations into mechanisms of immunosuppression. Various studies have shown that at least two T cell subpopulations must interact to generate antigen-specific suppression (13, 17, 21–23). Tada (13) reported the requirement of an Lyt-$1^+2^+3^+$, I-J^+, factor acceptor cell for suppression of T helper cells, implying the existence of T-T interactions between a suppressor-factor-producing T cell and a separate suppressor T cell from antigen-primed mice. This requirement for an antigen-primed I-J-bearing acceptor cell is consistent with evidence reported here and elsewhere (9, 10) for specific antigen-driven Ts^e-Ts_3 interactions. The Igh gene restriction demonstrated in several suppressor cell systems (2, 8, 17) is probably a consequence of idiotype-anti-idiotype interactions, as was previously detailed for suppression of NP-specific DTH response (3) and of NP-specific plaque-forming cell responses (24). Further, the Igh gene restriction

between idiotype-specific Ts^e and the immune lymphocyte population suggests that the Ts_3 population probably bears idiotypic determinants.

Therefore, based on cumulative findings in both the NP DTH and NP CS systems, we now propose a unifying hypothesis for a mechanism of immunosuppression after antigen exposure, as schematically depicted in Fig. 1. Antigen-coupled spleen cells trigger the proliferation of a cyclophosphamide-sensitivity, I-J-bearing, induction-phase suppressor T cell, termed Ts^i or Ts_1 in other systems, which has been shown to carry an idiotypic, antigen-binding receptor (13). Together with antigen, the Ts^i population induces the generation of I-J^+ effector-phase suppressors (Ts^e or Ts_2), which carry the corresponding anti-idiotypic receptor (3). Although the Ts^e population is active during the effector phase of the CS response, it is not the final effector cell.

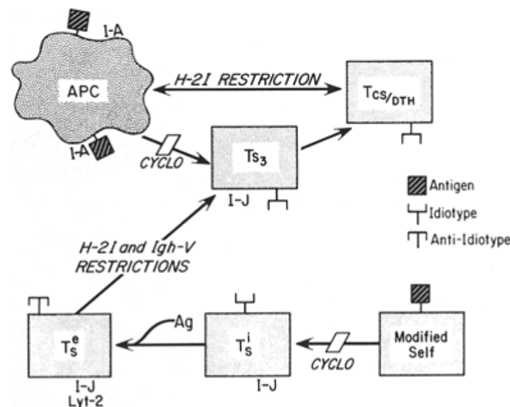

FIG. 1. Immunoregulation of NP-induced T cell responses. A schematic representation of the cell populations involved in the suppressor pathway. See text for details.

Thus, Ts^e alone are insufficient to directly suppress CS effector cells, but must interact with a third I-J^+ suppressor, termed Ts_3, present in the antigen-primed lymphocyte population. The Ts_3 population also functions in the effector phase of the immune response and may represent the actual effector cell. The Ts^e-Ts_3 interaction is restricted by both H-2 and Igh-region genes. These interactions could occur either through direct cell-cell contact or, more consistent with the findings in other systems, via a soluble suppressor factor (15). The dual genetic restriction may be the result of combined presentation of anti-idiotype along with Ia determinants on Ts^e to Ts_3. Alternatively, Ts^e (or its factor) may combine with two distinct Ts_3 populations, one via an Ia interaction, and the other via an idiotypic-anti-idiotypic interaction.

If a Ts_3 cell bears idiotypic receptors, it could form an antigen bridge with the CS or DTH effector T cells, or with helper T cells for antibody production, and transmit a suppressive signal. It is also possible that macrophages release suppressor factors after involvement in the Ts^e-Ts_3 interaction (25). However, we cannot rule out the possibility of more complex interactions involving multiple types of Ts_3 subsets, some idiotypic, others anti-idiotypic, which may be necessary to account for findings of idiotype-specific suppression in some other experimental systems (24, 26, 27).

Summary

In the current study, we examine the mechanism of suppression of cutaneous sensitivity (CS) responses to 4-hydroxy-3-nitrophenyl acetyl succinimide ester. Intra-

venous administration of haptenated syngeneic spleen cells induces a state of hapten-specific tolerance involving I-J-bearing suppressor T cells that function at either the induction phase or the effector phase of the CS response. The effector-phase suppressor cells (Ts^e) are genetically restricted by both Igh and H-2 region genes. However, a third cell population is also required in the immune lymphocyte population for immune suppression. This third cell population, termed Ts_3, is an I-J^+, cyclophosphamide-sensitive T cell, as shown by reconstitution experiments. Further, the Ts^e-Ts_3 interaction is restricted by genes in the H-2 and Igh gene complexes. The results are discussed with respect to the pathway of cellular interactions leading to immunosuppression.

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