

HAPTEN-SPECIFIC RESPONSES TO THE PHENYLTRIMETHYLAMINO HAPTEN

III. Mice Whose Delayed-type Hypersensitivity Responses Cannot Be Abrogated by the Presence of Anti-Idiotypic Suppressor T Cells Lack a Critical Modulatory T Cell Function*

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The immune system normally appears to exist in a stable, steady state. As proposed by Jerne (1), the stability of the immune system is in part the result of a network of interactions involving idiotypes (Id)¹ and anti-idiotypes (anti-Id). Several systems have described a critical requirement of Id recognition in the induction of antibody synthesis (2-6). Evidence has also accumulated in recent years (7, 8) indicating that Id-related mechanisms play a role in pathways among interacting T cells that eventually suppress hapten-specific delayed-type hypersensitivity (DTH). The down-regulation of the immune response by suppressor T cells (T_s) is complex and not fully understood. Recent reports describing the heretofore unrecognized immunoregulatory functions of T cells such as "contrasuppressors" (9) and "abrosuppressors" (10) add still more to the complexity involved in the biological function, immunoregulation.

In an effort to understand the role of the idiotypic network in the regulation of the immune response to a small synthetic antigen L-tyrosine-*p*-azophenyltrimethylammonium [tyr(TMA)], we have been studying the regulation of antibody and DTH responses directed to the TMA hapten. In the course of the study, we found that a single injection of tyr(TMA) in Freund's complete adjuvant (FCA) induced T_s that shut down only the cross-reactive Id⁺ component of the anti-TMA antibody response (11). Recently (12), we have characterized the tyr(TMA)-induced T_s and directly demonstrated the idio-type specificity of these cells by absorption procedures. Further, we demonstrated that these anti-idiotypic second-order T_s (T_{s2}) cells (8) can also shut down DTH reactions (12) in addition to Id⁺ antibody formation (11). The regulatory function of these cells on TMA-specific DTH responses was shown by adoptive transfer into naïve recipients. However, when the anti-idiotypic T_s-bearing mice were themselves immunized and tested for TMA specific DTH, they unexpectedly exhibited normal responses. Thus, although the anti-idiotypic T_s could readily suppress DTH upon transfer to normal recipients, these same cells could not function intrinsically.

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¹ *Abbreviations used in this paper:* BSS, balanced salt solution; C, complement; CY, cyclophosphamide; DTH, delayed-type hypersensitivity; FCA, Freund's complete adjuvant; IBC, idio-type-binding capacity; Id, Idiotypes; ILNC, immune lymph node cells; T_{DTH}, T cells that mediate DTH; TMA, trimethylaminoaniline; TMA-SC, TMA-coupled spleen cells; T_s, T suppressor cells; T_{s1}, first-order T_s; T_{s2}, second-order T_s; T_{s3}, third-order T_s; tyr(TMA), L-tyrosine-*p*-azophenyltrimethylammonium.

This anomaly led us to attempt to analyze a probable lesion in the suppressor pathway. The results reported here demonstrate the apparent loss of function of an idiotypic T_s essential for the anti-idiotypic T_s -mediated DTH suppression. This apparent loss of function of the idiotypic T_s in anti-idiotypic T_{s2} -bearing mice is discussed as a modulatory cell type critical for fine-tuning in immunoregulation.

Materials and Methods

Mice. Male A/J (H-2^a, Ig-1^e) mice were obtained from The Jackson Laboratory, Bar Harbor, ME and were 6–8 wk of age when used in these experiments.

Antigen Preparations. A 110-mM solution of trimethylaminoaniline (TMA) (Bachem Inc., Torrance, CA) diazonium salt was prepared as described earlier (13). The procedure for obtaining syngeneic spleen cells free of erythrocytes, and coupling with diazonium salt of TMA has already been described (14). In brief, syngeneic spleen cells devoid of erythrocytes were coupled with the diazonium salt of TMA at a final concentration of 10 mM at pH 8.2.

Immunization and Challenge. For the induction of DTH reactivity to TMA, routinely 3×10^7 freshly coupled syngeneic spleen cells (TMA-SC) were injected subcutaneously into separate sites on the dorsal flanks of the animal. In some experiments, anti-idiotypic antisera raised in rabbits were used for the induction of TMA-specific DTH. The induction, isolation, and purification of anti-Id and the determination of idio-type-binding capacity (IBC) of anti-Id have already been described (11, 13). 1.0 μ g IBC equivalent of anti-idiotypic antisera in 0.2 ml phosphate-buffered saline was injected subcutaneously to induce DTH (14). 5 d after immunization by either method, mice were challenged with 25 μ l of 10 mM diazonium salt in H₂O into the right footpad. The contralateral footpad was left uninjected and served as the control. The footpad thickness was measured with a Mitutoyo micrometer (Schlesingers for Tools Ltd., Brooklyn, NY) 24 h after the challenge. The magnitude of DTH reaction was expressed as the increment of footpad swelling between the challenged and unchallenged control footpads. Nonimmunized mice challenged similarly served as negative controls. In some experiments, for suppression of DTH, 20 μ g IBC equivalent of anti-Id was injected intravenously through a lateral tail vein 1 h before challenge as described previously (14).

Cyclophosphamide (CY) Treatment. Mice were treated intraperitoneally with (CY) (cytoxan; Mead Johnson & Co., Evansville, IN) reconstituted in sterile distilled H₂O and diluted in physiological saline. CY was administered 2 d before immunization if 200 mg CY/kg body weight was used, or 1 d after immunization if 20 mg CY/kg was used.

Induction of Suppressor Cells. Naïve A/J mice were inoculated intraperitoneally with 0.1 ml containing 100 μ g of tyr(TMA) and FCA (containing H37Ra, Difco Laboratories, Detroit, MI). Spleens were obtained from tyr(TMA)-inoculated mice 6–7 wk later, and a single cell suspension was prepared in sterile balanced salt solution (BSS). Viability was determined using trypan blue dye exclusion method. To adoptively transfer suppression, 6×10^7 viable splenic cells were inoculated intravenously through a lateral tail vein. To study the intrinsic unresponsiveness, tyr(TMA)-immunized mice were injected subcutaneously with TMA-SC or anti-Id, usually 6–7 wk after tyr(TMA) inoculation, except when specifically mentioned, and challenged in the footpad 5 d later.

Adoptive Transfer and Treatment of Lymph Node Cells. Mice were immunized with TMA-SC, and 5 d later the animals were killed. The superficial cervical, axillary, brachial, superficial inguinal, and mesenteric lymph nodes were removed, and a single-cell suspension was prepared using a fine mesh screen. After washing, viability was determined and desired number of cells were resuspended in BSS. Before adoptive transfer, the lymph node cells were treated with several reagents, washed extensively in BSS, and resuspended in BSS at a desired density.

The cells were suspended at 1×10^7 cells/ml concentration in BSS for treatment with several reagents. Anti-Thy 1 monoclonal antibody secreted by the hybridoma T24/40.7 (kindly provided by Dr. J. Kappler and Dr. P. Marrack, The National Jewish Hospital, Denver) was used at 1:30 dilution. Anti-Lyt.2 monoclonal antibody secreted by the hybridoma 2.43.1 and anti-Lyt.1 monoclonal antibody secreted by the hybridoma 78.10.1 were used at 1:50 dilution. Both hybridomas were obtained from Dr. Barbara Araneo (The Jewish Hospital, St. Louis) and were originally produced in Dr. Frank Fitch's laboratory, University of Chicago. Cells

were incubated with antisera for 1 h on ice, spun, and resuspended at 3.3×10^7 /ml concentration and incubated at 37°C for 30 min with low toxic rabbit complement (C) at 1:30 dilution. After incubation cells were extensively washed and used for transfer. Treatment of lymph node cells with anti-Id antisera and C was performed according to the method described by Sy et al. (15). Briefly, lymph node cells were suspended at 10^7 cells/ml in BSS and incubated on ice for 1 h with 25 µg IBC equivalent of anti-Id per every 10^8 cells. After incubation with anti-Id sera, cells were spun and resuspended in rabbit C (1:30) and incubated at 37°C for 30 min. All the lymph node cell preparations were injected intravenously through the lateral tail vein into appropriate recipients. The mice were challenged in the footpad within 1 h of cell transfer.

Statistical Analysis. All data were analyzed for significance by using a two-tailed Student's *t* test. Percent suppression was calculated using the formula: percent suppression = $\frac{[\text{positive control} - \text{experimental group}]}{[\text{positive control} - \text{negative control}]} \times 100$. More than 50% suppression was always highly significant.

Results

Transferable Suppression and Intrinsic Unresponsiveness. Mice were injected intraperitoneally with tyr(TMA) in FCA. These will be referred to as tyr(TMA)-immune mice. Spleen cells obtained from these mice 6 wk after tyr(TMA) injection when transferred intravenously into normal mice suppressed the DTH responses induced with TMA-SC (12). In conformity with this, we observed that tyr(TMA)-immune spleen cells suppressed the DTH responses of normal mice immunized either with TMA-coupled spleen cells (TMA-SC) or anti-Id antibodies (Table I) at the effector phase. However, when tyr(TMA)-immune mice were immunized with TMA-SC or anti-Id antibodies, no suppression was observed. This is referred to as the lack of intrinsic unresponsiveness. Thus, the tyr(TMA) inoculation of A/J mice led to the development of anti-

TABLE I
*Lack of Intrinsic Unresponsiveness in T_{s2} -bearing Mice**

Group	Treatment	Immuni- zation	Chal- lenge	Response	Percent suppres- sion
<i>mm ± 1 SEM</i>					
Intrinsic unresponsiveness					
1	—	TMA-SC	+	1.12 ± 0.10 (5)	—
2	tyr(TMA)	TMA-SC	+	1.45 ± 0.15 (4)	-46
3	—	Anti-Id	+	0.90 ± 0.10 (7)	—
4	tyr(TMA)	Anti-Id	+	0.84 ± 0.06 (7)	12
5	tyr(TMA)	—	+	0.53 ± 0.13 (4)	—
6	—	—	+	0.40 ± 0.04 (4)	—
Transferable suppression					
7	—	TMA-SC	+	0.78 ± 0.05 (4)	—
8	splenic suppressor cells	TMA-SC	+	0.43 ± 0.10 (4)	79
9	—	Anti-Id	+	0.83 ± 0.08 (11)	—
10	splenic suppressor cells	Anti-Id	+	0.56 ± 0.03 (8)	63
11	—	—	+	0.40 ± 0.03 (5)	—

* A/J mice were immunized with either 3×10^7 TMA-SC or 1.0 µg IBC equivalent of anti-Id and 5 d later challenged in the footpad with activated diazonium salt of TMA. Footpad swelling was measured 24 h later. Number of mice tested is given in parentheses. Percent suppression was calculated as described in Materials and Methods. In the experiment to study the intrinsic unresponsiveness, mice were inoculated intraperitoneally with 100 µg of tyr(TMA) + FCA 6 wk before immunization. The ability of suppressor cells generated by tyr(TMA) + FCA injection 6 wk before, was tested by transferring 6×10^7 splenic suppressor cells intravenously through a lateral tail vein into normal mice immunized with appropriate immunogens 24 h before challenge.

TABLE II
Kinetics of Intrinsic Unresponsiveness*

Period after tyr(TMA) inoculation	Response	Percent suppression
	<i>mm ± 1 SEM</i>	
Experiment A		
Positive controls	0.90 ± 0.04 (4)	—
1 wk	0.84 ± 0.08 (5)	10
2 wk	0.50 ± 0.06 (3)	64‡
3 wk	0.78 ± 0.05 (4)	19
4 wk	0.75 ± 0.06 (4)	23
6 wk	0.98 ± 0.12 (4)	-13
Negative controls	0.27 ± 0.03 (3)	—
Experiment B		
Positive controls	1.40 ± 0.10 (5)	—
2 wk	0.72 ± 0.10 (5)	68‡
8 wk	1.10 ± 0.10 (3)	30
9 wk	1.13 ± 0.06 (4)	27
10 wk	1.30 ± 0.10 (4)	10
Negative controls	0.40 ± 0.05 (5)	—

* A/J mice were immunized with 3×10^7 TMA-SC at different time intervals after 100 μ g of tyr(TMA) + FCA inoculation and challenged in the footpad 5 d later. Footpad swelling was measured 24 h after challenge. Number of mice tested is given in parentheses. Positive controls indicate the response of normal A/J mice that received no tyr(TMA) + FCA. Naïve mice not immunized with TMA-SC but challenged in the footpad served as the negative controls.

‡ Significantly different from positive controls; $P < 0.01$.

idiotypic T_{s2} (12), which could transfer suppression to normal mice without obviously impairing the ability of tyr(TMA)-immune mice to mount a normal DTH response when immunized with TMA-SC or anti-Id antibodies. Since no intrinsic suppression was noted at the 6-wk period, we next determined if tyr(TMA) inoculation led to intrinsic unresponsiveness at some earlier or later time point. Accordingly, mice were inoculated with tyr(TMA) in FCA and each week thereafter immunized with TMA-SC and assayed for DTH reactivity 5 d later. As seen in Table II, no significant suppression was seen at 3 wk and thereafter up to 10 wk after tyr(TMA) inoculation. The lack of suppression at the 1-wk period was not surprising, as we have previously shown (16) that this population does not contain suppressor activity, but in fact contains T cell mediated helper activity for anti-TMA antibody production. Clear-cut suppression was noted only at the 2-wk period, but this suppression is clearly mediated by idiotypic T_{s1} with different functional and phenotypic characteristics from that of anti-idiotypic T_{s2} induced 6 wk after tyr(TMA) injection (manuscript in preparation).

In summary, the data presented indicate that tyr(TMA) inoculation first leads to the appearance of idiotypic T_{s1} which can function intrinsically. At later time points (6 wk) anti-idiotypic T_{s2} emerge (12), which can only be detected by adoptive transfer experiments (see Table I). The failure of these T_{s2} to function intrinsically led us into a series of experiments to understand this apparent anomaly.

Lack of Intrinsic Unresponsiveness Is Not Caused by Compensatory Increases of Id^- T cells that Mediate DTH (T_{DTH}). Previous (14) as well as data presented herein indicate that

the T_{DTH} population bear surface Id^+ receptors. Given these facts, it was possible that the anti-idiotypic T_{s2} were suppressing Id^+ T_{DTH} , which then resulted in the expression of a compensating Id^- T_{DTH} population after TMA-SC inoculation. If this were the case, then inoculation of the anti-Id just before challenge should not block the DTH response in the tyr(TMA)-primed group. As shown in Table III, the DTH responses in the normal as well as the tyr(TMA)-immune mice were blocked equally well, suggesting an intact Id^+ T_{DTH} component. In addition, the blocking of DTH by anti-Id occurred even when mice were pretreated with high or low doses of CY. This suggests that the anti-Id-mediated suppression is not the result of the activation of CY-sensitive suppressor cells by the anti-Id, but rather a blockade or elimination of Id^+ T_{DTH} . Thus, it appears that the Id^+ component of the T_{DTH} is not remarkably influenced by the coexistence of anti-idiotypic T_{s2} in tyr(TMA)-immune mice.

Requirement of a CY-sensitive Cell for Anti-Idiotypic T_{s2} -mediated Suppression. Because anti-idiotypic T_{s2} function could only be demonstrated by transfer into naïve recipients, we reasoned that the recipients were supplying some needed cell type for suppressor function. Reports have recently emerged (17, 18) describing a CY-sensitive T cell that is necessary in some systems for effector-phase T_s function and is activated upon antigen priming. To test whether this described cell was operating in our system, we treated naïve mice with high or low doses of CY either before or after immunization with TMA-SC. 24 h before antigen challenge, spleen cells from 6-wk tyr(TMA)-immune mice were transferred into both the CY-treated and control mice. As seen in

TABLE III
Lack of Intrinsic Unresponsiveness Is Not caused by Compensating $CRI^- T_{DTH}^$*

Immunization	CY treatment	Anti-Id inoculation	Response	Percent suppression
	mg		$mm \pm 1 SEM$	
Normal mice				
TMA-SC	—	—	1.23 ± 0.05 (8)	—
TMA-SC	200	—	1.03 ± 0.08 (4)	22
TMA-SC	20	—	1.10 ± 0.13 (4)	14
TMA-SC	—	+	0.64 ± 0.06 (9)	66
TMA-SC	200	+	0.50 ± 0.05 (5)	81
TMA-SC	20	+	0.48 ± 0.13 (4)	83
tyr(TMA) immune mice				
TMA-SC	—	—	1.36 ± 0.07 (10)	—
TMA-SC	200	—	1.03 ± 0.03 (3)	32
TMA-SC	20	—	1.42 ± 0.12 (6)	-6
TMA-SC	—	+	0.74 ± 0.12 (8)	60
TMA-SC	200	+	0.52 ± 0.06 (5)	82
TMA-SC	20	+	0.72 ± 0.06 (5)	62
Negative Controls	—	—	0.33 ± 0.04 (11)	—

* Both normal A/J mice and mice inoculated intraperitoneally with tyr(TMA) + FCA 6 wk before were immunized with 3×10^7 TMA-SC and challenged in the footpad with the activated diazonium salt of TMA 5 d later. Response was measured 24 h after challenge, and the number of mice tested is given in parentheses. 200 mg CY was injected intraperitoneally 2 d before immunization with TMA-SC, and 20 mg CY was injected 1 d after immunization with TMA-SC. Mice were injected intravenously with 20 μ g IBC of anti-Id just before challenge. Negative controls indicate naïve mice challenged only.

Table IV, pretreatment of the recipients with either low or high doses of CY did not alter the DTH reaction induced by TMA-SC inoculation. However, these doses of CY markedly altered the ability of the transferred suppressor population to function when compared with the non-CY-treated control group (Table IV). Thus, the data suggest that the anti-idiotypic T_{s2} require a CY-sensitive cell, which is activated by antigen priming, to eventually suppress TMA-specific DTH.

tyr(TMA)-immune Mice Lack a Critical Cell Type Involved in T_{s2} -mediated Suppression. As previously pointed out, the CY-sensitive cell necessary for T_{s2} -mediated DTH suppression can be activated by priming normal mice with TMA-SC. Based on this fact, we tested whether this procedure could activate the necessary cell type in tyr(TMA)-immune mice. To assay for this activity, both control and tyr(TMA)-immune mice were primed with TMA-SC, and 5 d later their lymph nodes removed and the immune lymph node cells (ILNC) were transferred into naïve recipients with or without splenic T_s from 6-wk tyr(TMA)-immune mice. As seen in Table V, ILNC from normal mice (N-ILNC) transfer DTH but are suppressed by the addition of the splenic T_s population. No such suppression of DTH was observed when ILNC originated from the tyr(TMA)-immune mice. To test whether ILNC from tyr(TMA)-immune mice can be suppressed, ILNC from both sources were mixed and transferred along with the splenic suppressor cells into primed recipients. As seen in Table V, suppression of DTH was evident, indicating that the ILNC from the tyr(TMA) mice are indeed subject to suppression when mixed with N-ILNC.

Phenotypic Characteristics of the Modulatory T Cell. In this next series of experiments, we asked if the 6-wk tyr(TMA)-immune mice could be intrinsically suppressed if we

TABLE IV
Requirement of a CY-sensitive Cell Type Necessary for T_{s2} -mediated Suppression*

Immunization	CY treatment	Suppressor cells	Response	Percent suppression
	mg		mm + 1 SEM	
Experiment A				
TMA-SC	—	—	1.13 ± 0.07 (3)	—
TMA-SC	200	—	1.03 ± 0.08 (4)	14
TMA-SC	—	+	0.73 ± 0.09 (4)	55‡
TMA-SC	200	+	1.08 ± 0.06 (5)	7
Negative Controls	—	—	0.40 ± 0.12 (3)	—
Experiment B				
TMA-SC	—	—	0.80 ± 0.06 (3)	—
TMA-SC	20	—	0.75 ± 0.03 (4)	11
TMA-SC	—	+	0.40 ± 0.04 (4)	85
TMA-SC	20	+	0.78 ± 0.03 (6)	4
Negative Controls	—	—	0.30 ± 0.03 (3)	—

* Normal A/J mice were immunized and challenged as described before. 200 mg CY was injected 2 d before immunization, and 20 mg CY was injected 1 d after immunization. 6×10^7 splenic suppressor cells were inoculated intravenously through a lateral tail vein 24 h before challenge. Suppressor cells were obtained from mice inoculated intraperitoneally with 100 μ g of tyr(TMA) + FCA 6 wk before. Number of mice tested is given in parentheses. Negative controls indicate naïve mice challenged only.

‡ Significantly different from positive controls; $P < 0.02$.

TABLE V
*tyr(TMA)-immune Mice Lack a Critical Cell Type Needed for Suppression**

Cells transferred	Suppressor cells	Response	Percent suppression
		<i>mm ± 1 SEM</i>	
N-ILNC	-	0.76 ± 0.07 (5)	—
N-ILNC	+	0.40 ± 0.04 (4)	72
tyr-ILNC	-	0.90 ± 0.09 (5)	—
tyr-ILNC	+	0.79 ± 0.06 (7)	17
N-ILNC + tyr-ILNC	-	0.88 ± 0.04 (6)	—
N-ILNC + tyr-ILNC	+	0.46 ± 0.07 (9)	68
Negative controls	-	0.26 ± 0.02 (7)	—

* Both normal (N) and tyr(TMA) + FCA-injected (6 wk before) A/J mice were immunized with 3×10^7 TMA-SC, and 5 d later their immune lymph nodes were removed. 3×10^7 viable lymph nodes were transferred intravenously into naïve recipients with or without 6×10^7 splenic suppressor cells derived from A/J mice injected with tyr(TMA) + FCA 6 wk before. In some experiments, 1×10^7 N-ILNC and 2×10^7 tyr-ILNC were mixed immediately before transfer with or without additional splenic suppressor cells. All mice were challenged in the footpads within 1 h of cell transfer. Negative controls indicate the naïve mice challenged only. Number of mice tested is given in parentheses.

introduced ILNC from normal mice immunized with TMA-SC (N-ILNC) before challenge. Normal A/J mice were immunized with TMA-SC, and 5 d later ILNC were harvested and transferred into tyr(TMA)-immune mice, which had been previously primed with TMA-SC. 1 h after cell transfer, the recipients were challenged with the diazonium salt of TMA, and the footpad swelling was recorded 24 h later. As seen in Table VI, 6-wk tyr(TMA)-immune mice, when immunized with TMA-SC, give a normal DTH response. If N-ILNC are transferred just before challenge, the DTH (group 2) response is suppressed by 70%. To characterize the cells that convert the tyr(TMA) mice to intrinsic suppressors, the N-ILNC were pretreated with various antibody reagents plus C before transfer. These reagents were directed to the Thy-1, Lyt-2, and Lyt-1 antigens as well as the cross-reactive idiotypes associated with anti-TMA antibodies. As seen in Table VI, all of the antibody reagents, with the exception of the anti Lyt-1 (group 6), abolished the ability of the cells to mediate suppression. Furthermore, we show that transfer of tyr(TMA) (group 7) ILNC into the tyr(TMA) mice does not convert these mice, confirming our similar previous finding that tyr(TMA)-ILNC do not have modulatory cell activity when transferred into naïve animals (see Table V). To control for any specific or nonspecific suppressor element in the transfer system, either N-ILNC or ILNC from 6-wk tyr(TMA)-immune mice were transferred into normal mice immunized 5 d previously with TMA-SC. Transfer of these populations into normal mice (groups 17, 18) had no effect on the DTH reaction. Therefore, it would appear that the modulatory cell necessary for the function of T_{s2} is itself a T cell, bearing the Lyt-2 antigen as well as surface idiotypes.

Because the ILNC transfer DTH as well as modulatory cell activity, we wanted to determine if the cells mediating DTH could be phenotypically distinguished from the

TABLE VI
*Phenotype of the Modulatory Cells and T_{DTH}**

Group	Recipients	Cells transferred	Response	Percent suppression
			<i>mm ± 1 SEM</i>	
1	tyr(TMA)-immune mice	None	1.02 ± 0.03 (37)	—
2	tyr(TMA)-immune mice	N-ILNC + C	0.55 ± 0.03 (18)	70
3	tyr(TMA)-immune mice	N-ILNC + anti-Thy-1 + C	0.88 ± 0.09 (6)	21
4	tyr(TMA)-immune mice	N-ILNC + anti-Id + C	0.85 ± 0.03 (10)	25
5	tyr(TMA)-immune mice	N-ILNC + anti-Lyt-2 + C	0.82 ± 0.05 (6)	30
6	tyr(TMA)-immune mice	N-ILNC + anti-Lyt-1 + C	0.64 ± 0.02 (5)	57
7	tyr(TMA)-immune mice	tyr-ILNC + C	0.96 ± 0.09 (5)	9
8	Naive mice	None	0.35 ± 0.01 (30)	—
9	Naive mice	N-ILNC + C	0.72 ± 0.04 (20)	—
10	Naive mice	N-ILNC + anti-Thy-1 + C	0.26 ± 0.03 (7)	124
11	Naive mice	N-ILNC + anti-Id + C	0.43 ± 0.07 (8)	78
12	Naive mice	N-ILNC + anti-Lyt-2 + C	0.62 ± 0.03 (6)	27
13	Naive mice	N-ILNC + anti-Lyt-1 + C	0.28 ± 0.03 (6)	119
14	Naive mice	tyr-ILNC + C	0.91 ± 0.06 (9)	—
15	Naive mice	tyr-ILNC + anti-Id + C	0.54 ± 0.05 (8)	66
16	Normal mice	None	0.87 ± 0.07 (6)	—
17	Normal mice	N-ILNC + C	0.95 ± 0.03 (4)	-15
18	Normal mice	tyr-ILNC + C	0.78 ± 0.03 (4)	17

* tyr(TMA) + FCA injected (6 wk before) and untreated normal A/J mice were immunized with 3×10^7 TMA-SC, and 5 d later lymph nodes were removed from some of them. The remaining mice served as the recipients of lymph node cells. In addition, nonimmune, naïve A/J mice were used as recipients to monitor for the ability of treated lymph node cells to transfer DTH (line 9-15). The lymph node cells were treated with several reagents as indicated, washed extensively, and transferred intravenously into appropriate recipients. Nonimmune, naïve mice received no cells served as negative controls (line 8). All mice were challenged in the footpad within 1 h of cell transfer, and the footpad swelling was recorded 24 h later. Data from eight experiments were pooled. Number of animals tested is given in parentheses.

modulatory cell population. Accordingly, we treated the ILNC with the same reagents used in the previous experiments and then tested the ability of these treated cells to transfer TMA specific DTH. The transfer of DTH is abolished by (group 10, 11) anti-Thy-1 and the anti-Id reagent, the latter confirming our previous contention that T_{DTH} bear idiotypic determinants. However, the anti-Lyt-2 + C treatment did not affect the ability of ILNC to transfer DTH, whereas the anti-Lyt-1 + C completely abolished the transfer of DTH. In addition, the ability of ILNC from tyr(TMA) (group 15) mice to transfer DTH was greatly affected by treatment with anti-Id antibodies, confirming that the T_{DTH} present in tyr(TMA) mice are similar to the T_{DTH} in normal mice. However, the ability of ILNC from 2,4-dinitrofluorobenzene (DNFB)-painted A/J mice to transfer contact sensitivity was not abolished after treatment with anti-Id and C, confirming the specificity of the reagent (data not shown). Thus, the T_{DTH} are Id⁺, Lyt-1⁺, 2⁻; the modulatory T cells are Id⁺, Lyt-1⁻, 2⁺. In summary, it would appear that the inability of anti-idiotypic T_{s2}-bearing mice to exhibit intrinsic unresponsiveness is caused by the lack of function in an antigen-primed, Thy-1⁺, Id⁺, Lyt-1⁻, 2⁺ modulatory cell population.

Discussion

We have reported earlier that a single intraperitoneal injection of tyr(TMA) in FCA in A/J mice induced a suppressor cell population that was able to suppress the TMA-specific DTH responses when adoptively transferred into naïve mice (12). In addition, it was observed that these T_s (termed T_{s2} [8]) bear anti-idiotypic receptors, Lyt-2 alloantigen, and I-J subregion-encoded products and were both H-2- and Igh-V-restricted for their action (12). The data presented in this report demonstrate that T_{s2} can suppress not only the TMA-SC-induced DTH but also the anti-Id-induced DTH when adoptively transferred into normal recipients at the effector phase (Table I). However, when these T_{s2} -bearing tyr(TMA)-immune mice were intentionally immunized with either TMA-SC or anti-Id, they failed to exhibit intrinsic unresponsiveness. This paradoxical observation, wherein the presence of T_{s2} can only be demonstrated by adoptive transfer into normal recipients but failed to function intrinsically, is not caused by the inability of A/J mice in general to exhibit intrinsic unresponsiveness. This conclusion is based on the finding that 2 wk after tyr(TMA) + FCA injection, A/J mice exhibited intrinsic unresponsiveness (Table II). However, the T_s induced at this time were found to bear idiotypic, act only at the induction phase of DTH when adoptively transferred into normal recipients, and the factor extracted from these T_s found to act across MHC barriers (manuscript in preparation). Thus, it is clear that the reason for the failure to observe suppressor activity intrinsically in mice bearing anti-idiotypic T_{s2} is not caused by the general inability of A/J mice to exhibit intrinsic unresponsiveness. The fact that a normal DTH response can be evoked in T_{s2} -bearing mice with anti-Id (Table I) suggests that the DTH response in these mice is not qualitatively different from normal mice. This contention is further supported by two lines of evidence. Previously (14) we have shown that the intravenous administration of a large dose of anti-Id just before challenge blocked the DTH responses specific to TMA. In addition, it was observed that this mode of blocking of DTH occurred only in strains of mice possessing the same allotype as that of A/J strain, suggesting the presence of Id on the surface of T_{DTH} . To rule out the possibility that the anti-Id-induced blocking of DTH was not caused by the activation of suppressor cells, the mice were treated with doses of CY known to deplete suppressor T cell precursors (19–24). Irrespective of the treatment to mice with CY, DTH induced in normal as well as in tyr(TMA)-immune mice was effectively blocked with anti-Id (Table III), suggesting the presence of Id on T_{DTH} as observed in other systems (25, 26). Direct evidence for the function of the idiotypic component of T_{DTH} in tyr(TMA)-immune mice comes from experiments involving treatment of T_{DTH} with anti-Id + C before adoptive transfer into naïve recipients. Such a treatment abolished the ability of T_{DTH} derived from normal as well as tyr(TMA)-immune mice to transfer immunity into naïve recipients (Table VI, groups 9, 11, 14, and 15) equally well. Taken together, these results strongly indicate that the idiotypic component of T_{DTH} was not functionally deleted as a result of the coexistence of anti-idiotypic T_{s2} in tyr(TMA)-immune mice.

The notion that the auxiliary cells may help the manifestation of suppressor function has been previously suggested (8, 17, 18, 27–31). Such auxiliary (17) or T_{s3} (18, 30, 31) cells have been shown to be essential for the function of anti-idiotypic T_{s2} in nitrophenylacetyl-specific cutaneous hypersensitivity (18) and azobenzenearsonate-specific DTH (30) systems. Based on the fact that anti-idiotypic T_{s2} cannot intrinsi-

cally suppress the DTH, it was reasoned that this apparent inability of T_{s2} to function could be caused by the loss of the function of a cell type similar to an auxiliary or T_{s3} cell. Data in Table IV suggest that the ability of T_{s2} to suppress the DTH response when adoptively transferred into normal mice at the effector phase depends on an antigen-activated, CY-sensitive suppressor cell type in the recipients. This is in line with the findings that the CY-sensitive auxiliary (17) or T_{s3} cells (18) are needed for the manifestation of effector-phase suppressors. It is likely that an absence of this CY-sensitive suppressor cell activity, induced after TMA-SC immunization, is a major factor in the lack of expression of suppressor activity intrinsically in T_{s2} -bearing mice. Further experiments in fact indicated that the ILNC from tyr(TMA)-immune mice were not suppressed when adoptively transferred together with T_{s2} into naïve recipients (Table V). However, the addition of ILNC from normal mice to ILNC derived from tyr(TMA)-immune mice conferred the ability to be suppressed by T_{s2} . It is thus evident that the anti-idiotypic, $Lyt-2^+$, $I-J^+$ T_{s2} is not the effector suppressor, but the CY-sensitive cell type present in the antigen-activated immune lymphoid population could bring about suppression of TMA-specific DTH. Because the presence or absence of the function of the latter cell type determines whether suppression should occur, this cell type will be referred to as "modulatory" as suggested (32).

Because the transfer protocols do not really mimic the physiological situation, it was decided to see if we could convert the T_{s2} -bearing mice into intrinsically suppressed mice by providing activated modulatory cell populations from normal mice. The data show that it is possible to make the T_{s2} functional *in situ* by providing extraneous modulatory cells (Table VI). In addition, it was shown that the cells responsible for the function of T_{s2} are T cells that bear $Lyt-2$ alloantigen as previously shown for the T_{s3} (18) and amplifier of suppressor cells (28). Like the target cell of T_{s2} in another system (30), the modulatory cells involved in TMA-specific T_{s2} -mediated DTH suppression also bear the Id on their surface. It should be noted that the T_{s3} cells have been shown to bear the I-J subregion-encoded products and are restricted by H-2 and Igh-complex products (18). Thus, it appears that the modulatory cells, which are nonfunctional in anti-idiotypic T_{s2} -bearing mice, share many of the properties attributed to auxiliary (17), T_{s3} (18, 30), and amplifier of suppressor cells (28). The consensus from all these studies is that the modulatory cells are very critical for the expression of suppressor activity and may be the final cell type in the suppressor pathway (8, 30, 31). Because both T_{DTH} and modulatory cells appear to bear Id on their surface (Table VI), and the stimulation of T_{s2} or modulatory cell alone do not bring about any suppression, these data support the hypothesis that the T_{s2} and modulatory cells interact with each other by Id-anti-Id recognition, which releases a factor that directly or indirectly suppress the function of T_{DTH} (8, 30, 31).

We have previously shown (11) that if 6-wk tyr(TMA)-immune mice were inoculated with TMA coupled to a protein, the resulting anti-TMA antibody lacked the Id^+ component of the response, which normally constitutes on an average 50% of the response. Because this suppressor activity is T_{s2} mediated, an apparent difference in the ability of these mice to intrinsically modulate antibody vs. DTH responses exists. The discrepancy between the results would indicate that T_{s2} may act directly on Id^+ B cells through direct receptor interaction (33, 34) or through an antigen bridge. Alternatively, the immunization protocol used to generate antibody production could have activated the modulatory population. At present we cannot rule out any one of

these possibilities. Nevertheless, under a certain set of conditions the T_{s2} population is unable to shut down DTH but can mediate suppression at the humoral level. Taken together, the results indicate that the modulatory population can potentially play a strategic role in immune regulation. In this particular situation the modulatory population can split control-mediated by T_{s2} cells, allowing the suppression of antibody on one hand and intact DTH responses on the other. Rather than the necessity to regulate a variety of responses at the T_{s2} level, which would remove suppressor potential at both the antibody and DTH levels, the system via the modulatory cell is now capable of a more sophisticated form of control or "fine tuning." Thus, the induction of T_s using tyr(TMA) in FCA appears to offer the potential to study the fine regulation of antibody and DTH responses in a naturally controlled fashion.

Summary

A single intraperitoneal injection of the monovalent synthetic antigen, tyrosinated trimethylaminoaniline [tyr(TMA)] in Freund's complete adjuvant induces an anti-idiotypic second-order T suppressor (T_{s2}) cell population 6 wk later. This population was able to suppress TMA-specific delayed-type hypersensitivity (DTH) responses when adoptively transferred into normal syngeneic recipients. However, they failed to function intrinsically. The inability of the T_{s2} to function intrinsically was not caused by compensating idiotype-negative T cells that mediate DTH. Rather, this paradoxical observation was found to be caused by the absence or loss of function of a critical modulatory T cell population in the suppressor cell-bearing mice. This cell is functionally active in normal mice immunized for DTH responses and is sensitive to cyclophosphamide treatment. In addition, this cell type bears idiotype on its surface and is Thy-1⁺ and Lyt-1⁻,2⁺. It was demonstrated that by adoptively transferring the activated modulatory T cells from normal mice into tyr(TMA)-immune recipients, it was possible to observe suppressor cell function intrinsically. The potential importance of modulatory T cell function in the regulation of antibody and DTH responses is discussed.

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