AN ANTIGEN-SPECIFIC SIGNAL IS REQUIRED FOR THE ACTIVATION OF SECOND-ORDER SUPPRESSOR T CELLS IN THE REGULATION OF DELAYED-TYPE HYPERSENSITIVITY TO 2,4,6-TRINITROBENZENE SULFONIC ACID

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The interaction of multiple T cell subsets, in the down regulation of antigen-specific immune responses, have been demonstrated in many different experimental models (1, 2). However, due to the complexity by which the immune response is regulated and the 'subtle' variations in the experimental systems of different investigators, it has been difficult to compare results and to construct a general scheme that will encompass all the published findings.

Nevertheless, we proposed such a general scheme for a suppressor T cell pathway based mainly on the results obtained in our laboratories (3), with several models of immune responses by suppressor T cells, including cell-mediated immune response to simple haptens like azobenzenearsonate (ABA)[1] (4, 5, 6) and 4-hydroxy-3-nitrophenyl acetyl (NP) (7), and humoral immune response to the synthetic copolymer of L-glutamic acid-L-alanine-L-tryosine (GAT) (8, 9).

In our proposed scheme, we postulated that T cell-mediated suppression in murine system proceeds by a common major pathway involving the sequential interaction of three T cell subsets, each with distinct properties. We have named these three T cell subsets Ts-1, Ts-2, and Ts-3.

Based on our studies involving the suppression of delayed-type hypersensitivity (DTH) reaction to ABA and NP in mice, we concluded that intravenous injection of haptenated syngeneic spleen cells (SC) triggers a population of relatively cyclophosphamide-sensitive pre-Ts-1 cells, which then mature into antigen-reactive cells, or Ts-1. These Ts-1 cells are Lyt-1^+2^- and bear idiotypic determinants serologically cross-reactive (CRI) with those present in anti-ABA antibodies of appropriate strains of mice (10). A soluble suppressor T cell factor (TsF-1) is obtained from these Ts-1 cells or Ts-1 hybridoma cells. Similar to Ts-1 in receptor specificity, TsF-1 also binds antigen, and bears some CRI determinants. TsF-1 induces nonimmune T cells to become Ts-2. In contrast to Ts-1, Ts-2 are

* Supported by Mitsubishi Chemical Co., Tokyo, Japan.

‡ Supported in part by grants NIAID 16701 and AI-14732 from the National Institutes of Health.

[1] *Abbreviations used in this paper:* ABA, azobenzenearsonate; BSA, bovine serum albumin; CRI, cross-reactive idiotype; DTH, delayed-type hypersensitivity; GAT, L-glutamic acid-L-alanine-L-tyrosine; GT, L-flutamic acid-L-tyrosine; HBSS, Hanks' balanced salt solution; MHC, major histocompatibility complex; NP, 4-hydroxy-3-nitrophenyl; SC, spleen cells; TNP, trinitrophenyl; TNBS, trinitrobenzene sulfonic acid; Ts-1, 2, suppressor T cells 1 and 2; TsF-1, 2, suppressor T cell factors 1 and 2.

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/83/09/0932/14 $1.00
Volume 158 September 1983 932–945

antiidiotypic, Lyt-1^+2^+ cells, and function as efferent suppressors in the systems; for example, Ts-2 cells are able to suppress DTH reaction at the time of elicitation. Ts-2 cells release a soluble factor, TsF-2, which activates the final effector suppressor Ts-3. Ts-3 cells are Lyt-2^+3^+ and bind antigen.

In the ABA and NP systems, TsF-1 obtained either from Ts-1-containing SC or T cell hybridoma can trigger Ts-2 cells in the absence of any additional antigen. This is in sharp contrast with our earlier findings in the suppressor T cell pathway of GAT and L-glutamic acid-L-tryosine (GT). In the GAT and GT systems, antigens are absolutely required for the activation of Ts-2 cells by TsF-1 factors (8). It is highly unlikely that this discrepancy arises from differences in the assay systems. Furthermore, it has been reported recently that the Ts-2, induced in the presence of antigen in the GT system, is antigen specific rather than idiotypic specific (11). The exact reason why in some systems Ts-2 cells are antiidiotypic (12, 13, 14, 15), and yet in others they are antigen specific is not clear (11). It is possible, however, that the antiidiotypic nature of the ABA and NP Ts-2 cells and factors is due to the presence of a predominant CRI family in these two systems (12, 16). Since their humoral immune responses produce antibodies that lack a major predominant idiotype (17), systems like GAT may require both antigen-specific TsF-1 and an antigen signal to activate Ts-2.

To further clarify the importance of idiotypic-antiidiotypic interactions in the regulation of suppressor T cell interactions, and the role of antigen in triggering Ts-2 cells, in systems lacking major CRI, we investigated the regulation of DTH reaction to another simple hapten, trinitrobenzene sulfonic acid (TNBS), a system which has been studied extensively by Asherson and Zembala (18). Immune responses to TNBS lack a major predominant idiotype; thus the response provides us an opportunity to study the suppressor T cell pathway in a system where antigen, and not idiotypes, may mediate essential suppressor T cell interactions. The major points examined in our studies of suppressor T cells and their factors in the down regulation of DTH response to trinitrophenyl (TNP) were: (*a*) the binding specificity of the relevant cells and, in particular, whether their receptors are idiotypic (antigen specific) or antiidiotypic; (*b*) the antigen requirements for the activation of second-order Ts-2 cells; and (*c*) the mode of action of these suppressor T cells and their factors.

Materials and Methods

Mice. A/J (H-2^a), B10.BR (H-2^k), B6 (H-2^b), and DBA/2 (H-2^d) mice obtained from The Jackson Laboratory, Bar Harbor, ME. BALB/c (H-2^d) mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. All the 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).

Induction and Elicitation of DTH to TNP and ABA. To induce DTH to TNP, 0.2 ml of 10 mM TNBS pH 7.4 solution was injected subcutaneously into two separate sites on the dorsal flanks of the mice. Challenge was performed 6 d later by injecting 30 μl of 10 mM TNBS pH 7.4 solution into the right footpad. 24 h after the footpad challenge, DTH reactivity was assessed by measuring the swelling of the footpad with a Fowler micrometer.

The magnitude of the DTH was expressed as the increment of the thickness of the challenged right footpad as compared with the untreated left footpad. Responses were

given in units of 10^{-2} mm plus SE. Induction of DTH response to ABA was done exactly as described before (5). Each group consists of at least four mice.

Induction of Suppressor Cells and Preparation of Suppressor Cells. TNP-conjugated SC were prepared as described (19). To induce suppressor cells, normal mice were injected intravenously with 5×10^7 TNP-SC. 7 d later, these mice became the donors of Ts-1 cells. Spleens from these animals were removed, and a single-cell suspension was prepared in Hanks' balanced salt solution (HBSS). The cells were washed twice in HBSS and counted. To assay for the ability of such cells to inhibit TNP-specific DTH, 5×10^7 viable cells were injected intravenously into normal recipients that were then primed subcutaneously with TNBS and challenged 6 d later as described earlier.

Production of TsF-1. Production of TsF-1 was accomplished precisely as described earlier (4). Briefly, 1×10^8 to 5×10^8 washed SC in 1 ml of HBSS were subjected to alternate snap freezing at −78°C and thawing at 37°C. This was repeated four times and followed by centrifugation at 10,000 *g* for 90 min. The supernates were adjusted to 5×10^8 cell equivalents/ml and frozen until use at −80°C. To test the ability of TsF-1 to inhibit TNP-specific DTH, the extract was injected intravenously into normal mice beginning at the time of immunization with TNBS. 2×10^7 cell equivalents of the factors were administered each day for five successive days. 2 d later, on day 7, these animals were challenged and their footpad responses were measured.

Induction of Ts-2. To induce Ts-2, TsF-1 (2×10^7 cell equivalents/d) was injected intravenously into normal mice for five successive days. On the first day of TsF-1 injection, the mice were also injected with 0.2 ml s.c. of 10 mM TNBS solution. On day 7, SC were assayed for Ts-2 activity by adoptive transfer to normal recipients that were immunized and challenged as described above. To assay for the ability of Ts-2 to inhibit the effector phase of the DTH reaction, 5×10^7 putative Ts-2 were transferred into animals that had been immunized 6–7 d earlier. Within 2 h after cell transfer, all the recipients and their appropriate controls were challenged in the footpad with TNBS as described, and the increase in footpad swelling determined 24 h postchallenge.

Antiserum Treatment. 1×10^8 SC were incubated with 1 ml of 1:20 dilution of anti-Thy-1,2 hybridoma antibody for 45 min at 0°C, washed once in HBSS, and then incubated again with 1 ml of a 1:6 dilution of low-tox rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY) for 30 min at 37°C. The cells were then washed twice in HBSS, recounted, and resuspended for cell transfer.

Plate Purification of Suppressor T Cells. The spleens from BALB/c mice injected with TNP-SC or TsF-1 were teased and washed in phosphate-buffered saline with 5% fetal calf serum, and erythrocytes were lysed with 0.83% Tris-NH_4Cl. As described elsewhere (13), the B cells were removed by incubation of the whole SC population on plastic petric dishes coated with affinity-purified rabbit anti-mouse Ig. The cell population that did not adhere to the anti-Ig–coated dishes contained 5–8% surface Ig-bearing cells as judged by immunofluorescence using fluoresceinated rabbit anti-mouse Ig.

The cells were then incubated on TNP-bovine serum albumin (BSA)-coated plastic petri dishes as described previously (13). After 1 h of incubation at room temperature, nonadherent cells were removed from such plates with three gentle washes. After a 30-min incubation and cold shock at 4°C, the adherent cells were vigorously removed with three washes. The cells were washed once in HBSS, counted, and resuspended in the appropriate volume for either transfer.

Results

Suppressor Cells Induced by Intravenous Injection of TNP-SC Are T Cells and Are Antigen Specific in their Action. To investigate the regulation of T cell-mediated DTH reaction to TNP, we first examined the induction of suppressor cells by intravenous injection of TNP-SC. The results of this experiment are shown in Table I. As can be seen, intravenous injection of TNP-SC readily induces suppressor cells in the animals, as demonstrated by adoptive transfer experiment

TABLE I
Suppressor Cells Induced by Intravenous Injection of TNP-SC Are T Cells and Are Antigen Specific in Their Action

Expt.	Inducers of Ts*	Number of cells transferred‡	Immunization§	Challenge‖	Footpad swelling ($\times 10^{-2}$ mm ± SE)	P value¶
1	—	—	TNBS	TNBS	30.0 ± 3.5	—
	TNP-SC	5×10^7	TNBS	TNBS	13.0 ± 2.9	<0.01
	—	—	—	TNBS	6.7 ± 3.0	<0.01
	—	—	TNBS	TNBS	54.9 ± 2.2	—
	TNP-SC	5×10^7	TNBS	TNBS	28.7 ± 2.5	<0.01
	TNP-SC	5×10^7 ($\alpha\theta$ + complement)	TNBS	TNBS	48.8 ± 3.1	NS**
2	TNP-SC	5×10^7 (complement)	TNBS	TNBS	29.9 ± 3.0	<0.01
	—	—	—	TNBS	14.1 ± 2.4	<0.01
3	—	—	ABA	ABA	39.8 ± 5.7	—
	TNP-SC	5×10^7	ABA	ABA	49.5 ± 3.1	NS
	—	—	—	ABA	7.0 ± 1.0	<0.01

* To induce suppressor T cells, normal BALB/c mice were injected with 5×10^7 TNP-SC i.v.
‡ 7 d after intravenous injection of TNP-SC, these mice were the donors of suppressor T cells, and 5×10^7 SC were transferred intravenously into groups of naive recipients.
§ Within 2 h after receiving suppressor T cells, all the recipients and the appropriate controls were immunized subcutaneously with 0.2 ml of a 10 mM TNBS solution or 0.2 ml of a 10 mM ABA salt.
‖ 6 d after immunization, all the experimental groups including the controls were challenged in the footpad as described in Materials and Methods. The increase in footpad swelling was measured 24 h postchallenge.
¶ As compared with positive control.
** Not significant.

(experiment 1 and 2). In addition, in vitro treatment of these putative suppressor cells with a monoclonal anti-Thy-1 antibody and complement completely abrogated their ability to transfer unresponsiveness. Thus, the passive transfer of suppressor activity is a T cell-dependent phenomenon. Moreover, these suppressor T cells are antigen specific, since Ts induced by TNP-SC failed to suppress the development of DTH reaction to an unrelated antigen, ABA (experiment 3).

Suppressor T Cells Induced with TNP-SC Bind Antigen. Earlier studies from our laboratories (1, 3) have provided evidence for the participation of both antigen-binding and non–antigen-binding suppressor T cells in the ABA- and NP-specific suppressor T cell pathway. To further characterize the TNP-specific suppressor T cells, we investigated their binding specificity using an experimental protocol that has been used extensively in our earlier studies (13). In the ABA system, we have shown that antigen-specific Ts-1 cells can be highly enriched on antigen-coated plates. Thus, we investigated whether TNP-specific suppressor T cells can be enriched on antigen-coated plates.

The results of such an experiment are shown in Table II. Incubation of suppressor T cells on petri dishes coated with TNP-BSA resulted in a significant enrichment of suppressor T cells, as compared with unfractionated whole SC.

TABLE II

Fractionation of TNP-Specific Suppressor T Cells on Antigen-coated Plates

Fractionation*	Number of cells transferred‡	Immunization§	Footpad swelling ($\times 10^{-2}$ mm ± SE)‖
—	—	TNBS	32.2 ± 3.1
—	5×10^7	TNBS	11.0 ± 4.0
—	1×10^7	TNBS	26.2 ± 2.7
TNP-BSA adherent	5×10^5	TNBS	8.0 ± 1.6
TNP-BSA nonadherent	5×10^6	TNBS	29.0 ± 3.0
—	Challenge only		3.7 ± 1.2

* To induce suppressor T cells, normal BALB/c mice were injected with 5×10^7 TNP-SC i.v. 7 d later, these mice were donors of suppressor T cells. Before transfer to a naive recipient, they were then fractionated first on rabbit anti-mouse immunoglobulin plates to remove B cells and then again on TNP-BSA-coated plates to enrich for antigen-binding suppressor T cells as described in Materials and Methods.

‡ Various numbers of suppressor T cells were then transferred to groups of naive recipients.

§ All recipients and their appropriate controls were immunized as described in Materials and Methods.

‖ 6 d after immunization, all the experimental groups including the controls were challenged in the footpad as described, and increases in footpad swelling were determined 24 h postchallenge.

Furthermore, incubation of suppressor T cells on plates coated with an unrelated antigen failed to show any significant enrichment (data not shown). Therefore, similar to the results obtained in the ABA system, suppressor T cells induced by intravenous inoculation of TNP-SC also bind antigen.

Antigen-specific TsF Can Be Obtained from TNP-SC-induced Ts and Is Strain Specific in its Action. Since TsF have been shown to play a critical role in the propagation of suppressor pathways in many different experimental systems (1–4), we prepared TsF using a protocol we had used earlier in the ABA system (14). This putative TsF was then given to groups of mice that had been immunized with TNBS to test for their suppressor activity. The results of this experiment are shown in Table III.

Injection of TsF obtained from BALB/c (H-2^d) mice inhibited the development of TNP-specific DTH in syngeneic BALB/c mice. The same TsF preparation also inhibited the development of DTH reaction in H-2-identical DBA/2 (H-2^d) mice. Nevertheless, these TsF failed to suppress the DTH reaction in H-2-distinct C57BL/6 (H-2^b) and B10.Br (H-2^k) mice. Furthermore, they also failed to inhibit DTH reaction in A/J mice (H-2^a) that are identical to the BALB/c mice in the I-C,D subregion of the H-2 complex. Thus, TNP-specific TsF obtained from BALB/c mice appears to be restricted in its action by the left-hand side of the major histocompatibility complex (MHC) complex (K, I-A, I-B, I-J, I-E).

Failure of TNP-specific TsF to Induce Ts-2 in Naive Mice. One of the many features of antigen-specific TsF is their ability to induce second-order suppressor T cells known as Ts-2 (1, 3, 4). In addition, our previous experiments suggest that in some experimental systems the participation of antigen in conjunction

TABLE III
Strain Specificity of TNP-specific TsF-1

Strain	Immunization*	Treatment‡	Footpad swelling ($\times 10^{-2}$ mm ± SE)§	*P* value
BALB/c	TNBS	—	37.7 ± 1.9	
(H-2[d])	TNBS	BALB/c TsF	16.0 ± 2.3	<0.001
	—	—	5 ± 0.58	
DBA/2	TNBS	—	33.6 ± 1.5	
(H-2[d])	TNBS	BALB/c TsF	16.7 ± 1.3	<0.001
	—	—	4.6 ± 0.8	
C57BL/6	TNBS	—	30.0 ± 1.5	
(H-2[b])	TNBS	BALB/c TsF	30.3 ± 0.3	0.8 (NS)‖
	—	—	4.5 ± 0.5	
B10.BR	TNBS	—	23.7 ± 1.9	
(H-2[k])	TNBS	BALB/c TsF	22.5 ± 1.5	0.6 (NS)
	—	—	8.0 ± 1.7	
A/J	TNBS	—	31.7 ± 2.7	
(H-2[a])	TNBS	BALB/c TsF	27.5 ± 1.8	0.24 (NS)
	—	—	4 ± 0.5	

* Different strains of mice were immunized with TNBS as described.
‡ Suppressor factor (TsF-1) was prepared as described in Materials and Methods. TsF were injected intravenously into immunized animals beginning on the day of immunization at 2×10^7 cell equivalent in 0.2 ml/d for five successive days.
§ 2 d after the last injection of TsF, all the mice and their appropriate controls were challenged in the footpad with TNBS, and the increase in footpad swelling was determined 24 h postchallenge.
‖ Not significant.

with TsF is required for the induction of Ts-2 (i.e., GAT system). Nevertheless, in the ABA and NP systems, TsF alone appears to be able to induce Ts-2 in the absence of any antigen. To investigate whether TsF obtained from TNP-specific Ts will induce Ts-2 in naive mice, we injected TsF into groups of normal BALB/c mice for five successive days. 2 d after the last injection, they became the donors of putative Ts-2 cells. The results of a typical experiment are shown in Table IV.

TNP-specific TsF, when injected into sensitized mice beginning on the day of immunization, inhibited the development of TNP-specific DTH reaction. However, injection of the same TsF into naive mice failed to induce second-order suppressor cells in these animals as demonstrated by adoptive transfer experiment. Therefore, we proceeded to examine the possibility that the induction of Ts-2 in the TNP system may require the participation of antigen.

Induction of Ts-2 in the TNP System But Not in the ABA System Requires Both TsF-1 and Antigen. To clarify whether the induction of Ts-2 in the TNP system may require antigen, we performed the following experiment. Normal BALB/c mice were either immunized subcutaneously with TNBS or injected intravenously with TNP-specific TsF. In addition, a third group of mice were immunized with TNBS on the first day and were also given TsF for five successive days. 7 d later, 2 d after the last injection of TsF, 5×10^7 SC from these animals were transferred to groups of naive recipients that were then immunized. The results of such an experiment are shown in Table V.

It was clearly demonstrated that SC obtained either from animals immunized

TABLE IV

TsF-1 Obtained from TNP-specific Ts-1 Inhibits the Development of TNP-specific DTH, But the Injection of TsF-1 into Naive Animals Failed to Induce Second-order Ts-2

Immunization*	Treatment‡	Footpad swelling (× 10^{-2} mm ± SE)	*P* value
TNBS	—	43.0 ± 2.1	—
TNBS	TsF_1	27.6 ± 4.3	<0.01
Challenge only		15.3 ± 1.2	<0.01

Inducer of Ts-2§	Number of cells transferred‖	Immunization	Footpad swelling (× 10^{-2} mm ± SE)	*P* value
—	—	TNBS	46.00 ± 3.0	—
TsF_1	5 × 10^7	TNBS	49.25 ± 3.7	NS
	Challenge only		10.33 ± 1.4	<0.01

* Normal BALB/c mice were immunized subcutaneously with 0.2 ml of a 10 mM TNBS solution.

‡ TsF-1 prepared as described in Materials and Methods was injected intravenously into immunized animals beginning on the day of immunization at 2 × 10^7 cell equivalent in 0.2 ml/d for 5 d.

§ To induce Ts-2, normal BALB/c mice were injected with 2 × 10^7 i.v. cell equivalent of TsF-1 in 0.2 ml/d for 5 d. On day 7, they were the donors of putative Ts-2 cells.

‖ 5 × 10^7 SC from animals treated with TsF-1 were transferred intravenously into groups of naive animals that were then immunized subcutaneously with TNBS.

with TNBS alone or from animals injected with TsF alone were unable to transfer suppression. However, SC obtained from animals that had been immunized with TNBS and then given TsF for five successive days showed potent suppressor activity. In contrast, experiment 3 in Table IV demonstrated that, in the ABA system, injection of ABA-specific TsF-1, without simultaneous administration of antigen, induced significant levels of Ts-2 activity. Thus, we concluded from these experiments, that the induction of Ts-2 in the TNP, but not the ABA system, required the participation of antigen in BALB/c mice.

Antigen Specificity of the Second Signal Required for the Activation of Ts2. To further characterize the nature of the second signal required for the activation of TNP-specific Ts-2, we investigated whether the delivery of the second signal was an antigen-specific event. The results of this experiment are shown in Table VI. SC obtained from animals treated with TNP-specific TsF-1 and TNBS provided strong suppressor T cell activity. In contrast, SC obtained from animals treated with TNP-specific TsF-1 and an unrelated antigen, ABA, failed to activate TNP-specific Ts-2 cells. Thus the second signal required for the activation of Ts-2 in the TNP system was antigen specific.

Suppressor Cells (Ts-2) Induced With TsF-1 and Antigen Are Efferent Suppressors. One of the main features of Ts-2 cells in other well-characterized systems was their ability to suppress at the efferent stage of the immune response (15, 20). Therefore, we next examined whether Ts-2 cells in the TNP system were also efferent suppressors. In these experiments, Ts-2 cells were induced as described earlier. However, in contrast to earlier experiments, these Ts-2 cells

TABLE V

Induction of Ts-2 in the TNP System But Not in the ABA System Requires Both TsF-1 and Antigen

Expt.	Treatment of Ts-2 donors*	Number of Ts-2 transferred‡	Immunization§	Footpad swelling ($\times 10^{-2}$ mm ± SE)‖	*P* value
1	—	—	TNBS	46.0 ± 3.08	—
	TsF	5 × 10^7	TNBS	49.2 ± 3.7	NS¶
	TsF + TNBS	5 × 10^7	TNBS	19.3 ± 2.2	<0.01
	—	Challenge only		10.3 ± 1.4	<0.01
2	—	—	TNBS	34.5 ± 2.5	—
	TNBS	5 × 10^7	TNBS	33.2 ± 1.0	NS
	TsF	5 × 10^7	TNBS	37.6 ± 4.0	NS
	TNBS + TsF	5 × 10^7	TNBS	11.0 ± 2.6	<0.01
	—	Challenge only		6.0 ± 2.5	<0.01
3	—	—	ABA	34.5 ± 1.5	
	ABA	5 × 10^7	ABA	33.0 ± 3.2	NS
	TsF	5 × 10^7	ABA	17.7 ± 1.5	0.01
	ABA × TsF	5 × 10^7	ABA	23.0 ± 2.8	0.012
	—	Challenge only		7.0 ± 1.1	<0.01

* Normal BALB/c mice were either immunized subcutaneously with 0.2 ml of a 10 mM TNBS solution, given TNP-specific TsF for five successive days, or were given TNBS and TsF both. For groups receiving both, TNBS was given only on the first day.

‡ 7 d later, mice were donors of putative Ts-2 cells, and 5 × 10^7 SC from various groups were transferred to groups of naive recipients that were then immunized subcutaneously.

§ Mice were immunized subcutaneously with 0.2 ml of a 10 mM TNBS solution.

‖ Challenge was done 6 d after immunization, and the increase in footpad swelling measured 24 h postchallenge.

¶ Not significant.

TABLE VI

Antigen Specificity of the Second Signal Required for the Activation of Ts-2

Treatment of Ts donors*	Number of cells transferred‡	Immunization§	Footpad swelling ($\times 10^{-2}$ mm ± SE)‖	*P* value
—	—	TNBS	31.7 ± 2.6	—
TsF	5 × 10^7	TNBS	25.5 ± 1.2	0.8 (NS)¶
TsF + TNBS	5 × 10^7	TNBS	13.7 ± 1.6	0.001
TsF + ABA	5 × 10^7	TNBS	28.7 ± 2.6	0.5 (NS)
Challenge only			5.3 ± 2.9	0.001

* Normal BALB/c mice were either injected with TNP-specific TsF, given both TsF and TNBS, or given TNP-TsF and 0.2 ml of a 10 mM ABA solution, as described in Materials and Methods.

‡ 7 d later mice were the donors of putative Ts-2 cells and 5 × 10^7 SC from various groups were transferred to another group of naive recipients that were then immunized subcutaneously.

§ Mice were immunized subcutaneously with 0.2 ml of a 10 mM TNBS solution.

‖ Challenge was done 6 d later and the increase in footpad swelling measured 24 h postchallenge.

¶ Not significant.

were transferred into animals at the day of challenge rather than at the time of immunization, and the results are shown in Table VII. SC obtained from animals treated with TNP-specific TsF and TNBS were effective in suppressing the TNP-specific DTH reaction even when given at the day of challenge. Therefore, Ts-2 in the TNP system were efferent suppressors.

Ts-2 Cells in the TNP System Bind Antigen. The requirement for antigen in the activation of Ts-2 cells prompted us to investigate the binding specificity of these Ts-2. Similar to the experiment described earlier, we examined whether Ts-2 cells can be enriched on antigen-coated plates. The results of such an experiment are shown in Table VIII. As can be seen, similar to Ts-1 induced by TNP-SC, Ts-2 cells induced by TsF-1 and antigen could be readily enriched on antigen-coated plates.

TABLE VII

Ts-2 Cells in the TNP System Are Efferent Suppressors

Treatment of Ts-2 donors*	Number of cells transferred‡	Immunization	Day of transfer§	Footpad swelling (× 10^{-2} mm ± SE)	P value
—	—	TNBS	—	39.8 ± 2.3	—
TsF	5 × 10^7	TNBS	6	32.5 ± 1.7	NS‖
TsF + TNBS	5 × 10^7	TNBS	6	23.3 ± 2.7	<0.01
	Challenge only			5.7 ± 1.9	<0.01

* Normal BALB/c mice were either injected with TsF or with TsF and TNBS as described.
‡ 7 d later, mice were the donors of Ts-2 cells.
§ 5 × 10^7 Ts-2 cells were transferred into groups of recipients that had been immunized with TNBS 6 d earlier. Within 1 h after transfer, all the recipients were challenged in the footpad with TNBS.
‖ Not significant.

TABLE VIII

Fractionation of TNP-Specific Ts-2 on Antigen-coated Plates

Fractionation*	Number of cells transferred‡	Footpad swelling (× 10^{-2} mm ± SE)§	P value
—	—	31.7 ± 2.0	—
—	5 × 10^7	9.7 ± 2.7	<0.01
B cells	5 × 10^7	23.0 ± 4.0	0.08 (NS)‖
T cells	1 × 10^7	14.0 ± 2.8	<0.01
TNP-BSA adherent	1 × 10^6	13.0 ± 0.9	<0.01
TNP-BSA nonadherent	5 × 10^6	21.2 ± 4.6	0.08 (NS)

* Normal BALB/c mice were injected with TNBS and TsF-1 to induce Ts-2. Before transfer to naive recipients, Ts-2 were then fractionated first on rabbit anti-mouse Ig plates to remove B cells and then again on TNP-BSA plates to enrich for antigen-binding suppressor T cells as described.
‡ Various number of suppressor T cells were then transferred to groups of naive recipients that had been immunized with TNBS 6 d earlier.
§ Within 2 h after transfer, all recipients were challenged in the footpad and the increase in footpad swelling determined 24 h postchallenge.
‖ Not significant.

Discussion

The results reported in this paper represent an extension of our earlier studies (4–7) on suppressor T cells and their soluble products that regulate the development of DTH reaction to simple haptens. Using another simple hapten, TNBS, we have uncovered evidence of the involvement of a Ts-1-like cell in this system. Suppressor T cells induced with TNP-SC can be enriched on antigen-coated plates, and are afferent suppressors and Lyt-1^+ (data not shown). Furthermore, they produced soluble TsF that are active in vivo. Thus, the Ts-1 cells in the TNP system are very similar to the Ts-1 cells in other systems.

Nevertheless, three major differences were noted in the current studies. First, when we examined the strain specificity of TNP-specific TsF-1, to our surprise, we found that TsF-1 obtained from TNP-SC-induced suppressor T cells is MHC-restricted. Second, injection of TNP-specific TsF-1 into naive mice was unable to induce Ts-2 unless additional antigen was injected. Third, the Ts-2 cells induced by administration of both TsF-1 and TNBS were antigen specific rather than antiidiotypic as were the ABA and NP systems (13, 15).

We shall first compare these results in the TNP system with our earlier findings in the ABA and NP systems and then with results from other investigators. In contrast to results obtained earlier in the ABA and NP systems, in which Ts-1 cells and their factors (TsF-1) could function without any genetic restriction (15, 18), TsF-1 in the TNP system are MHC restricted in their activity. Thus, TsF-1 obtained from BALB/c (H-2^d) mice were effective in suppressing the DTH reaction in syngeneic BALB/c, H-2-identical DBA/2 (H-2^d), and B10.D2 (H-2^d) mice (data not shown). However, the same TsF-1 preparation was unable to suppress DTH reaction in allogeneic B10.Br (H-2^k) or C57BL/6 (H-2^b) mice. It also failed to suppress in semiallogeneic A/J (H-2^a) mice. Therefore, the activity of TNP-specific TsF-1 appeared to be restricted by genes located in the left-hand side of the MHC (H-2 K, I-A, I-B, I-J, I-E). Experiments are now in progress using H-2-congeneic mice to locate and explore the exact H-2 subregion(s) that govern the activity of TNP-specific TsF-1.

The exact reason why TsF-1 in the ABA and NP systems are not H-2 restricted, while TNP-specific TsF are H-2 restricted, was puzzling. In our initial studies in the ABA system, we found that ABA-specific TsF-1 did not suppress Igh-1-different mice directly. Our immediate interpretation was that TsF-1 in the ABA system was restricted by Igh-1-linked genes. However, further analysis revealed that these factors induced Ts-2 cells appropriately in Igh-1-mismatched recipients, and that the failure to observe suppression was due to the role of Igh-1-linked genes in controlling idiotypic interactions further along the suppressor pathway (21).

In the TNP system, we have so far only tested TNP-specific TsF-1 directly in different strains of animals. We have not yet designed experiments to address the possibility that, despite the failure of TNP-specific TsF to induce suppression in allogeneic animals, they may still be able to induce a second population of Ts-2 cells in these animals. However, due to the lack of appropriate interaction further along the suppressor pathway, these Ts-2 cells may remain immunologically silent. In addition, experiments are in progress to determine whether TNP-specific TsF binds antigen and bears any MHC determinants. To facilitate further

characterization of these TsF, we have recently successfully fused these Ts-1 cells with a T cell thymoma, BW5147, and generated Ts-1-like T cell hybridomas. Some of these hybridomas produce biologically active material that functions in vivo in the inhibition of TNP-specific DTH and in vitro in the generation of TNP-specific cytotoxic T cell response.

The finding that TNP-specific TsF is MHC restricted in its activity is not a new finding. Many different groups, including ourselves, have reported a role for H-2 gene products in Ts or TsF activity. Miller et al. (22) have shown that suppression of DTH reaction to 1-fluoro-2,4-dinitrobenzene requires identity between Ts donors and recipients at the H-2D locus. Moorhead (23) found that TsF induced with dinitrobenzene sulfonic acid (DNBS) was H-2K- or H-2D-restricted in its interaction with its target cell. Asherson and Zambala (24) found that TsF induced with TNBS is restricted by the I-J subregions of the MHC gene products. Thus, H-2 genetic restrictions are common in suppressor systems, although the subregion involved and the restriction site appear to differ in various experimental models.

In an attempt to further characterize the suppressor T cell pathway in the TNP system, we came across another surprising finding. We found that even though in vivo administration of TNP-specific TsF was able to suppress the DTH reaction, it was unable to induce Ts-2 in naive animals, unless additional antigen was provided. This requirement for antigen in the activation of Ts-2 cells could not be overcome by increasing the dose of TsF injected (i.e., using 1×10^8 cell equivalent/d for 5 d) or the number of cells transferred (i.e., 1×10^8 SC from a TsF-treated animal) (data not shown).

The requirement for antigen in the induction of Ts-2 was first demonstrated in the regulation of antibody response to GAT by suppressor T cells (8). The reason for this requirement remains ambiguous. One possibility is that the interaction of antigen and TsF complex results in the formation of new antigenic determinants that favor the activation of suppressor cells. Another possibility is that the presence of antigen may serve as a focusing signal to direct TsF to its appropriate target. An additional intriguing possibility was raised by the recent finding of Ptak et al. (25). These investigators found that interactions between two molecules released by two different T cell subsets may yield a complete factor with suppressor activity. In a study very similar to ours, they found that sensitizing an animal with TNBS results in the activation of Lyt-1^+ T cells that secrete an antigen-binding material, while intravenous injection of TNBS activates Lyt-2^+ T cells to produce a second subunit that does not see antigen. These two subunits by themselves do not have any biological activity. However, the interaction of these two molecules results in the formation of biologically active suppressor molecules. It is possible that our TsF-1 may represent one of these two molecules, while the immunizing antigen activates another T cell to release the second subunit, thus creating a functional suppressor factor with the ability to induce second-order suppressor T cells (Ts-2). Experiments are now in progress to address these issues.

Our findings that Ts-2 induced in the TNP system are antigen specific rather than idiotype-specific is similar to the findings in the GAT system. However, it is in sharp contrast with our earlier findings in the ABA and NP systems. The

most obvious difference between these systems is the presence of a major dominant CRI in the ABA and NP systems (10, 16), but not in the TNP and GAT systems (17). It is reasonable to postulate that in systems where a major idiotype is involved, there may be regulatory T cells preprogrammed to recognize certain idiotypic determinants; therefore, the role of idiotype-antiidiotype interaction is predominant. Such interactions represent the major scheme of the network theory of immunoregulation, postulated by Jerne (26). In systems like those of TNP and GAT, in the absence of a major idiotype, the idiotypic determinants that are unsually involved in cellular interaction may not be "immunogenic" enough to provide the necessary signal for the activation of antiidiotypic Ts-2. Thus, to complete the suppressor pathway, the system has to use an alternative suppressor pathway using both suppressor factors and antigen. If this interpretation is correct, the second-order suppressor cells should be antiidiotypic in all experimental systems that involve a major idiotypic family, while in systems lacking a predominant idiotype, the Ts-2 induced should be idiotypic and should always require antigen. Another interesting finding with respect to the role of idiotype in T cell interaction is that in systems where a major idiotypic family does exist in the B cell compartment (e.g., ABA and NP), some of these determinants can also be detected in their T cell subpopulations (4, 7). The most obvious explanation for these observations is that both B and T cells use some identical genetic information to encode for part of their receptors.

Nevertheless, it is also possible that B and T cells use different genetic information to encode for their receptors. In some yet undefined mechanisms, however, B cells may be able to select or influence the generation of the T cell repertoire. This would result in the generation of a T cell subpopulation with receptors that are the mirror image of those present in B cells.

Thus far, we have only studied the induction of Ts-1 and Ts-2 cells in the TNP system. Yet, it has been established that an additional suppressor T cell, Ts-3, is involved in the efferent limb of the suppressor T cell circuit. Asherson and Zambala (24) and our laboratories (27) have shown that in the TNP and ABA systems, Ts-3 represents the final effect suppressors which can mediate suppression in an antigen nonspecific function. Experiments are now in progress in our laboratories to further characterize this additional suppressor T cell in the TNP system.

Summary

Suppressor T cells (Ts-1) induced with trinitrophenyl (TNP)-conjugated syngeneic spleen cells (TNP-SC) can be enriched on antigen-coated plates and are afferent suppressors. In addition, these suppressor cells produced soluble suppressor factors (TsF) that were active in vivo. Therefore, the Ts-1 cells in the TNP system are very similar to the Ts-1 cells in other systems we have studied earlier. Further characterization of these TsF-1 revealed that TsF-1 obtained from TNP-SC-induced Ts-1 is major histocompatibility complex restricted in its activity. Injection of TNP-specific TsF-1 into naive mice did not induce Ts-2 unless additional corresponding antigen was provided. Moreover, the Ts-2 cells induced by administration of both TsF-1 and trinitrobenzene sulfonic acid were

antigen specific rather than antiidiotypic.

Received for publication 31 May 1983.

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