ANTIGEN- AND RECEPTOR-DRIVEN REGULATORY MECHANISMS

IV. Idiotype-bearing I-J$^+$ Suppressor T Cell Factors Induce Second-Order Suppressor T Cells Which Express Anti-Idiotypic Receptors*

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Regulation of the immune response to antigen involves the interactions of many cell types. There is substantial evidence attesting to the decisive role of suppressor T cells in the inhibition of both humoral (1) and cell-mediated (2) immune reactivity to a variety of antigens. In addition, regulatory molecules derived from such suppressor T cells have been identified in a number of experimental systems (reviewed in 3, 4).

Suppressor T cells (Ts)[1] operative in regulating responses to many antigens have been shown to express certain Lyt differentiation markers, as well as structures encoded by the I subregion of the H-2 major histocompatibility complex (MHC) (5). Furthermore, antigen-specific Ts-derived suppressor factors (TsF) have been found in many different systems to bear determinants encoded by the I-J subregions of the H-2 MHC (3, 4). Despite the above mentioned information, the mechanisms by which Ts and TsF exert their inhibitory effect is complex and not completely understood. Moreover, the precise number of phenotypically distinct Ts and TsF has not been determined. As a result, it has not been possible to formulate a single, all encompassing scheme which can explain related data from many different laboratories, each employing distinct experimental systems.

In an attempt to elucidate the role of antigen-specific Ts and their factor(s) in the regulation of cell-mediated immune response, over the past 3 y, our laboratory has been studying delayed-type hypersensitivity (DTH) to the p-azobenzenearsonate (ABA) hapten (6-8). Conjugates of this hapten evoke an anti-ABA antibody response in certain strains of mice, in which the antibody molecules bear cross-reactive idiotypic (CRI) structures and can be detected by anti-idiotypic antiserum.

[1] *Abbreviations used in this paper:* ABA, p-azobenzenearsonate; ABA-BSA, p-azobenzenearsonate-conjugated bovine serum albumin; ABA-SC, p-azobenzenearsonate-coupled syngeneic spleen cells; anti-CRI, anti-cross-reactive idiotypic antibodies; CRI, cross-reactive idiotype common to anti-ABA antibodies of A/J mice; DTH, delayed-type hypersensitivity; GAT, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GT, L-glutamic acid^{50}-L-tyrosine^{50}; HBSS, Hanks' balanced salt solution; IBC, idiotype-binding capacity; MHC, major histocompatibility complex; NMS, normal mouse serum; NRS, normal rabbit serum; PBS, phosphate-buffered saline; PBS-5, phosphate-buffered saline containing 5% fetal calf serum; RAMIg, rabbit antimouse immunoglobulin; TNP, trinitrophenol; TS, suppressor T cells; Ts$_1$, suppressor T cells induced by antigen; TsF, suppressor T cell factors derived from Ts$_1$; Ts$_2$, suppressor T cells induced by TsF; V$_H$, variable portion of the Ig heavy chain.

Previous studies have revealed that syngeneic cells conjugated with ABA and injected subcutaneously stimulate hapten-specific DTH. In contrast, a state of hapten-specific tolerance and the induction of ABA-specific Ts results when hapten-coupled syngeneic cells are injected i.v. (6). These Ts can be enriched on p-azobenzenearsonate-coupled syngeneic spleen cells (ABA-BSA)-coated dishes. Moreover, in vitro treatment of such ABA-specific Ts with well-defined anti-cross-reactive idiotypic (anti-CRI) antibodies and complement before transfer results in a complete loss of their suppressive activity (Dietz, M. H. et al. Manuscript in preparation.). Thus, Ts induced by antigen-coupled spleen cells (ABA-SC) bear idiotypic determinants on their surface and these determinants presumably mediate hapten binding. Furthermore, TsF can be obtained from these Ts either by in vitro culture or by physical disruption (7). In addition to I-J-controlled specificities, ABA-specific TsF also bear idiotypic determinants, the expression of which is linked to the heavy-chain allotype linkage group of genes (8). Therefore, it has been postulated that TsF utilize the variable portion of the Ig heavy chain (V_H) gene products in the construction of the antigen-binding site.

Another intriguing aspect of the immune regulatory suppressor pathway is the observation that many TsF, when administered to naive recipients or co-cultured with normal spleen cells, appear to induce second-order Ts. This phenomenon has been studied extensively with TsF specific for the synthetic copolymers L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT), and L-glutamic acid^{50}-L-tyrosine^{50} (GT) (9–11), and independently with carrier specific TsF by Tada and his colleagues (3, 12).

Based on these observations, it was postulated that administration of antigen under appropriate conditions will activate first-order suppressor T cells (Ts_1), capable of producing antigen-specific TsF. This TsF in turn stimulates another population of regulatory cells to develop into a mature second-order suppressor cell, referred to as Ts_2 (11, 13). However, the communications within this regulatory T cell circuit remain imprecisely defined (11–13).

The experiments presented in this paper document that in the ABA-specific suppressor system, I-J$^+$ TsF obtained from antigen-induced ABA-specific Ts_1, when injected into normal animals, also activate second-order suppressor T cells. In this system, a comparative analysis could be made of the respective specificity of the factor-producing Ts_1 and the factor-induced Ts_2. In contrast with antigen-induced Ts_1, which bears idiotype, these Ts_2 are shown to express anti-idiotypic receptors. Thus, the interaction between TsF and its target, Ts_2, are mediated by idiotype and anti-idiotype recognition. Both ABA-specific Ts_1 and anti-CRI-bearing Ts_2 suppress the generation of ABA DTH equally. The significance of these findings in relationship to the network theory of the immune response and the role of Ts_2 in suppression will be discussed.

Materials and Methods

Mice. A/J (H-2^a, Igh-1^e) female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. All animals in these experiments were 10–12 wk old at the time of the experiment. All experimental groups consisted of at least four animals per group.

Preparation of Antigen and Antigen-coupled Spleen Cells. These have been described in detail elsewhere (6). Briefly, a 40 mM solution of ABA diazonium salt was prepared from arsanilic acid (Eastman Kodak Co., Rochester, N.Y.). The ABA solution was activated as previously described and conjugated to single cell suspensions of erythrocyte-free spleen cells at a final concentration of 10 mM ABA. After washing twice in Hanks' balanced salt solution (HBSS), the ABA-SC were used to induce DTH or Ts as described hereafter.

Induction and Elicitation of DTH to ABA. Briefly, to induce DTH to ABA, 3×10^7 ABA-SC were injected subcutaneously into two separate sites on the dorsal flanks of mice. Challenge was administered 5 d later by injecting 30 μl of a solution of 10 mM ABA diazonium salt into the left footpad. 24 h later, DTH reactivity was assessed by measuring the footpad swelling using a Fowler micrometer (Schlesinger's Tools, Brooklyn, N.Y.). The magnitude of the DTH reaction was expressed as the increment of thickness of the challenged left footpad as compared with the untreated right footpad in 10^{-3} in ± SEM.

Induction and Assay of Ts and TsF. Normal A/J mice were injected intravenously with 5×10^7 ABA-SC. 7 d later, these mice served as donors of Ts. Spleens from such animals were removed, and a single cell suspension was prepared in HBSS. The cells were washed twice in HBSS and counted. To assay for the ability of such cells to inhibit ABA-specific DTH, 5×10^7 viable cells were injected i.v. into normal recipients, which were then primed s.c. with ABA-SC and challenged 5 d later. Production of TsF was done precisely as described earlier (7). Briefly, 1–5 $\times 10^8$ washed spleen cells 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 was followed by centrifugation at 10,000 g for 90 min and/or at 100,000 g for 45 min. The supernatants were adjusted to 5×10^8 cell equivalents/ml and frozen until use at $-40°C$. To test the ability of TsF to inhibit ABA-specific DTH, TsF was injected i.v. into normal mice beginning at the time of immunization with 3×10^7 ABA-SC. 2×10^7 cell equivalents of factor were administered each day for 5 successive d, at which time the animals were challenged and their footpad responses measured (7). To induce suppressor cells, TsF (2×10^7 cell equivalents/day) was injected i.v. into normal A/J mice for 5 successive d (13). On day 7, spleen cells were assayed for suppressive activity by adoptive transfer to normal recipients immunized and challenged as described above.

Antiserum Treatment. Anti-Thy 1.2 hybridoma antiserum was kindly provided by Dr. P. Lake, University College, London, England. Antisera to the CRI of A/J anti-ABA antibodies were prepared and quantitated as described (14). 1×10^8 spleen cells were incubated with 1 ml of 1:20 dilution of anti-Thy 1.2 hybridoma antibody or 25 μg idiotype-binding capacity (IBC) of the anti-CRI antibodies in 1 ml of 45 minutes at 0°C, washed once in HBSS, and then incubated again with 1 ml of a 1:10 dilution of Low Tox rabbit complement (Cedarlane Laboratories Ltd., London, Ontario, Canada) for 30 min at 37°C. The cells were then washed twice in HBSS, recounted, and resuspended for cell transfer.

Affinity Chromatography of ABA Suppressor Factor. Solid-phase immunoadsorbent columns were prepared and characterized as described earlier (7, 8). Anti I-J^k antiserum was prepared by immunizing (DBA/2 × B10.A[3R])F₁ mice with B10.A(5R) lymphocytes and was kindly provided by Dr. M. E. Dorf, the Department of Pathology, Harvard Medical School, Boston, Mass. Soluble ABA suppressor factor(s) were fractionated on immunoadsorbents in the following manner. 5-ml plastic columns containing Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.) conjugated with the relevant immunoglobulin were prepared and extensively washed with phosphate-buffered saline (PBS), pH 7.2, immediately before loading of the ABA suppressor factors. The absorption of the factor was performed at 4°C by allowing 5×10^8 cell equivalents of suppressor factor in 1 ml to enter the gel matrix; the suppressor factor was allowed to remain in the column for at least 60 min at 4°C. The column was then washed with at least five times its own void volume of PBS, pH 7.2. Such effluents were termed filtrates. Materials that remained adsorbed to the column were rapidly eluted with five bed volumes of a glycine-HCl buffer, pH 2.8. The collected eluate was immediately neutralized to pH 7.0 with 1 N NaOH as the material emerged from the column bed. Both the filtrate and the eluate were concentrated to the original volume by negative pressure dialysis at 4°C and were thereafter frozen at $-40°C$. Such materials were thawed immediately before use.

Cell Fractionation; Removal of B Cells. B cells were removed by a modification of the method described by Mage et al. (15). 4 ml of rabbit antimouse immunoglobulin (RAMIg) prepared as described (16) at a concentration of 0.7–1.0 mg/ml in PBS were added to 100 × 15 mm polystyrene petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 1 h at room temperature. Plates were washed extensively with PBS, pH 7.2, and then incubated for 20 min with 4 ml PBS with 5% phosphate-buffered saline containing 5% fetal calf serum (PBS-5) before addition of splenic cells. After removal of PBS-5, 60–75 × 10⁶ spleen cells depleted of erythrocytes by incubation with 0.83% (Tris) NH₄Cl were added to each plate in a volume of 5 ml PBS-5. After 30 min at 4°C, the plates were gently rocked, and after 1 h

incubation, nonadherent cells were removed by gently swirling and washing the plates with PBS-5. These nonadherent populations contained approximately 5–8% surface immunoglobulin-bearing cells as judged by immunofluorescence using fluorescinated-RAMIg.

Purification of Ts on ABA-BSA and Idiotype-coated Plates.   Specific suppressor cells were separated according to a modification of the method of Taniguchi and Miller (17). ABA-BSA was prepared as described (18). ABA-BSA coated plates were prepared by overnight incubation of 4 ml ABA-BSA at a concentration of 1 mg/ml on 100 × 15 mm polystyrene Petri dishes at 4° C. The ABA-BSA solution was then removed, the plates washed as indicated above for RAMIg, and after removal of the PBS-5, rinsed with PBS before addition of T cells. A/J anti-ABA antibodies which contain 20–70% CRI were prepared as described (19). CRI-coated dishes were prepared according to a modification of the method of Abbas et al. (20). Briefly, 4 ml of CRI (0.5–1.0 mg/ml in PBS) were incubated on 100 × 15 mm polystyrene Petri dishes for 2–3 h at room temperature. After removal of immunoglobulin solutions, the plates were washed with PBS, incubated 20–30 min with PBS-5, and washed extensively with PBS before the addition of T cells. 60–65 × 10^6 plate-purified T cells were added to antigen-coated plates or idiotype-coated plates in a total volume of 5 ml PBS-5. These cells were incubated for 1 h at 20°C. ABA-BSA plates were gently rocked at the midpoint of incubation. Nonadherent cells were then removed from ABA-BSA plates with three cycles of swirling and washing with warm (20°C) PBS-5. Nonadherent cells were removed from CRI-coated plates with two such cycles. Then 5 ml of chilled PBS-5 was added to all plates, which were then placed at 4°C for a further 30 min. The adherent cells were suspended by vigorous pipetting. After two additional chilled PBS-5 washes with vigorous pipetting, the adherent cells were pooled, spun at 200 g for 10 min, resuspended in PBS-5, and counted. The cells were divided into groups, washed two times with chilled HBSS, and brought to appropriate volume before transfer. These separation procedures routinely yielded a T cell population equal to 35–50% of the original spleen cells added to the RAMIg plates; cells adherent to ABA-BSA or CRI-coated dishes comprised 5–15% of the applied T cell population or ~2–7% of the total spleen cell population.

Statistical Analysis.   Analysis of the differences of data obtained was performed with the Wang programmable computer. The means and standard error of the mean (SEM) were expressed, as well as the relevant P value obtained with the two-tailed Student's t test.

Results

Induction of Ts by TsF.   Previous studies have revealed that subcellular antigen binding materials obtained from T cells of animals treated 7 d earlier with ABA-SC but not from T cells of normal animals, can suppress ABA-specific DTH (7). To characterize further the mechanism of action of TsF, we investigated whether the inhibitory effect of TsF was due to their ability to induce Ts_2 in the recipients, a property shared by many suppressor factors in other systems (3, 4, 13).

TsF were injected intravenously into groups of normal A/J mice for 5 successive d (2 × 10^7 cell equivalents/d) as described in Materials and Methods (7). On day 7, these mice served as donors of putative Ts_2. Single cell suspensions were prepared from the spleen, and before transfer to naive syngeneic recipients, two of the groups were treated in vitro with either normal mouse serum (NMS) or anti-Thy 1.2 antiserum and complement. The recipients of such spleen cells were immunized with ABA-SC subcutaneously and challenged 5 d later.

The results of a representative experiment are depicted in Fig. 1. As can be seen, spleen cells obtained from normal untreated animals were unable to suppress ABA DTH upon transfer. However, spleen cells obtained from animals which have been treated with TsF for 5 successive d produced a significant inhibition of DTH reactivity upon transfer to normal recipients. Furthermore, treatment with anti-Thy 1.2 and complement, but not NMS and complement resulted in a complete loss of their suppressive activities. Therefore, the phenomenon of suppression is T cell dependent.
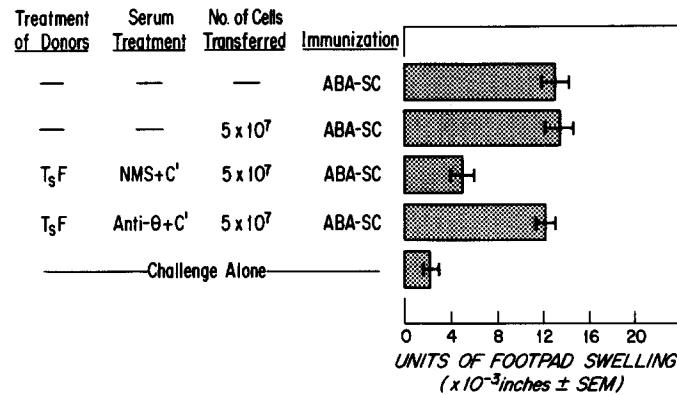
Fig. 1.   Induction and characterization of suppressor cells with TsF. Normal A/J mice were injected with 2×10^7 cell equivalents/d of TsF i.v. for 5 successive d. On d 7, 5×10^7 viable spleen cells from these animals were transferred to naive recipients. Two of the groups were treated in vitro with either anti-Thy 1.2 antiserum or normal mouse serum (NMS) and complement before transfer. All recipients and appropriate controls were immunized with 3×10^7 ABA-SC subcutaneously within 2 h of cell transfer. 5 d later, they were challenged in the footpad with 30 μl of diazonium salt of p-arsanilic acid, and the increase in footpad swelling was measured 24 h postchallenge. Bars represent the mean footpad swelling of at least four mice ± SEM.

In accord with earlier observations (9–12), the in vivo treatment with TsF induces Ts2.

Characterization of TsF Which Induces Ts2.   We have demonstrated previously that TsF which inhibits ABA-specific DTH does not bind to antibody, or to immunoglobulin C_H or C_L determinants. However, TsF activities can be removed upon passage through anti-idiotypic or appropriate anti-H-2 immunoadsorbent columns (7, 8). To delineate further whether the suppressive principle associated with the above mentioned TsF molecule is the same which is responsible for the activation of Ts2, we passed TsF from A/J mice through a variety of immunoadsorbent columns. Thereafter, the filtrate and the acid eluate from such columns were tested for their ability to induce Ts2.

The results of one such experiment are shown in Fig. 2. The data clearly demonstrate that the ability to induce Ts2 in normal mice was present only in the filtrate and not in the acid eluate fractions obtained when TsF was passed through a RAMIg-Sepharose immunoadsorbent column. In contrast, Ts2-inducing activity can be recovered in the acid eluate fraction when TsF was passed on either an anti-idiotypic or an anti-I-J^k immunoadsorbent column. From these observations, we conclude that the molecules responsible for the suppressive activities studied previously, and for Ts2-inducing ability share similar structural features including a lack of Ig constant region determinants, and cannot be distinguished at this time.

In Contrast with Ts1, Ts2 Are Resistant to Lysis with Anti-Idiotypic Antibodies and Complement.   Experiments from many different experimental systems have provided evidence that Ts1 bear idiotypic determinants on their surface (16, 21). However, there are also data indicating that Ts with similar suppressive activity may carry anti-idiotypic receptors (22–24). Recently, we have found that in vitro treatment of Ts induced by ABA-SC in A/J mice with anti-CRI and complement results in a complete loss of their suppressive activities. In contrast, when Ts were induced with
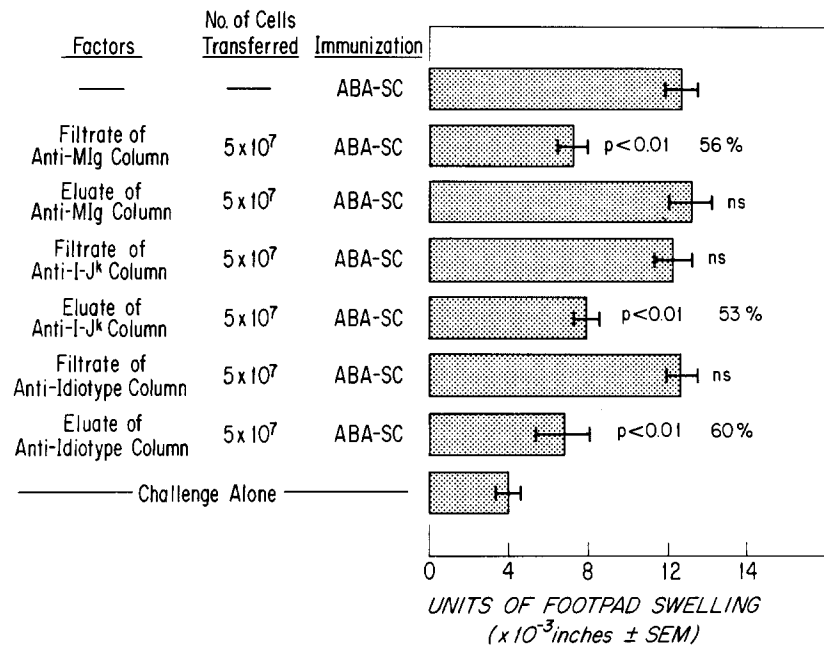
Factors	No. of Cells Transferred	Immunization		
——	——	ABA-SC		
Filtrate of Anti-MIg Column	5×10^7	ABA-SC	$p<0.01$	56%
Eluate of Anti-MIg Column	5×10^7	ABA-SC	ns	
Filtrate of Anti-I-J^k Column	5×10^7	ABA-SC	ns	
Eluate of Anti-I-J^k Column	5×10^7	ABA-SC	$p<0.01$	53%
Filtrate of Anti-Idiotype Column	5×10^7	ABA-SC	ns	
Eluate of Anti-Idiotype Column	5×10^7	ABA-SC	$p<0.01$	60%
———— Challenge Alone ————				

0   4   8   12   14

UNITS OF FOOTPAD SWELLING
($\times 10^{-3}$ inches $\pm$ SEM)

Fig. 2. Characterization of the principal components in the TsF complex which is responsible for the induction of Ts₂. 5×10^8 cell equivalents of TsF in 1 ml were passed through different immunoadsorbent columns as described in Materials and Methods. Both the filtrate and eluate from each column were concentrated back to the original volume, and injected into separate groups of normal A/J mice over 5 successive d. On day 7, 5×10^7 viable spleen cells from each individual groups were transferred to groups of naive recipients. All recipients and appropriate controls were immunized with 3×10^7 ABA-SC subcutaneously within 2 h of cell transfer. 5 d later, they were challenged in the footpad with 30 μl of diazonium salt of p-arsanilic acid, and the increase in footpad swelling was measured 24 h postchallenge. Bars represent the mean footpad swelling of at least four mice ± SEM. The number in parentheses indicates the percentage of suppression transferred. It was calculated according to the following formula:

$$\text{Percent tolerance} = \left(\frac{\text{positive control} - \text{experimental}}{\text{positive control} - \text{negative control}} \right) \times 100.$$

different haptenated cells (i.e., trinitrobenzenesulfonic acid-derivatized cells [TNP-cells]) or in animals which do not possess the necessary genetic information to express CRI determinants (i.e., BALB/c) these Ts were insensitive to the same treatment (Unpublished results.). In an attempt to compare Ts induced by antigen to those induced with Ts₁-derived TsF, we examined the effect of anti-idiotypic antibodies and complement treatment on these two types of Ts.

The results of a typical experiment are depicted in Fig. 3. Ts₁ induced by ABA-SC are sensitive to treatment with anti-idiotypic antibodies and complement. In contrast, the same treatment did not discernibly affect the ability of Ts₂ induced by TsF to transfer suppression. Thus, Ts induced by antigen (ABA-SC) bear idiotypic determinants on their surface whereas Ts₂ appear to lack these determinants.

Enrichment of Ts₂ with Idiotype-coated Dishes. The ability of Ts to bind directly to either antigen columns (25) or antigen-coated plates (17) has permitted the positive selection of such antigen-induced Ts. Similarly, Ts induced by intravenous injection
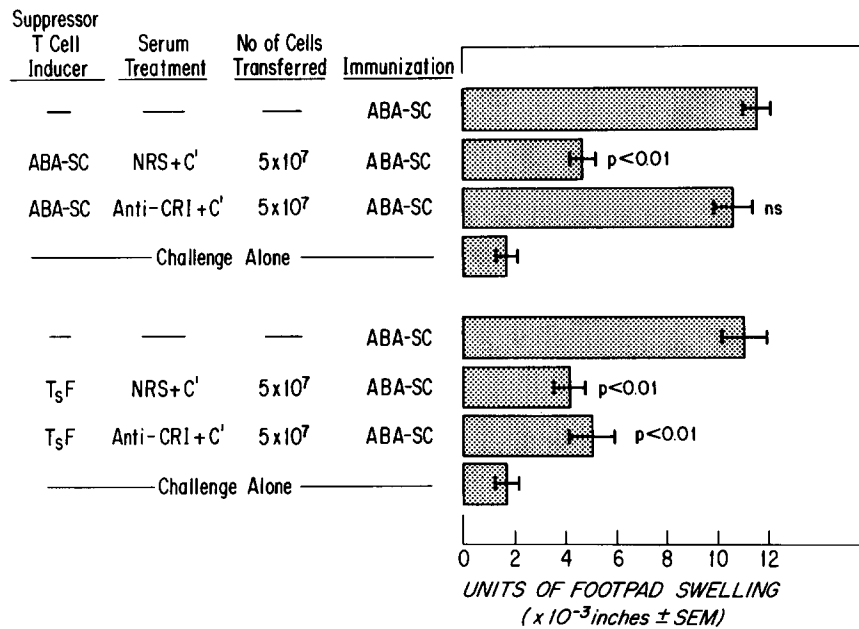
Fig. 3.   Characterization of Ts_1 and Ts_2 with respect to their sensitivity to in vitro treatment with anti-CRI antibodies and complement. Normal A/J mice were injected with 5×10^7 ABA-SC intravenously and were the donors of Ts_1. Ts_2 were induced by injection of TsF over 5 successive d $(2 \times 10^7$ cell equivalents/day). On day 7, single cell suspensions were prepared from their spleens and, before transfer to naive recipients, the cells were treated in vitro with either normal rabbit serum (NRS) or anti-CRI antibodies and complement. 5×10^7 viable cells were then transferred to different groups of naive recipients. All recipients and appropriate controls were immunized with 3 $\times 10^7$ ABA-SC subcutaneously within 2 h of cell transfer. 5 d later, they were challenged in the footpad with 30 μl of diazonium salt of p-arsanilic acid and the increase in footpad swelling was measured 24 h later. Bars represent the mean footpad swelling of at least four mice $\pm$ SEM.

of ABA-SC can be positively enriched on ABA-BSA but not TNP-BSA-coated plates. Such procedures usually produce a 16–50-fold enrichment of Ts when compared with the whole spleen population (Dietz, M. H. Manuscript in preparation.).

To investigate the specificity of Ts_2 induced by CRI^+ TsF as compared with antigen-specific Ts_1 induced by antigen, we examined whether Ts_2 bind to idiotype-coated plates.

The results of such an experiment are presented in Fig. 4. Ts induced by intravenous injection of ABA-SC can be enriched on ABA-coated plates, since cells which adhere to ABA-BSA-coated plates transfer significant suppression to naive recipients. However, ABA-SC-induced Ts do not adhere to idiotype-coated plates. In contrast with this binding characteristic of Ts induced by antigen, Ts_2 induced by TsF adhere to idiotype-coated plates but not to antigen-coated plates. In addition, the Ts_2 are not enriched on normal mouse Ig plates (Data not shown.). Thus, Ts induced by antigen bind to antigen, while Ts induced by CRI^+ TsF bind to idiotype, indicating an anti-idiotypic specificity for these Ts_2, consistent with their failure to be lysed by anti-CRI plus C.
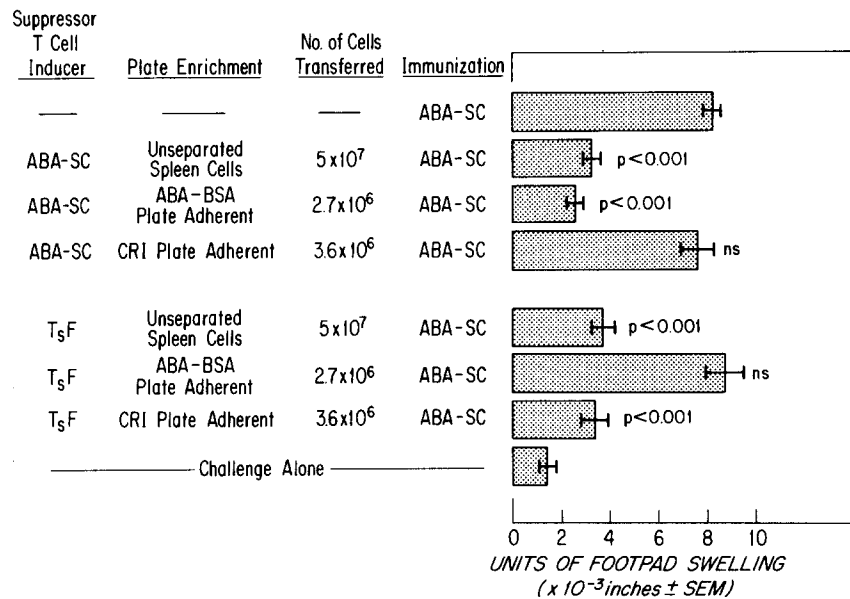
Suppressor T Cell Inducer	Plate Enrichment	No. of Cells Transferred	Immunization	
—	—	—	ABA-SC	
ABA-SC	Unseparated Spleen Cells	5×10^7	ABA-SC	p<0.001
ABA-SC	ABA-BSA Plate Adherent	2.7×10^6	ABA-SC	p<0.001
ABA-SC	CRI Plate Adherent	3.6×10^6	ABA-SC	ns
T_sF	Unseparated Spleen Cells	5×10^7	ABA-SC	p<0.001
T_sF	ABA-BSA Plate Adherent	2.7×10^6	ABA-SC	ns
T_sF	CRI Plate Adherent	3.6×10^6	ABA-SC	p<0.001
Challenge Alone				

0 2 4 6 8 10
UNITS OF FOOTPAD SWELLING
$(\times 10^{-3}$ inches $\pm$ SEM)

FIG. 4. Antigen induced Ts_1 binds to antigen plates while Ts_2 induced by TsF binds to CRI plates. Normal A/J mice were injected with 5×10^7 ABA-SC intravenously once or 2×10^7 cell equivalents of TsF per day for 5 successive d. On day 7, they were the donors of Ts_1 and Ts_2, respectively. Single cell suspensions were prepared from their spleen and a portion of the spleen cells were separated on different plates as described in Materials and Methods. Cells adhering to ABA-BSA or CRI plates were collected and transferred to different groups of naive recipients in various numbers. All recipients and appropriate controls were immunized with 3×10^7 ABA-SC subcutaneously within 2 h of cell transfer. 5 d later, they were challenged in the footpad with 30 μl of diazonium salt of p-arsanilic acid and increase in footpad swelling was measured 24 h later. Bars represent the mean footpad swelling of at least four mice $\pm$ SEM.

Discussion

The data presented above serve both to extend previous observations on the activity of soluble TsF (3, 4, 7–13, 25–30) by demonstrating that ABA-TsF also act to induce Ts_2 when administered to an immunologically naive recipient, and to provide new insight into the role of idiotypic interactions in cell to cell communication between T lymphocyte subsets, by directly demonstrating the ability of a nonimmunoglobulin, T cell-derived product to initiate an anti-idiotypic response.

Antigen-specific Ts regulate immunity at various levels. Previous studies have indicated that (a) the generation of immune cells (27) or (b) their functional expression (28, 29) can be limited by Ts in different systems. These have been termed afferent or efferent suppression, respectively. The studies herein do not directly address which phase of the ABA-specific immune response is dampened by either Ts_1 or Ts_2 cells. Nevertheless, many aspects of suppression in at least three well investigated systems are striking in their similarities, with respect to both the functional and structural characteristics of their responsible T cells and suppressor factors. Thus suppressor systems in the mouse specific for the synthetic copolymers GAT (4, 9, 10, 30), GT (11), and ABA (7, 8) are all characterized by the presence of a cyclophosphamide-sensitive antigen-specific cell which develops into the Ts_1 cell. This cell produced TsF which, in both systems, bears cross-reactive idiotypic determinants as well as I-J-encoded specificities. In previous studies in the ABA system (7, 8) polyvalent rabbit

anti-mouse F(ab')$_2$ with specificity for μ, γ, α, λ, κ determinants was unable to remove any of the ABA-specific TsF activity. Similarly, anti-H-2 subregion antiserum (exclusive of anti-I-J antibodies) could not deplete TsF of its suppressive capacity. Similar work in the GAT system has revealed that only anti-idiotypic (anti-cross-reactive idiotype of anti-GAT antibodies) or relevant I-J antibodies could interact with the TsF molecule elaborated by GAT specific Ts$_1$ (30). Furthermore, the active supernatants obtained from Ts$_1$ in the GAT and ABA as well as other suppressor systems (29) are characterized by the ability of these materials to induce the production of Ts$_2$. Thus, there are many concordant features of suppression in distinct systems, one operative to limit the DTH reactivity to ABA, and the other to limit the production of antibodies to GAT.

The major questions that were raised during these studies were (a) is the active material which suppresses ABA-specific immunity identical with that which induces Ts$_2$ and (b) can suppressive activity of TsF result in part or entirely from its ability to stimulate the generation of Ts$_2$? The data obtained in these studies strongly suggest that the two materials are identical and produced by similar, if not the same, cell. Ts$_1$ are idiotype-positive cells as deduced by their ability to be lysed by anti-idiotype and complement and furthermore bind selectively to ABA-coated plates. Recently, we have shown in preliminary experiments that Ts$_1$ cells which elaborate TsF used to induce Ts$_2$, also bind to ABA-coated plates and are sensitive to anti-idiotype and complement lysis (Unpublished result.). Hence, the cellular source of both suppressive materials is the same. Immunochemical analysis of the TsF endowed with the capacity to stimulate Ts$_2$ studied herein was accomplished by passage through well characterized immunoadsorbent columns. Such treatments clearly indicated that the material studied here has the same structural features as the TsF previously characterized. These features are the presence of CRI$^+$ determinants and I-J-encoded specificities. Hence, by these criteria, the material which stimulates Ts$_2$ and that which inhibits specific ABA reactivity are identical.

We have as yet not entirely defined the respective roles of Ts$_1$ or Ts$_2$. However, in experiments to be reported elsewhere, we have observed that Ts$_1$ cells do not suppress ABA DTH late in the immune response whereas Ts$_2$ cells do (Unpublished results.). Thus, Ts$_1$ may only function by virtue of the generation of Ts$_2$ cells. More detailed analysis of the functional properties of these Ts$_1$ or Ts$_2$ cells, however, is needed to elucidate their respective roles in immune regulation. It should be noted that a similar idiotype-anti-idiotype-determined T cell suppression occurs in the regulation of the immune response to the 4-hydroxy-3-nitrophenyl hapten in mice of the appropriate Igh-1 allotype (31). Furthermore, both idiotype- and anti-idiotype-bearing T$_s$F have been identified in A/J mice suppressed for the expression of the CRI antibodies (32).

Another important issue is the respective specificities of Ts$_1$ cells and Ts$_2$ cells in this suppressor pathway, a matter which could be adequately investigated in the ABA system. The data presented establish (a) that antigen-specific suppressor factor produced by Ts$_1$ cells induces idiotype-specific Ts$_2$ cells, and (b) that these idiotype specific Ts$_2$ cells can, upon adoptive transfer to a normal recipient, suppress its ability to develop ABA-specific DTH just as well as the passive transfer of an equal number of antigen-induced Ts$_1$ cells. However, before these data could be considered firmly established, in view of the ability of cell-bound antibody to induce similar anti-idiotype suppressor cells in the ABA system, as reported earlier by this laboratory

(33), certain critical controls had to be carried out to verify that the generation of Ts$_2$ cells was indeed stimulated by the TsF in the spleen supernate used. The ability to stimulate TsF could not be absorbed by anti-Ig immunoadsorbent columns but could be removed by and eluted from anti-I-J^k immunoadsorbent columns; these are properties which are characteristic of the immunochemical nature of the aforementioned suppressor factor in contrast with antibody. We conclude, therefore, that the CRI$^+$ TsF present, in amounts too small to detect so far by other than functional tests, is highly effective in generating anti-idiotype Ts$_2$. It remains to be determined whether in the GAT and GT systems that Ts$_2$ induced by TsF are also anti-idiotype, a matter which is presently under investigation. Similarly, Eardley et al. (34) have suggested that the V$_H$ restriction observed between inducer and target suppressor T cell in the feedback suppression model may reflect idiotype-anti-idiotype interactions.

Another prediction which could be made on the basis of the findings reported here is the existence of genetic restrictions controlled by V$_H$ genes, but not by MHC in the ability of ABA TsF to induce suppression in recipient mice. Preliminary results utilizing allotype congenics and H-2 congenic strains of mice indicate that such restrictions indeed exist.

The finding that Ts$_2$-induced CRI-TsF bear anti-idiotype receptors is in agreement with the earlier observations of Owen et al. (22, 23) who reported that Ts specific for the cross-reactive idiotype can be detected among T cells from suppressed A/J mice hyperimmunized with ABA-keyhole limpet hemocyanin. Similarly, Bona and Paul (24) have recently identified naturally occurring T suppressor cells with anti-idiotypic receptors specific for the MOPC 460 myeloma protein. In our own laboratory, we have also determined, as stated above, that the suppressor T cell induced by intravenous immunization with CRI$^+$ anti-ABA antibody conjugated to normal A/J spleen cells bear anti-idiotype receptors (Sy., M.-S. et al. Manuscript in preparation). The accumulated results from these and other laboratories demonstrating the involvement of idiotypic regulation at the T cell level extend considerably the importance of the idiotype and anti-idiotype regulatory network first proposed by Jerne (35), because the T lymphocytes appear at the center of critical regulatory interactions in the immune system. It is interesting also to speculate whether anti-idiotype Ts$_2$ produce an I-J+ suppressor factor and whether such a factor is capable of inducing suppressor T cells which would be anti-anti-idiotype or possibly antigen specific. Alternatively, the factor produced by Ts$_2$ could be acting directly upon its target.

The results in the ABA system presented in this and other reports from our laboratory lead to an interesting paradox. Both suppressor cells induced by ABA suppressor factor documented in this study and those induced by CRI bearing anti-ABA antibody coupled to syngeneic cells are anti-idiotype. Yet, they are able to suppress upon adoptive transfer a DTH response to ABA in syngeneic A/J mice, the effector cells of which appear not to bear idiotypic determinants, since they cannot be lysed by anti-idiotype and complement, whereas anti-idiotype-induced DTH effectors cells bear ABA CRI (36). This apparent contradiction could be explained by (a) the presence of idiotype in the precursors of the DTH effector cells, (b) the requirement for idiotype-specific helper T cells which could be the target of the suppression, (c) the requirement for the presence of another idiotype-positive T cell subpopulation other than suppressor T cells, T$_{DH}$ cells, or T helper cells. This situation has been shown to occur in the 2,4-dinitro-1-fluorobenzene contact sensitivity system (37) in which T

auxiliary cells are required for suppression; and an additional reason, (d) the ability of small amounts of idiotype-positive anti-ABA antibody concomitantly produced to bind with the T_{DH} cells after their interaction with antigen; such antibody could focus anti-idiotype Ts cells on the relevant T_{DH} cell. Experiments are now in progress to examine the aforementioned possibilities.

Lastly, if, as we are proposing, the stimulation of Ts_2 idiotypic determinants of the TsF has any in vivo relevance, we should be able to detect in the same animal first the idiotype-bearing Ts_1, followed by the appearance of anti-idiotypic Ts_2 in the course of tolerization with ABA-SC.

Summary

Administration of azobenzenearsonate (ABA)-coupled syngeneic spleen cells intravenously to A/J mice leads to the generation of suppressor T cells (Ts_1) which exhibit specific binding to ABA-bovine serum albumin (BSA)-coated dishes. These Ts_1 share idiotypic determinants with the major cross-reactive idiotype (CRI) of the anti-ABA antibodies of A/J mice, and also produce a soluble suppressor factor (TsF) bearing CRI and I-J subregion-coded determinants. Injection of this TsF into naive A/J mice elicits a second set of specific suppressor cells (Ts_2) which are not lysed by anti-CRI antibody plus C, and which do not bind to ABA-BSA-coated dishes. However, in contrast with Ts_1, these Ts_2 do bind to plates bearing CRI^+ anti-ABA immunoglobulin. Thus, Ts_2 exhibit anti-idiotypic specificity. These data indicate that antigen elicits the production of a soluble T cell product bearing both variable portion of the Ig heavy chain (V_H) and I-J subregion-coded determinants which serves to communicate between T cell subsets to establish an idiotype-anti-idiotype regulatory pathway.

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