ANTIGEN-SPECIFIC SUPPRESSOR T CELL INTERACTIONS

II. Characterization of Two Different Types of Suppressor T Cell Factors Specific for L-Glutamic Acid-S°-L-Tyrosine-S° (GT) and L-Glutamic Acid-S°-L-Alanine-3°-L-Tyrosine-1° (GAT)*

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The cellular interactions involved in antigen-specific suppressor T cell pathways are the subject of intense investigation. Although portions of the pathways have been elucidated in a variety of systems, there is no clear-cut information from these systems concerning the number of T cell subsets involved and the extent of interactions among these subsets. Our studies of suppressor T cell subsets have focused on responses to the synthetic polymers L-glutamic acid-S°-L-alanine-3°-L-tyrosine-1° (GAT) and L-glutamic acid-S°-L-tyrosine-S° (GT). Both of these antigens stimulate suppressor T cells in certain strains of mice (1, 2) and extracts of these suppressor T cells contain factors (TsF) that inhibit GAT- or GT-specific antibody responses by normal spleen cells or proliferative responses by primed T cells (3, 5). The TsF found in these extracts are antigen-binding proteins that bear determinants encoded by the I-J subregion of the H-2 gene complex (6, 7) and determinants that cross-react with idiotypic determinants on antibodies of the same specificity (8). No determinants encoded by IgCH region genes have been detected on these factors. GAT-TsF and GT-TsF specifically inhibit responses by allogeneic mice (4, 9, 10); this suppression is correlated with the induction of a second set of suppressor T cells (Ts2) (11-13). Furthermore, induction of these Ts2 cells by TsF is antigen dependent (11-13).

We have previously demonstrated that T cell hybridomas can be constructed which constitutively produce GAT-TsF or GT-TsF that are functionally and serologically identical to factors extracted from or secreted by suppressor T cells (14, 15). In addition, we have shown that monoclonal GT-TsF can induce specific unresponsiveness in vivo or in vitro and that this unresponsiveness correlates

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Abbreviations used in this paper: ABC, antigen-binding chain; DTT, dithiotreitol; GA, random linear copolymer of L-glutamic acid-S°-L-alanine-3°; GAT, random linear terpolymer of L-glutamic acid-S°-L-alanine-3°-L-tyrosine-1°; GT, random linear copolymer of L-glutamic acid-S°-L-tyrosine-5°; MBSA, methylated bovine serum albumin; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PFC, plaque-forming cell; SE, sheep erythrocyte; TsF, T cell-derived suppressor factor.

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with the development of antigen-specific suppressor T cells (Ts2) (16). T cell hybridomas have been constructed by fusion of BW5147 with GT-TsF1-induced Ts2 cells, and clones that produce suppressor factor (GT-TsF2) were isolated and characterized. GT-TsF2, like GT-TsF1, is antigen specific in suppressive activity and also in its antigen-binding capacity (14). However, GT-TsF2 differs from the GT-TsF1 used to induce it in that GT-TsF1 acts across allogeneic barriers, whereas GT-TsF2 does not. This restriction is controlled by genes in the H-2 complex and maps to the I-J subregion. Thus, GT-TsF2 closely resembles the carrier-specific, I-J*, genetically restricted factor described by Tada and Okumura (17) and the hapten-specific third-order factors (TsF3) described by Okuda et al. (18). Hybridomas producing monoclonal GAT-TsF1 and GAT-TsF2 were also generated when spleen cells from mice injected with syngeneic GAT-pulsed macrophages as neonates were fused with BW5147 as adults (15). These factors have properties identical to the GT-TsF1 and GT-TsF2 counterparts described above.

Studies reported in this manuscript demonstrate that these GT- and GAT-specific TsF1 and TsF2 are also structurally distinct suppressor factors. These molecules bear antigen-binding sites and determinants encoded by the I-J subregion; however, these two determinants reside on separate polypeptide chains and are linked by disulfide bonds in TsF2, whereas both determinants appear to reside on a single polypeptide chain in TsF1. We have prepared hybridomas producing monoclonal TsF2 from mice bearing three different H-2 haplotypes (a, b, and q) and have analyzed the ability of isolated antigen-binding chains (ABC*) and I-J* chains from these three TsF2 molecules to function when recombined with chains derived from other haplotypes. Results reported herein demonstrate that mixtures of heterologous chains are not functional, suggesting a restriction in the ability of the chains to form biologically active recombinant molecules. Furthermore, indirect evidence has been obtained which suggests that this restriction is due to the failure of the chains to interact with one another rather than the inability of the target cells to recognize both chains.

Materials and Methods

Mice. C57BL/10 (H-2b), B10. BR (H-2a), B10.Q (H-2q), BALB/c (H-2a), B10.S (H-2o), (B10 × B10.Q)F1 (H-2kq), and (B10 × CBA)F1 (H-2wk) mice were bred in the Animal Resources Facility at the Jewish Hospital of St. Louis. Mice were maintained on laboratory chow and water ad libitum and used when 3–6 mo old. Mice were vaccinated with the IHD-T strain of vaccinia virus at 5 wk of age.

Antigens. GAT (lot 111) and methylated bovine serum albumin (MBSA) were purchased from Sigma Chemical Co., St. Louis, MO; GT, lot 10, was purchased from Miles Laboratories Inc., Research Products Div., Elkhart, IN. GAT, GAT-MBSA, and GT-MBSA complexes were prepared to use as antigens in culture as previously described (19).

Anti-In (I-J) Antibodies and Immunoabsorbents. Monoclonal anti-I-J*, WF 9.40.5 [B10.A(5R) anti-B10.A(3R)] and anti-I-J*, WF8.C12.8 [B10.A(3R) anti-B10.A(5R)] were provided by Dr. Carl Waltenbaugh, Northwestern University, Evanston, IL (20). Monoclonal antibody that recognizes suppressor factors from H-2b mice (putative anti-I-J*; Ky 35) but not those from H-2k mice was prepared by Dr. Vera Haupfeid by immunizing AKR mice with the T cell hybridoma, 258C4.6, a sister hybridoma isolated from the same fusion as 258C4.4 (21). A monoclonal antibody that reacts with suppressor factors from several H-2b T cell hybridomas (putative anti-I-J*; Ky 81) was prepared from (B10.BR × AKR)F1 mice that had been immunized with a GAT-specific T cell hybridoma from
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C57BL/10 mice (21 and Hauptfeld, manuscript in preparation). Spleen cells from immunized mice were fused to the non-Ig-secreting myeloma line Sp2/0 Ag14 and clones producing antibodies were selected by immunofluorescence and cytotoxicity against the immunizing T cell hybridomas. Supernatant fluids from these hybridomas (Ky 35, Ky 81, WF9.40.5, and WF8.C12.8) were coupled to cyanogen bromide-Sepharose 4B (Sigma Chemical Co.) according to the manufacturer's directions. We found that mixing equal volumes of swollen beads with supernatant fluids diluted 1:5 in phosphate-buffered saline (PBS) reproducibly resulted in >95% of the protein coupling to the beads. Further, these beads were capable of absorbing factors bearing the appropriate I-J haplotype. GAT and GT were individually coupled to aminohexyl Sepharose 4B (Sigma Chemical Co.) at 2 mg/ml as previously described (6).

Undiluted supernatant fluids from T cell hybridomas were applied to antigen-coupled beads at a ratio of 5 vol of supernatant fluid to 1 vol of Sepharose beads in the presence or absence of 5 mM dithiothreitol (DTT). After reacting for 1 h at 4°C, the beads were washed with PBS and the effluent was collected; the adsorbed TsF was eluted by reaction with 2 M KCl as previously described (6). After dialysis against PBS (pH 7.0) to remove the KCl and DTT, TsF was diluted 1:5 with PBS and reacted with anti-I-J-Sepharose 4B in a ratio of 5:1 as above. The beads were washed with PBS, the effluent was collected, and adsorbed material was eluted with 2 M KCl. Amounts of TsF added to cultures have been corrected for dilution during these procedures as far as possible.

T Cell Hybridomas. Production of GAT- and GT-specific TsF1- and TsF2-producing hybridomas has been described in detail (14–16). Once the T cell hybridomas were selected and cloned, they have been maintained as frozen stocks in liquid nitrogen.

Cell Culture and Assay of T Cell Hybridoma Supernatant Fluids. Development of primary splenic plaque-forming cell (PFC) responses was measured in vitro under modified Mishell-Dutton conditions as described previously (15, 19). PFC responses in cultures stimulated with 5 μg/ml GAT, GAT-MBSA, GT-MBSA, or 10^7 sheep erythrocytes (SE) were assayed on day 5 using GAT-SE and SE as indicator cells in a slide modification of the Jerne hemolytic plaque assay.

Supernatant fluids from hybridoma cultures were diluted and added to assay cultures at initiation unless otherwise stated. Unpurified supernatant fluids from suppressor T cell hybridomas routinely inhibit specific responses by 75–100% at final dilutions of 1:10,000 (14–16). Data are presented as GAT-specific IgG PFC/culture or percent control response. As a semiquantitative measure of suppressive activity, in some experiments the supernatant fluids have been titrated and the data plotted as percent suppression versus the log_{10} of the inverse of the dilution; those data have been reported as S_{50} U/ml, the inverse of the dilution that caused 50% suppression derived by interpolation. Active supernatants suppress responses by 75–100% whereas supernatants that suppress responses by <20% were considered negative and the titer recorded as less than the inverse of the lowest dilution tested, e.g., <10,000. The specificity of these supernatant fluids was verified by their addition to cultures stimulated with an irrelevant antigen (SE); none of the factors inhibited the PFC response to SE and these data have not usually been shown. The experiments presented have been performed several times and the results are very reproducible. Thus, data shown are from representative experiments.

Results

Derivation and Characterization of T Cell Hybridomas and Suppressor Factors. The characteristics of the T cell hybridoma-derived suppressor factors used in these experiments are summarized in Table I. Most of these results have been reported elsewhere (14–16, 22, 23). T cell hybridomas producing GT-TsF1 (367A5.4) and GAT-TsF1 (258C4.4) were derived by fusing GT- or GAT-primed splenic T cells with BW5147 (14). The GT-TsF2-producing hybridoma (469B5.A5) was derived by fusion of T cells from B10.A mice that had been injected with a mixture of monoclonal GT-TsF1 plus 10 ng GT (16). For studies reported here,
TABLE 1

Characteristics of T Cell Hybridomas and Suppressor Factors*

| Hybridoma | Strain | H-2 | Type TsF | MHC restriction | Antigenic specificity | Idiotype |
|-----------|--------|-----|----------|-----------------|----------------------|----------|
| 367A5.4   | CBA    | k   | GT-TsF1  | none            | +        | NT       |
| 469B5.A5  | B10.A  | a   | GT-TsF2  | I-J            | +        | NT       |
| 258C4.4   | DBA/1  | q   | GAT-TsF1 | none            | +        | NT       |
| 762B3.7   | B10.Q  | q   | GAT-TsF2 | H-2k           | +        | NT       |
| 372B3.5   | B10    | b   | GAT-TsF1 | none            | +        | NT       |
| 372D6.5   | B10    | b   | GAT-TsF2 | I-J            | +        | NT       |

* All hybridomas were produced by fusion of antigen-primed splenic T cells with the AKR thymoma BW5147.Ag.1.4.

1 Binding specificity was determined by filtration through antigen-Sepharose beads as described in Materials and Methods. Suppressive activity was completely removed (+) or not removed at all (−) (14, 15, 22).

2 Expression of determinants that cross-react with mouse anti-GAT antibodies. The CGAT idiotype is expressed by GT-binding antibodies; the GA-1 idiotype is expressed by GA-binding antibodies (22). The antibodies were coupled to Sepharose and the suppressor factors were filtered through; suppressive activity was completely removed (+) or not removed at all (−) (15, 22).

3 Not tested.

we constructed an H-2k-restricted GAT-TsF2 (762B3.7) by fusing T cells from B10.Q mice previously injected with GAT-TsF1 (258C4.4) plus 10 ng GAT. GAT-specific suppressor T cells from C57BL/10 mice, which are responders to GAT, were induced by injection of neonatal mice with GAT-pulsed macrophages. Restimulation of spleen cells from these mice as adults by incubation with GAT in vitro produced activated T cells that, when fused with BW5147, gave rise to two types of hybridomas (15). One of these is antigen-specific but genetically unrestricted (372B3.5), a GAT-TsF1 by analogy with 367A5.4 and 258C4.4. The other type of suppressor T cell hybridoma is represented by 372D6.5, which is antigen specific and genetically restricted and therefore referred to as GAT-TsF2.

As mentioned above, all of the suppressor factors derived from these hybridomas are antigen specific in suppressive activity and all have binding sites specific for the inducing antigen. Analysis of the fine specificity of these factors has been determined by the ability of these factors to bind Sepharose beads coupled with the related polymers, GAT, GT, or L-glutamic acid-L-alanine (GA). All of these factors bind to GAT whether they are induced by GT or GAT. The factors induced by GT bound GT but not GA, whereas the factors induced by GAT segregated into two classes: those which bound GAT and GA (258C4.4 and 762B3.7) and those which bound GAT and GT (372B3.5 and 372D6.5). The expression of determinants on these suppressor factors that cross-react with idiotypic determinants on antibodies to GAT and GA has been assessed only in a limited fashion. Nevertheless, the emerging pattern indicates that all of the GAT- and GT-binding factors that have been tested are bound by guinea pig anti-CGAT antibodies (15, 22, 23) and that the one GT(GAT) binding factor (469B5.A5) tested for binding to anti-GA-1 does not. On the other hand, the GA-binding factor, 258C4.4, was bound by both anti-CGAT and anti-GA-1.
Since the anti-CGAT is now known to contain small amounts of anti-GA-1 activity (24), the reactivity of 258C.4.4 with anti-CGAT is probably the result of interaction with the contaminating antibodies. The correlation between GT-binding activity and the expression of the CGAT idiotype and the GA-binding activity with the expression of the GA-1 idiotype was originally defined for GAT-specific antibodies (25). Interestingly, this pattern is also reflected in the limited number of monoclonal suppressor factors that have been examined.

**Affinity Purification of TsF1 and TsF2 Under Reducing and Nonreducing Conditions.** GT-TsF1 (367A5.4) and GT-TsF2 (469B5.A5) were filtered through GT-Sepharose beads in the presence or absence of 5 mM DTT. The effluents were collected, the beads washed extensively, and the bound material was then eluted with 2 M KCl. All fractions were dialyzed and assayed for biological activity in cultures of B10.BR spleen cells stimulated with GT-MBSA or SE (Table II). It is clear that GT-TsF1 binds to GT-Sepharose and that 2 M KCl elutes the biologically active material in the presence or absence of reducing agent. By contrast, GT-TsF2 binds to and can be eluted from GT-Sepharose in the absence of reducing agent, but in the presence of 5 mM DTT, neither the effluent nor the eluate alone are active. Adding both the effluent and eluate to culture reconstitutes the suppressive activity of TsF2. This suggests that, in the native state, GT-TsF2 is a complex of at least two polypeptide chains, one of which is antigen binding, held together by disulfide bonds. The same results have been obtained with TsF1 and TsF2 derived from H-2^a and H-2^b hybridomas (not shown).

The next experiment demonstrated that the determinant encoded by the I-J subregion of the H-2 complex is on the same polypeptide chain as the antigen-binding site in TsF1 (Fig. 1), but is on the non-antigen-binding polypeptide in TsF2 (Fig. 2). The adsorption of TsF1 and TsF2 with GAT-Sepharose in the presence of 5 mM DTT was performed as described in the previous experiment.

**Table II**

| TsF          | GT-Sepharose fraction | S_50 U/ml* |
|--------------|-----------------------|------------|
|              |                       | Minus DTT  | Plus DTT   |
| GT-TsF1 (367A5.4) | Unfractionated       | 13,000     | 21,000     |
|              | Effluent A            | <1,000     | 4,400      |
|              | Eluate B              | 75,000     | 75,000     |
|              | A + B                 | 46,000     | 75,000     |
| GT-TsF2 (469B5.A5) | Unfractionated       | 100,000    | 80,000     |
|              | Effluent A            | <5,000     | <5,000     |
|              | Eluate B              | 130,000    | <10,000    |
|              | A + B                 | 90,000     | 104,000    |

* Hybridoma supernatant fluids containing GT-TsF1 or GT-TsF2 were filtered through GT-Sepharose in the presence or absence of 5 mM DTT. Unfractionated factors, effluents, and material eluted with 2 M KCl were dialyzed, and various dilutions were added to spleen cells from B10.BR mice stimulated with GT-MBSA or SE. No suppression of SE PFC was observed (data not shown).
Figure 1. GAT-TsF1 (372B3.5) was fractionated over GAT-Sepharose in the presence of DTT, and the effluent and KCl eluate were subsequently filtered through anti-I-Jα Sepharose beads in the absence of DTT. Effluents and KCl eluates were collected, dialyzed, and added to cultures of spleen cells from C57BL/10 mice. Cultures were stimulated with GAT or SE (not shown) and the results expressed as the percent of the control PFC response which was 365 PFC/culture in this experiment.

Figure 2. GAT-TsF2 (372D6.5) was fractionated and assayed as described in legend to Fig. 1. The control PFC response to GAT in this experiment was 395 PFC/culture.

After dialysis against PBS, the effluents and eluates were separately applied to anti-I-Jα Sepharose beads; the effluents were collected, the beads washed with PBS, and the adsorbed factors eluted with 2 M KCl. These fractions were again dialyzed against PBS and added at a 1:10,000 final dilution to C57BL/10 spleen cell cultures stimulated with GAT or SE. The results, as percent of the control response to GAT, are shown in parentheses. GAT-TsF1 (372B3.5) activity is eluted from GAT-Sepharose and this activity can subsequently be adsorbed to and eluted from anti-I-Jα Sepharose (Fig. 1). Our interpretation of this experiment is that GAT-TsF1 is composed of a single polypeptide chain bearing both the antigen-binding site and I-J determinant. GAT-TsF2 (372D6.5), as shown in Table II, is dissociated into two chains by the reduction of disulfide bonds with DTT; one of these chains is antigen binding while the other is not. Subsequent filtration of the isolated chains through anti-I-Jα Sepharose demonstrated that
the non-ABC is adsorbed to and eluted from anti-I-J Sepharose, whereas the ABC is not adsorbed by anti-I-J⁰ Sepharose (Fig. 2). Thus, we conclude that GAT-TsF2 is composed of two polypeptide chains; one is antigen-binding and I-J⁺ and the other is non-antigen-binding but I-J⁺. Identical results have been observed with GAT-TsF2 from H-2¹ and GT-TsF2 from H-2² T cell hybridomas (data not shown).

Since a mixture of the isolated chains of GAT- (or GT-) TsF2 reconstituted the biological activity of this factor, we wondered whether the isolated chains actually reassocciated in a covalently linked form by reformation of the disulfide bridge, a phenomena that has been reported for Ig heavy and light chains (26). To examine this possibility, isolated ABC⁻,I-J⁺ and ABC⁺, I-J⁻ chains from 469B5.A5 GT-TsF2 were mixed together and refiltered through GT-Sepharose in the absence of reducing agents. If the chains had reassocciated in a stable covalent linkage, the biological activity would have been detected in the KCl eluate, whereas if the chains did not reassociate, then neither the effluent nor the eluate would be active and biological activity would require mixture of the effluent and eluate. The results, shown in Table III, display the latter pattern and thus indicate that biological activity can be retained by isolated chains of TsF2 in the absence of covalent linkage between the chains.

Although the data in Table III suggest that reformation of the interchain disulfide bonds is not essential to the biological activity of TsF2, the relative importance of disulfide bonds and/or sulfhydryl groups of TsF2 is illustrated in Table IV. In this experiment, TsF1 and TsF2 were reduced with 5 mM DTT and adsorbed to anti-I-J⁰-Sepharose beads. The effluents and KCl eluates were collected and a portion of each was incubated with 10 mM iodoacetamide to alkylate the sulfhydryl groups. After dialysis, the suppressive activities of the effluents and eluates were assayed. Reduction and alkylation had little or no effect on the biological activity of GAT-TsF1, but abolished the ability of the I-J⁺ and I-J⁻ chains of GAT-TsF2 to suppress the response to GAT (Table IV).

### Table III

| Isolated chains* | GT-Sepharose absorption† | S₅₀ U/ml | Exp. 1 | Exp. 2 |
|------------------|--------------------------|----------|--------|--------|
|                  |                          | GT-MBSA  | SE     |        |
|                  |                          |          |        |        |
|                  |                          | GT-MBSA  | SE     |        |
| + +              | Effluent (A)             | <4,000   | <4,000 | NT     |
| + +              | Eluate (B)               | <4,000   | <4,000 | <3,000 |
| + +              | (A) + (B)               | 80,000   | <4,000 | <3,000 |

* TsF2 from 469B5.A5 were separated into two chains by filtration through GT-Sepharose in the presence of 5.0 mM DTT.

† Isolated chains from 469B5.A5 were dialyzed to remove DTT, mixed together, and filtered through GT-Sepharose in the absence of DTT. The effluent, eluate, and mixtures of the two were assayed for suppressive activity.

‡ Not tested.
TABLE IV

Effect of Reduction and Alkylation on TsF1 and TsF2 Activity

| TsF          | Anti-I-Jb Sepharose fractionation + DTT* | Percent control PFC response* |
|--------------|-----------------------------------------|-------------------------------|
|              | Untreated                               | Alkylated                     |
| GAT-TsF1 (372B3.5) | Unfractionated                          | <10                           | NT*                           |
|              | Effluent A                              | 74                            | 103                           |
|              | Eluate B                                | <10                           | 38                            |
|              | A + B                                   | NT                            | NT                            |
| GAT-TsF2 (372D6.5) | Unfractionated                          | 15                            | NT                            |
|              | Effluent A                              | 108                           | 147                           |
|              | Eluate B                                | 97                            | 205                           |
|              | A + B                                   | <10                           | 110                           |

* Unfractionated factors and the isolated chains were added to cultures of spleen cells from B10 mice at a final dilution of 1:10,000. Cultures were stimulated with GAT or SE; none of the fractions inhibited responses to SE. The data shown represent the percent of the control response to GAT in the absence of TsF, which was 405 PFC/culture.

** TsF1 and TsF2 were equilibrated with 5 mM DTT and filtered through anti-I-Jb Sepharose; adsorbed material was eluted with KCl. The isolated chains were dialyzed to remove DTT and KCl and either not treated further or alkylated by incubation with 10 mM iodoacetamide at room temperature for 20 min followed by dialysis and testing.

Not tested.

TABLE V

Kinetics of Suppression by TsF2

| Group | Day added to culture* | ABC+ I-J- | ABC- I-J+ | PFC/Culture |
|-------|-----------------------|-----------|-----------|-------------|
| A     | -                     | -         | -         | 435         | 9,990       |
| B     | 0                     | -         | -         | 365         | 6,015       |
| C     | -                     | 0         | 0         | 420         | 8,400       |
| D     | 0                     | 0         | 0         | 70          | 6,285       |
| E     | 1                     | 1         | 1         | 580         | 7,055       |
| F     | 2                     | 2         | 2         | 530         | 6,210       |
| G     | 1                     | 0         | 0         | 560         | 4,580       |
| H     | 2                     | 0         | 0         | 600         | 6,975       |
| I     | 0                     | 1         | 4         | 40          | 8,205       |
| J     | 0                     | 2         | <5        | 8,295       |
| K     | 0                     | 3         | 20        | 11,600      |
| L     | 0                     | 4         | 524       | 10,340      |

* Isolated chains from 372D6.5 GAT-TsF2 were added to spleen cells from B10 mice at 1:10,000 final dilution at culture initiation (day 0) or on day 1, 2, 3 or 4. Cultures were stimulated with GAT or SE, and IgG PFC responses against GAT and SE were assayed on day 5.

Kinetics of the Addition of Isolated TsF2 Chains to Culture. After observing that the isolated chains from TsF2 could suppress immune responses without disulfide linkage between the chains, we asked whether both chains needed to be added to the culture simultaneously. The results of one such experiment are shown in Table V. Neither I-J+ nor I-J- chains suppressed singly, and, when mixed
together, suppressed GAT-specific responses only when added at culture initiation and not if added 24 or 48 h later. Similarly, unfractionated TsF2 suppressed responses only when added at culture initiation (not shown). Addition of the I-J$^+$ chain at time 0 and the ABC at 1 or 2 d did not reconstitute suppressive reactivity. However, if the ABC is added to cultures at initiation, then suppression can be reconstituted by the addition of the I-J$^+$ chain on day 1, 2, or 3.

**Are Heterologous Mixtures of the Isolated Chains from TsF2 Factors from Different H-2 Haplotypes Immunosuppressive?** The last issues to be addressed in this report are (a) a comparison of the restriction patterns in suppressive activity between TsF1 and TsF2, and (b) whether ABC from one TsF2 can be recombined with I-J$^+$ chains from another TsF2 with reconstitution of suppressive activity, and, if so, whether the restriction pattern is dictated by the haplotype of the I-J$^+$ chain. The PFC responses to GAT or GAT-MBSA are inhibited by GAT-TsF1 derived from mice that are syngeneic or allogeneic to the responding spleen cells (Table VI). There is no significant difference in the degree or extent of suppression observed in responses by strains of mice that are responders or nonresponders to GAT. Moreover, GAT-TsF1 from responder mice (372B3.5) suppressed responses to GAT-MBSA by nonresponder B10.Q and B10.S spleen cells and responses to GAT and GAT-MBSA by responder BALB/c and B10 spleen cells. Similarly, GAT-TsF1 from nonresponder mice (258C4.4) suppressed responses by spleen cells from both responder and nonresponder mice.

By contrast, intact two-chain factors have been shown to be restricted in suppressive activity by the I-J subregion of the H-2 gene complex (15, 16). The same restriction is observed when isolated TsF2 chains are mixed together in homologous pairs and added to cultures of syngeneic or allogeneic spleen cells (Table VII). Because all of the strains to be tested are able to respond to GAT-MBSA and all of the factors can bind to GAT, the cultures in these experiments were all challenged with GAT-MBSA rather than some with GAT-MBSA and

**Table VI**

*Suppression by TsF1 Is Not MHC-Restricted*

| Responding spleen cells | H-2 | GAT-TsF1 added* | Day 5 IgG PFC/culture† |
|-------------------------|-----|----------------|------------------------|
|                         |     | GAT | GAT-MBSA | SE |
| B10                     | b   |     |           |    |
| 372B3.5                 |     | <10 | <10       | 1345       |
| 258C4.4                 |     | <10 | <10       | 2000       |
| B10.Q                   | q   |     |           |    |
| 372B3.5                 |     | <10 | <10       | 1310       |
| 258C4.4                 |     | <10 | <10       | 1320       |
| BALB/c                  | d   |     |           |    |
| 372B3.5                 |     | <10 | 40        | 815        |
| 258C4.4                 |     | <10 | 25        | 850        |
| B10.S                   | s   |     |           |    |
| 372B3.5                 |     | 20  | 320       | 1450       |
| 258C4.4                 |     | 15  | 15        | 1020       |

* GAT-TsF1 (372B3.5 [H-2b] and 258C4.4 [H-2d]) were added to spleen cell cultures at initiation, at a final dilution of 1:10,000.

† Cultures were stimulated with GAT, GAT-MBSA, or SE, and PFC responses to GAT and SE were assayed on day 5.
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TABLE VII

Are Heterologous Mixtures of TsF2 Chains Immunosuppressive?

| Experiment | Haplotype* | S<sub>90</sub> U/ml in response to GAT-MBSA<sup>b</sup> |
|------------|------------|--------------------------------------------------|
|            | I-J<sup>+</sup> | ABC<sup>+</sup> | B10 | B10.BR |
| 1          | a          | a          | <10,000 | 54,000 |
|            | b          | b          | 73,000  | <10,000 |
|            | a          | b          | <10,000 | <10,000 |
|            | b          | a          | <10,000 | <10,000 |
|            | B10.Q      | B10.BR     | <10,000 | <10,000 |

a a <10,000 54,000
b b 73,000 <10,000
a b <10,000 <10,000
b a <10,000 <10,000
B10.Q B10.BR

* Isolated chains from 469B5.A5 (H-2<sup>a</sup>), 372D6.5 (H-2<sup>b</sup>), and 762B3.7 (H-2<sup>q</sup>) were prepared as described in Table II.

* Isolated chains were mixed, diluted, and added to cultures of spleen cells from the indicated strains, which were stimulated with GAT-MBSA. PFC responses to GAT were assayed on day 5.

The first combination to be tested was isolated chains from 372D6.5 (H-2<sup>b</sup>) and 469B5.A5 (H-2<sup>a</sup>) (experiment 1, Table VII). It is clear that the suppression of responses by B10 spleen cells occurred only when the I-J<sup>+</sup> and ABC<sup>+</sup> chains from 372D6.5 were mixed together and that suppression of responses by B10.BR spleen cells occurred only when both the I-J<sup>+</sup> and ABC<sup>+</sup> chains derived from 469B5.A5 were mixed. The same phenomenon occurred when chains from 469B5.A5 (H-2<sup>a</sup>) and 762B3.7 (H-2<sup>q</sup>) were mixed (experiment 2, Table VII). A small degree of suppression, ~10% of the suppressive activity of 762B3.7, has occasionally been observed in the responses by B10.Q spleen cells incubated with I-J<sup>+</sup> chain of H-2<sup>q</sup> and the ABC<sup>+</sup> chain from H-2<sup>b</sup>.

There are two straightforward interpretations of these data. First, there could be steric hindrance or restrictions in the ability of two chains from different haplotypes to deliver a functional suppressive signal. Alternatively, chains from two different haplotypes could form a functional unit, but the target cells used might not bear appropriate receptors that recognize both chains. To choose between these alternatives, we tested the ability of these recombinant molecules to inhibit responses by spleen cells from F<sub>1</sub> hybrid mice (Table VIII). These data confirm a previous observation that TsF2 molecules can suppress responses of F<sub>1</sub> mice if one of the parental mice in the F<sub>1</sub> is syngeneic with the TsF2 donor (16). However, none of the putative recombinant TsF2 suppressed responses by spleen cells from F<sub>1</sub> mice.

**Discussion**

We previously presented evidence that two functionally different types of GAT/GT-specific suppressor factors could be produced by T cell hybridomas (14-16). Both types of factors were antigen specific in suppressive activity and both had antigen-specific binding sites. Nevertheless, these factors could be others with GT-MBSA. The first combination to be tested was isolated chains from 372D6.5 (H-2<sup>b</sup>) and 469B5.A5 (H-2<sup>a</sup>) (experiment 1, Table VII). It is clear that the suppression of responses by B10 spleen cells occurred only when the I-J<sup>+</sup> and ABC<sup>+</sup> chains from 372D6.5 were mixed together and that suppression of responses by B10.BR spleen cells occurred only when both the I-J<sup>+</sup> and ABC<sup>+</sup> chains derived from 469B5.A5 were mixed. The same phenomenon occurred when chains from 469B5.A5 (H-2<sup>a</sup>) and 762B3.7 (H-2<sup>q</sup>) were mixed (experiment 2, Table VII). A small degree of suppression, ~10% of the suppressive activity of 762B3.7, has occasionally been observed in the responses by B10.Q spleen cells incubated with I-J<sup>+</sup> chain of H-2<sup>q</sup> and the ABC<sup>+</sup> chain from H-2<sup>b</sup>.

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**Table VIII**

Are Heterologous Mixtures of TsF2 Chains Immunosuppressive When Tested on F1 Spleen Cells?

| Experiment | Haplotype* | $S_{50}$ U/ml in Response to GAT-MBSA1 |  |
|------------|------------|----------------------------------|---|
|            | I-J*       | ABC*                             | B10 | B10.Q | (B10 × B10.Q)F1 |
| 1          | q          | q                                | <10,000 | 35,000 | 56,000 |
|            | b          | b                                | 43,000 | <10,000 | 51,000 |
|            | q          | b                                | <10,000 | <10,000 | <10,000 |
|            | b          | q                                | <10,000 | <10,000 | <10,000 |
|            |           |                                  | B10  | B10.BR | (B10 × CBA)F1 |
| 2          | a          | a                                | <10,000 | 80,000 | 34,000 |
|            | b          | b                                | 36,000 | <10,000 | 47,000 |
|            | a          | b                                | <10,000 | <10,000 | <10,000 |
|            | b          | a                                | <10,000 | <10,000 | <10,000 |

* Isolated chains from 762B3.7 (H-2q), 372D6.5 (H-2b), and 469B5.A5 (H-2") were prepared as described in Table II.

* Isolated chains were mixed, diluted, and added to cultures of spleen cells from the indicated strains, which were stimulated with GAT-MBSA. PFC responses to GAT were assayed on day 5.

Distinguished by the pattern of suppressive activity on responses by allogeneic mice. The factors we call first-order factors (TsF1) suppress in a genetically unrestricted manner; in previous studies, TsF1 obtained as extracts from mice that are nonresponders to GAT suppressed responses by all other allogeneic nonresponder mice (9). Furthermore, responses of mice that were responders to GAT could be suppressed by extract TsF1 from nonresponders only if the spleen cells were pretreated with TsF1 before antigenic stimulation (9, 12). The data in Table VI clearly show that hybridoma-derived TsF1 suppressed across allogeneic barriers when added to cultures at initiation, whether the spleen cell cultures were derived from responder or nonresponder mice. The reason for the discrepancy in the ability of nonresponder-derived TsF1 to suppress responder strains is unknown, but may be related to differences in extracted and hybridoma-derived TsF1 or to the fact that the mice used are healthier now that Sendai virus has been eliminated from our mouse colony.

By contrast to TsF1, the second-order factors (TsF2) are genetically restricted and suppression is only manifest when the strain of origin of TsF2 and the target-responding spleen cells are syngeneic at the I-J subregion (15, 16). Data presented above clearly demonstrate that the molecular structures of these two types of factors are also different. As previously suggested (27), TsF1 molecules are composed of a single polypeptide chain that bears both the antigen-binding site and I-J determinant, as demonstrated by the failure of reduction of disulfide bonds to separate these markers during subsequent immunoadsorption procedures (Table IV). Furthermore, reduction and alkylation failed to significantly reduce the biological activity of TsF1 (Table IV), which suggests that if disulfide bonds or sulfhydryl groups are present in TsF1, they are not essential to the biological activity. By contrast, undenatured TsF2 bears both the antigen-binding site and I-J determinant, but these determinants can be separated by reduction
of disulfide bonds (Table II). It is interesting that both the antigen-binding activity and binding by anti-I-J antibodies are detectable with the intact, covalently associated chains as well as the isolated chains. Thus, it appears that conformational effects of the association of the two chains do not contribute significantly to the antigen-binding activity or to the I-J structure. Although the isolated chains of TsF2 do not reassociate by reformation of the disulfide bonds (Table III), the disulfide bonds and/or sulfhydryl groups are relevant to the biological activity of TsF2 since reduction and alkylation inactivates this factor (Table IV). Whether the inactivation is due to the fact that alkylation indirectly alters the activity by changing the conformation of the molecule, or to some other mechanism, is not known.

The identification of two distinct antigen-binding suppressor factors that regulate immune responses to the same antigen provides some new insights into the relationship among the numerous suppressor factors described in the literature. GAT/GT-TsF2 molecules resemble the carrier-specific, I-J*, genetically restricted factors described by Taniguchi and colleagues (28, 29). Because GAT/GT-TsF2 factors were induced by GAT/GT-TsF1 factors, we suggest that TsF1-producing cells are an early cell in the pathway of suppression and that this cell is required for the activation of cells producing antigen-specific, major histocompatibility complex (MHC)-restricted TsF2. Whether antigen-specific, TsF1-producing cells are required for the generation of suppressor pathways that regulate responses to other antigens is not yet known. However, several other factors have been described that inhibit responses in a genetically unrestricted manner (30–34) and some of these mediators are known to bear I-J determinants (31–33). The molecular structures of most of these mediators have not been reported and it may be that some of these factors are analogous to GAT/GT-TsF1. To test this possibility indirectly, we have produced monoclonal antibodies that recognize TsF1 molecules and other monoclonal antibodies that recognize all of the TsF2 molecules tested. We are in the process of determining whether these antibodies abrogate development of suppressor T cells to other antigens. Although some genetically unrestricted factors may be single polypeptide chains, there is already evidence that at least one other antigen-specific, genetically unrestricted suppressor factor has been identified that is composed of two polypeptide chains, one of which is antigen binding and the other I-J* (35). Clearly, resolution of the relationship between these suppressor factors awaits further analysis.

Induction of MHC-restricted, I-J* suppressor factors by stimulation with other suppressor factors also has been demonstrated in the hapten-specific systems of Dietz et al. (36) and Okuda et al. (37). However, in these systems it is thought that injection of unrestricted suppressor factors in the absence of antigen induces antidiotypic suppressor T cells whose factors ultimately stimulate third-order, antigen-binding, MHC-restricted (TsF3) molecules. On the other hand, Taniguchi et al. have found that the antigen-specific, MHC-restricted, two-chain factor interacts with an antidiotypic T cell that actually mediates suppression (38). We have not yet found an obligate need for an antidiotypic suppressor T cell in our system, but such a cell may well exist. There is, in our opinion, insufficient information to say whether a central common pathway of suppression
involving all of the described suppressor T cells exists or whether there are multiple separate pathways, each of which can cause specific inhibition. However, based on the fact that isotype-specific suppressor T cells and factors (39, 40) as well as allotype-specific suppressor T cells and factors (41) are also known to regulate immune responses, we favor the interpretation that there are multiple suppressor pathways that are complex and potentially interacting systems.

The observation that mixtures of the isolated chains of TsF2 are immunosuppressive in the absence of covalent association (Table II) raises interesting questions concerning the mechanism by which TsF2 molecules inhibit immune responses. One issue that we investigated was whether the two chains must be present simultaneously to cause suppression. The experiment shown in Table V demonstrates that the ABC must be added at culture initiation, but that the I-J+ chain can be added at any time up to day 3 of a 5-d culture period. Although this was an unexpected finding, it is consistent with the observation that covalent association between the two chains is not essential to the biological activity. As yet, we have no indication of how these chains interact with the target cell, or cells, or whether the chains actually reassociate to deliver the inhibitory signal, but the fact that the chains can be added separately provides the opportunity to dissect the system further.

The last two experiments reported above do provide indirect evidence supporting the interpretation that the two chains must interact to deliver the suppressive signal. The first observation is that none of the isolated antigen-binding and I-J+ chains from hybridomas derived from three independent haplotypes were able to suppress responses of parental strains when mixed in nonhomologous pairs. Second, the heterologous mixtures of chains from different haplotypes cannot suppress responses by spleen cells from F1 hybrid mice even though the F1 mice are suppressed by both homologous pairs of chains. Since F1 mice have receptors for, or the ability to interact with, TsF2 derived from both parental mice, there must be a further constraint on the system. Although there are several conceivable explanations for this observation, the simplest is that there is a requirement for the chains to interact at some level to form a functional suppressor signal and that there is a restriction in the ability of isolated chains from different mice to interact. The extent to which these restrictions will apply to other MHC-restricted suppressor factors is not known. However, a similar restriction was reported for the I-J+ chains of the unrestricted suppressor factors reported by Lei et al. (35).

Summary

We have previously reported that two types of suppressor T cell factors (TsF) specific for L-glutamic acid\textsuperscript{10}-L-tyrosine\textsuperscript{10} (GT) or L-glutamic acid\textsuperscript{60}-L-alanine\textsuperscript{30}-L-tyrosine\textsuperscript{10} (GAT) can be distinguished based upon differences in their ability to suppress responses by allogeneic mice. Injection of GAT or GT induces a suppressor T cell subset that produces an antigen-binding, I-J+, genetically unrestricted, specific suppressor factor (TsF1). Injection of this factor plus small amounts of antigen induces a second-order suppressor T cell that produces an antigen-binding, I-J+, genetically restricted, specific suppressor factor (TsF2). In this report, we demonstrate that these two factors are also biochemically distinct.
Monoclonal TsF1 molecules are composed of a single polypeptide chain that bears both the antigen-binding site and I-J determinant, whereas TsF2 molecules are composed of two disulfide-linked polypeptide chains, one of which is antigen-binding and I-J+, and the other, nonantigen-binding, I-J-. The antigen-binding chain must be added at culture initiation to achieve suppression, but the I-J+ chain can be added as late as day 3 with complete suppression observed. However, isolated chains from TsF2-producing hybridomas derived from three different haplotypes were unable to suppress immune responses when chains from heterologous TsF2 were mixed. Indirect evidence is presented that suggests that this restriction is because the chains fail to interact rather than the inability of the target cells to recognize both chains.

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