PURIFICATION AND CHARACTERIZATION OF AN L-GLUTAMIC ACID^{60}-L-ALANINE^{30}-L-TYROSINE^{10} (GAT)-SPECIFIC SUPPRESSOR FACTOR FROM GENETIC RESPONDER MICE*

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Regulation of immune responses by T cells is mediated by the release of soluble factors in several systems (1–3). These factors have the same function, antigenic specificity, and genetic restrictions as the cells from which they are derived and may represent a soluble or shed form of the T cell receptor for antigen. One of the best-characterized T cell factors is generated following challenge of genetic nonresponder mice with the antigen L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)[1]; this factor is derived from suppressor T cells and is termed GAT-TsF (4). Until recently, detailed information about the chemistry and mechanism of action of this and related molecules has been hindered by the inability to obtain sufficient amounts of material in a pure form. The development of hybridoma techniques has allowed construction of T cell hybrids constitutively producing sufficient quantities of factor for analysis. Recently, efficient methods for purifying GAT-TsF to chemical homogeneity using immunoabsorbents and high performance liquid chromatography (HPLC) techniques have been reported (5). We have used modifications of these techniques for the purification of a GAT-specific suppressor T cell factor, termed GAT-TsF$_R$, from genetic responder mice to GAT (6). GAT-specific suppressor T cells (GAT-Ts$_R$ cells) were induced in responder mice by injection of neonates with GAT-pulsed syngeneic macrophages (GAT-Mø), restimulated with GAT in vitro and hybridomas were constructed by fusion to the AKR thymoma, BW5147. The GAT-TsF$_R$ purified from hybridoma culture supernatant fluids is compared with GAT-TsF derived

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[1] *Abbreviations used in this paper:* GA, random polymer of L-glutamic acid^{50}-L-alanine^{50}; GAT, random polymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-TsF, GAT-specific suppressor T cell factor from nonresponder mice; GAT-TsF$_R$, GAT-specific suppressor T cell factor from responder mice; GAT-Ts$_R$, GAT-specific suppressor T cell from responder mice; GT, random polymer of L-glutamic acid^{50}-L-tyrosine^{50}; HPLC, high performance liquid chromatography; Mø, macrophage(s); PAGE, polyacrylamide gel electrophoresis; PFC, plaque-forming cell(s); PNA, peanut agglutinin; SDS, sodium dodecyl sulfate; SRBC, sheep erythrocyte; Ts cell, suppressor T cell.

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from hybridomas of nonresponder suppressor T cells and the concept of a family of suppressor T cell molecules is discussed.

Materials and Methods

Mice. C57BL/10 mice were bred in the Animal Resources Facility of the Jewish Hospital of St. Louis, maintained on water and laboratory chow *ad libitum* and used at 10–20 wk of age. Mice were vaccinated at 5 wk of age with the IHD-T strain of vaccinia virus.

Antigens and Immunizations. GAT (45,000 mol wt, Vega Biochemicals, Tucson, AZ) was prepared for use as antigen in culture (7), preparation of GAT-Mø (8, 9) and coupling to sheep erythrocytes (SRBC) for use as indicator cells in the hemolytic plaque assay (7), as described. Neonatal mice were injected with 2×10^6 syngeneic GAT-Mø for the induction of GAT-Ts_R, as previously described (6). Spleens were removed from these animals at 10–20 wk of age and used for the generation of T cell hybridomas.

Generation of T Cell Hybridomas. Spleen cells from C57BL/10 mice (5×10^6/well) injected as neonates with syngeneic GAT-Mø were restimulated with GAT in vitro and fused with the AKR thymoma BW5147, as previously described (10–13). Individual clones were screened for GAT-specific suppressive activity and the biological activity of supernatant fluids from the hybridoma to be discussed, 372B3.5, has been described in detail (6). Supernatant fluids from BW5147 and other wells from the same fusion were consistently negative for both GAT-specific and nonspecific suppressive activity.

Culture System and Hemolytic Plaque Assay. Single cell suspensions of spleen in completely supplemented Eagle's minimal essential medium with 10% fetal calf serum (lot 52101, Reheis Chemical Co., Kankakee, IL) were incubated at 5×10^6 cells in 0.5 ml in 16-mm wells of a 24-well tissue culture plate (76-033-05; Linbro Div., Flow Laboratories, Inc., Hamden, CT) for 5 d at 37°C in an atmosphere of 10% CO_2, 7% O_2, 83% N_2 under modified Mishell-Dutton conditions (7, 8). Soluble GAT (2 μg/culture) or 10^7 SRBC and appropriately diluted samples of GAT-TsF_R were added to the spleen cells at culture initiation. GAT-specific IgG and SRBC-specific IgM and IgG antibody responses were assayed using the slide modification of the Jerne hemolytic plaque assay (7, 14). Data are expressed as plaque-forming cells (PFC)/culture or as the inverse of the final dilution of factor yielding 50% suppression of the PFC response (S_{50} U).

High Performance Liquid Chromatography (HPLC). Hybridoma supernatant fluids containing GAT-TsF_R were adjusted to 30% 1-propanol (vol/vol); the resulting precipitate was removed by centrifugation (10,000 *g*). The propanol extract was adjusted to pH 5.5 with 2 M acetic acid and applied to a preparative column (2.25 cm × 22.5 cm) containing Lichroprep RP-8 (25–40 micron) (E. Merck, Darmstadt, Federal Republic of Germany). Proteins were eluted with a stepwise reverse phase gradient of 0–60% 1-propanol in 0.5 M acetic acid/1.0 M pyridine, pH 5.5 buffer at a flow rate of 50 ml/min. Fractions were assessed for suppression of GAT-specific PFC responses; biologically active fractions were pooled and adjusted to 20% 1-propanol. This material was applied to a Lichrosorb RP-8 (10 m) analytical column (0.45 cm × 22.5 cm) (Merck & Co., Rahway, NJ) and eluted with a stepwise gradient of 1-propanol in 0.5 M acetic acid/1.0 M pyridine, pH 5.5 buffer at a flow rate of 0.22 ml/min. Biologically active fractions were identified, pooled, adjusted to 20% 1-propanol, and rechromatographed on a Lichrosorb RP-8 (10 μm) column under the same buffer conditions and flow rates, but using an expanded propanol gradient to increase resolution. Column effluents were monitored for protein using fluorescamine (Hoffmann-LaRoche, Nutley, NJ) and an automated detection system (15). Molecular weight was determined by applying GAT-TsF_R to a tandem column containing BIO-SIL TSK 125 and TSK 250 resins (Bio-Rad Laboratories, Richmond, CA). Proteins were eluted with 0.5 M acetic acid/1.0 M pyridine, pH 5.5 buffer using a flow rate of 0.25 ml/min. In one series of experiments, hybridoma supernatant fluids were concentrated by lyophilization, applied to Sephacryl S-200 columns (1.4 cm × 50.0 cm), and eluted with phosphate-buffered saline (0.15 M, pH 7.5) or sodium acetate (0.5 M, pH 5.0). Fractions of 2 ml were collected and tested for biological activity.

Gel Electrophoresis. Purified samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or nonreducing conditions in 12.5% polyacrylamide gels (16). Isoelectric focusing gels were performed as described by Briles and Davie (17) using a pH gradient of 4 to 10. Gels were fixed in methanol/acetic acid/water, 50:10:40 (vol/vol/vol) and stained with silver by the method of Morrisey (18).

Amino Acid Composition Analyses. Purified GAT-TsF_R from hybridoma 372B3.5 was subjected to SDS-PAGE (12.5% gel) that was stained with Coomassie Brilliant Blue; individual bands were cut from the gel, eluted and dried in vacuo. The samples were hydrolyzed in 6 M HCl/0.1% thioglycollic acid at 110°C for 24 h. After drying, the samples were dissolved in 0.2 M sodium citrate/0.1% thiodiglycol, pH 2.2 and analyzed in a fluorescamine amino acid analyzer (19).

Immunoadsorbents. GAT-, L-glutamic acid^{50}-L-tyrosine^{50}-(GT-) and L-glutamic acid^{50}-L-alanine^{50}-(GA-) Sepharose were provided by Dr. J. Kapp (Jewish Hospital of St. Louis, Washington University Medical School). Peanut agglutinin (PNA) agarose was purchased from E-Y Laboratories, San Mateo, CA. Immunoadsorbents of monoclonal anti-I-J^b, anti-I-J^q, and an antiidiotype (guinea pig anti-mouse anti-GAT antibody), anti-cGAT, were prepared as described previously (6). Purified GAT-TsF_R was applied to these immunoadsorbents; the effluent and material eluted with 2.0 M KCl were tested for biological activity.

Results

Purification of GAT-TsF_R. Supernatant fluids from 40 l of 372B3.5 cells grown to a density of 1.4×10^6 cells/ml were divided into 10-liter portions and adjusted to 30% 1-propanol (vol/vol). The precipitate was separated by centrifugation, the propanol-extract was applied to a preparative Lichroprep RP-8 column and eluted with a stepwise gradient of 1-propanol in 0.5 M acetic acid/1.0 M pyridine, pH 5.5 buffer. Samples were removed from individual fractions and tested for their ability to suppress an in vitro GAT-specific PFC response by spleen cells from C57BL/10 mice. The data in Fig. 1 *A* show that GAT-specific suppressive activity eluted in 40% propanol with one of several protein peaks. Similar results were obtained for all four of the 10-liter preparations (data not shown).

Biologically active fractions were pooled, adjusted to 20% 1-propanol, and chromatographed on an analytical Lichrosorb RP-8 column in acetic acid/pyridine buffer at a flow rate of 0.22 ml/min and a 0–60% 1-propanol gradient. Biological activity eluted 25–35 min into the 40% 1-propanol step (Fig. 1 *B*); these fractions were pooled and rechromatographed on the Lichrosorb RP-8 column under the same buffer conditions using a more shallow 0–60% 1-propanol gradient. Biologically active protein again eluted at a position 25–40 min into the 40% 1-propanol step and was spread over several fractions, but was within the single protein peak (Fig. 1 *C*). A summary of the purification scheme, yield, and specific activity of GAT-TsF_R at each step of purification of 40 l of hybridoma supernatant fluid is in Table I. 40 l of supernatant fluid containing 6.8×10^{-3} S_{50} U/ng protein was purified in three steps to yield a final specific activity of 28.4×10^3 S_{50} U/ng protein representing a 4.2×10^6-fold purification over the starting material. Crude supernatant fluid and HPLC-purified, biologically active protein (peak from Fig. 1 *C*) from hybridoma 372B3.5 were tested for antigen-binding capacity and presence of serologic determinants by applying samples to various immunoabsorbents, collecting effluents and material eluted by 2 M KCl, and testing for biological activity. The data in Table II show that

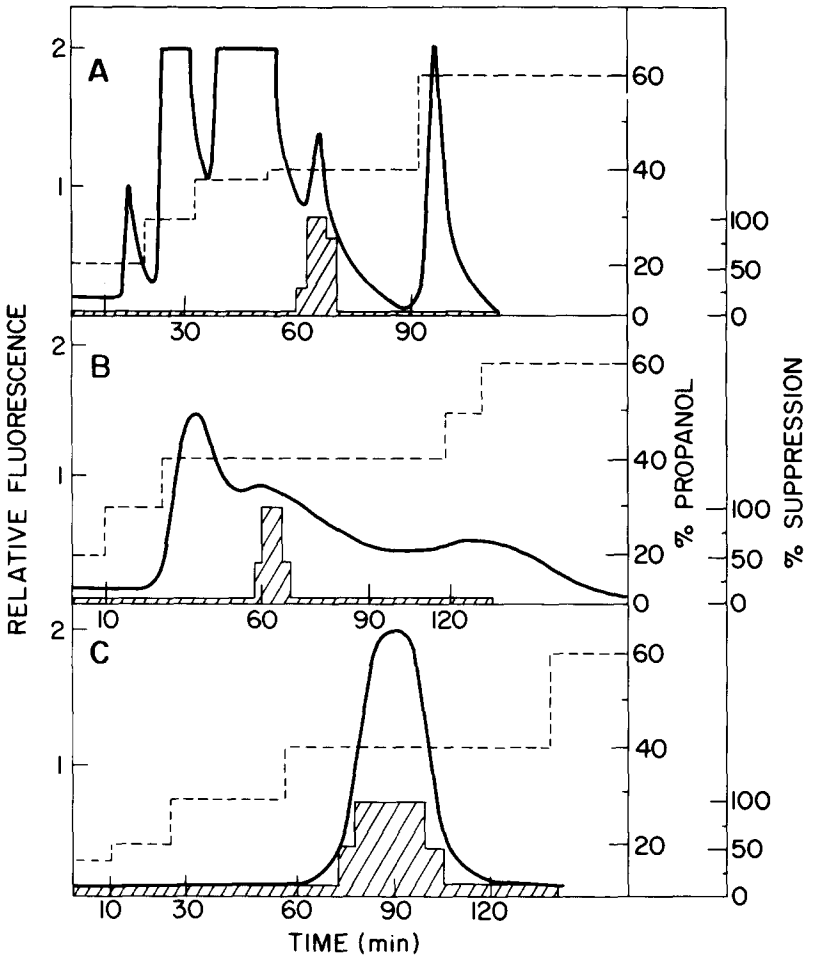

FIGURE 1. Purification of GAT-TsF$_R$ by reverse phase HPLC. (*A*) Supernatant fluids from a 10-liter culture of hybridoma 372B3.5 were precleared by precipitation with 30% (vol/vol) 1-propanol, applied to a 2.25 cm × 22.5 cm Lichroprep RP-8, 25–40-μm bead size preparative column and eluted with a stepwise gradient of 0–60% 1-propanol in 0.5 M acetic acid/1.0 M pyridine, pH 5.5 buffer at a flow rate of 50 ml/min. Individual fractions were tested for biological activity at a final dilution of 1/1,000 in culture. (*B*) Biologically active fractions from *A* were pooled, adjusted to 20% 1-propanol, applied to a 0.45 cm × 22.5 cm analytical Lichrosorb RP8 (10 μm) column, and eluted with a propanol gradient in the pH 5.5 buffer at a flow rate of 0.22 ml/min. (*C*) Biologically active fractions from *B* were rechromatographed with an expanded propanol gradient in the pH 5.5 buffer at a slow flow rate (0.22 ml/min). Protein concentration is estimated using fluorescamine and an automated detection system (15).

GAT-specific suppressive activity in both preparations was absorbed by and eluted from GAT-, GT-, monoclonal anti-J^b-, PNA-, and anti-cGAT-Sepharose, but not GA-Sepharose or monoclonal anti-J^q Sepharose. Responses to SRBC were unaffected by any of these materials (not shown).

Molecular Weight Analysis of GAT-TsF$_R$.

In a previous publication (6) we reported the apparent molecular weight of GAT-TsF$_R$ from hybridoma 372B3.5 to be 48,000–63,000 as determined by

TABLE I

Purification Summary of GAT-TsF$_R$ from Hybridoma 372B3.5

Step	Total protein*	Bioactivity	Specific activity	Purification factor
	ng	S_{50} *U*‡	S_{50} *U/ng protein*	
Crude supernate	5.88×10^{11}	400×10^7	6.8×10^{-3}	1
Lichroprep RP-8	1.22×10^8	220×10^7	1.8×10^{-1}	2.6×10^3
Lichrosorb RP-8	9.3×10^5	500×10^7	53.8×10^2	7.9×10^5
Lichrosorb RP-8	8.8×10^5	$2{,}500 \times 10^7$	28.4×10^3	4.2×10^6

*Protein concentration determined by fluorescamine and an automated detection system (15).
‡ Biological activity is measured by the ability to suppress a GAT-specific PFC responses in vitro and is expressed as S_{50} units, the reciprocal of the dilution required to suppress the response by 50%.

TABLE II

Partial Serological Characterization of 372B3.5

Immunoabsorbent*	GAT-specific PFC/Culture			
	Crude 372B3.5		Purified 372B3.5	
	Effluent	Eluate	Effluent	Eluate
None	83		35	
GAT-Sepharose	450	13	435	<10
GT-Sepharose	488	75	430	<10
GA-Sepharose	43	435	<10	405
mc Anti-J^b-Sepharose‡	460	23	480	<10
mc Anti-J^q-Sepharose‡	105	533	<10	450
PNA-Agarose	505	25	550	<10
Anti-cGAT-Sepharose	433	85	370	<10

*Crude and HPLC-purified culture supernatant fluids from hybridoma 372B3.5 were reacted with the indicated immunoabsorbent; the effluent and material eluted with 2.0 M KCl were tested for biological activity at a final dilution in culture of 1/10,000. Data are presented as IgG GAT-specific PFC/culture. Response in the absence of added GAT-TsF$_R$ was 503 PFC/culture.
‡ *mc*, monoclonal anti-I-J reagents.

chromatography on Sephacryl S-200 at physiological pH and ionic strength. The molecular weight of GAT-TsF$_R$ was determined under different conditions by three independent methods. Supernatant fluids were applied to a Sephacryl S-200 column and analysis of individual fractions for biological activity showed that GAT-TsF$_R$ eluted with phosphate-buffered saline (0.15 M, pH 7.5) had a molecular weight in the range from ~46,000 to 68,000 (Fig. 2 *A*) whereas, when eluted with high ionic strength acetate buffer (0.5 M sodium acetate, pH 5.5), GAT-TsF$_R$ had a molecular weight of 16,000–21,000 (Fig. 2 *B*). This observation suggests that GAT-TsF$_R$ aggregates under physiological conditions (see Discussion). Molecular sieve chromatography by HPLC using TSK resins and a 0.5 M acetic acid/1.0 M pyridine pH 5.5 buffer yielded a molecular weight of 18,000–21,000 for GAT-TsF$_R$ (Fig. 3).

The degree of purity and estimation of molecular weight of GAT-TsF$_R$ from 372B3.5 was also determined at each step of purification by SDS-PAGE under reducing and nonreducing conditions. Biologically active fractions from HPLC were lyophilized in the presence of 0.5% SDS to prevent adherence to the tube and applied to a 12.5% acrylamide gel. Fig. 4 represents the electrophoretic

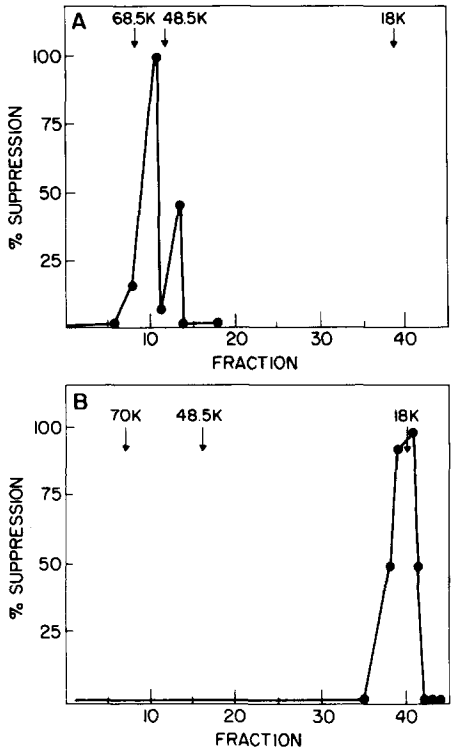

FIGURE 2. Supernatant fluid from 372B3.5 was concentrated by lyophilization, applied to a 14 cm × 50 cm Sephacryl S-200 column and eluted with either 0.15 M phosphate-buffered saline, pH 7.5 (Panel *A*) or 0.5 M sodium acetate, pH 5.5 (panel *B*). Individual fractions of 2 ml were collected and tested for biological activity at a final dilution of 1/1,000. Arrows indicate the elution volume of proteins of known molecular weights.

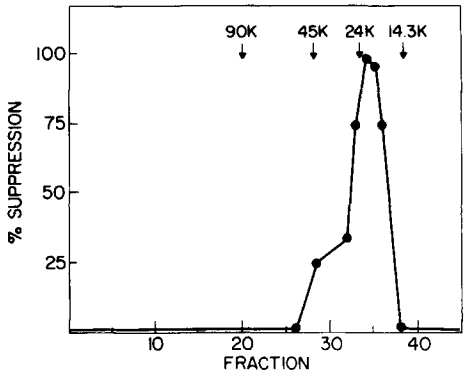

FIGURE 3. Supernatant fluid from 372B3.5 was concentrated by lyophilization, applied to a tandem array of TSK 125 → TSK 250 molecular sieve HPLC columns and eluted at a flow rate of 0.25 ml/min with 0.5 M acetic acid 1 M pyridine, pH 5.5 buffer. Individual fractions were tested for biological activity at a final dilution of 1/1,000. Arrows indicate the elution volume of proteins of known molecular weights.

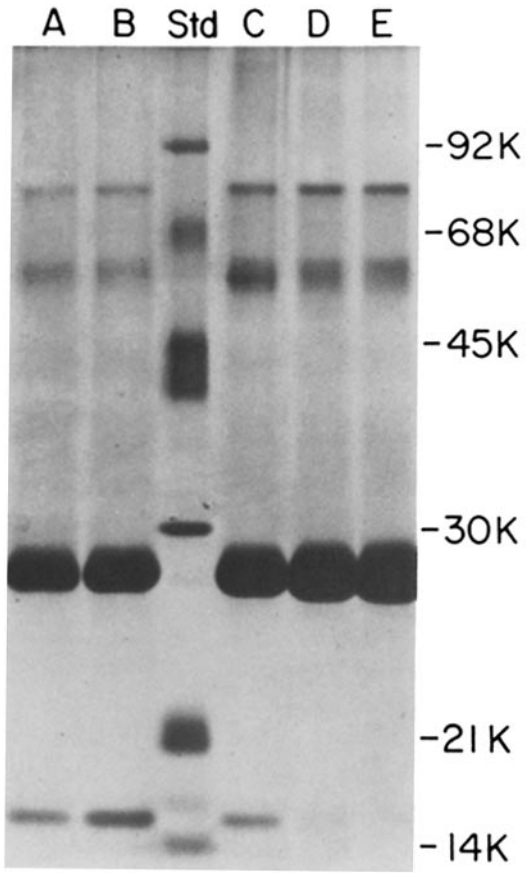

FIGURE 4. SDS-PAGE under nonreducing conditions of GAT-TsF_R purified by HPLC. Lanes *A–E* represent the five biologically active fractions from the second Lichrosorb RP-8 HPLC (panel *C*, Fig. 1). Molecular weight standards are indicated by arrows. Protein samples were applied to a 12.5% acrylamide resolving gel and bands were visualized by staining with silver.

pattern under nonreducing conditions of the five individual fractions with bioactivity from the second RP-8 HPLC (Fig. 1 *C*); four bands were observed after staining with silver with molecular weights of 18,000, 28,000, 64,000, 84,000. Amino acid composition analyses (Table III) of these individual bands eluted from a 12.5% gel were essentially identical suggesting aggregation of lower molecular weight species into the larger forms. Similar results were obtained by SDS-PAGE analysis under both nonreducing and reducing conditions (data not shown), suggesting that GAT-TsF_R is irreversibly aggregated or that aggregation is not dependent upon disulfide bond formation.

The issue of aggregation was investigated further by applying purified GAT-TsF_R to two adjacent lanes of a 12.5% acrylamide gel and electrophoresing under nonreducing conditions. Protein in one lane was visualized by silver staining: the other lane was divided into 3-mm slices that were eluted with phosphate-buffered saline and the material was tested for suppression of GAT-

TABLE III

*Amino Acid Composition of the Four Biologically Active Molecular Weight Species of GAT-TsF$_R$ from Hybridoma 372B3.5**

	18,000§	28,000	64,000	84,000
Asx	8.1	7.5	7.5	7.9
Thr	2.4	2.7	3.0	2.2
Ser	2.8	2.0	2.9	2.0
Glx	16.3	16.0	16.0	16.1
Pro	ND‡	ND‡	ND‡	ND‡
Gly	12.1	12.0	11.0	11.0
Ala	7.5	7.9	7.0	9.0
Val	6.8	6.0	6.3	6.3
Met	<1.0	<1.0	<1.0	<1.0
Ile	1.9	1.3	1.0	1.8
Leu	14.5	14.2	15.0	14.5
Tyr	1.4	1.1	1.1	1.6
Phe	2.6	3.0	2.6	2.6
His	3.0	2.8	2.6	2.8
Lys	10.0	9.8	9.8	10.0
Arg	5.8	5.1	5.9	5.0
Cys	0.0	0.0	0.0	0.0
Try	ND‡	ND‡	ND‡	ND‡

*Purified 372B3.5 (~1 μg) was applied to a 12.5% SDS-acrylamide gel and electrophoresed, and the gel was stained briefly by Coomassie Brilliant Blue. Individual bands were cut out of the gel (see Fig. 4), eluted and subjected to amino acid analysis as described in Materials and Methods. Values reported are mole percent of each amino acid recovered.

‡ *ND*, not determined.

§ Molecular weight species.

specific PFC responses at several dilutions. The results in Fig. 5 demonstrate that GAT-specific suppressive activity was associated with all of the protein bands seen by silver staining and was not found in areas of the gel where there was no protein. Further analysis of the fractions of GAT-TsF$_R$ eluted from the SDS-PAGE demonstrated that each molecular weight form was absorbed by and eluted from a monoclonal anti-I-J^b immunoabsorbent, demonstrating the presence of I-J determinants on all molecular weight forms (data not shown). The question of aggregation was also addressed by applying samples of purified GAT-TsF$_R$ to adjacent lanes of an isoelectric focusing gel with a pH gradient of 4 to 10. One lane was divided into 3-mm slices, eluted, and material from individual slices was tested for suppression of GAT-specific PFC responses; the protein in the other lane was visualized by silver staining. Purified GAT-TsF$_R$ focused as a single band with a pI of 6.4 and specific biological activity was associated only with this band (Fig. 6). Finally, chemical purity of GAT-TsF$_R$ was also indicated by the detection of a single amino acid, aspartic acid, by N-terminal analysis and partial sequence data (C. M. Sorensen, C. W. Pierce, and D. R. Webb, manuscript in preparation).

Discussion

Fusion of GAT-specific suppressor T cells from genetic responder C57BL/10 mice with the AKR thymoma, BW5147, yielded a hybridoma, 372B3.5, which

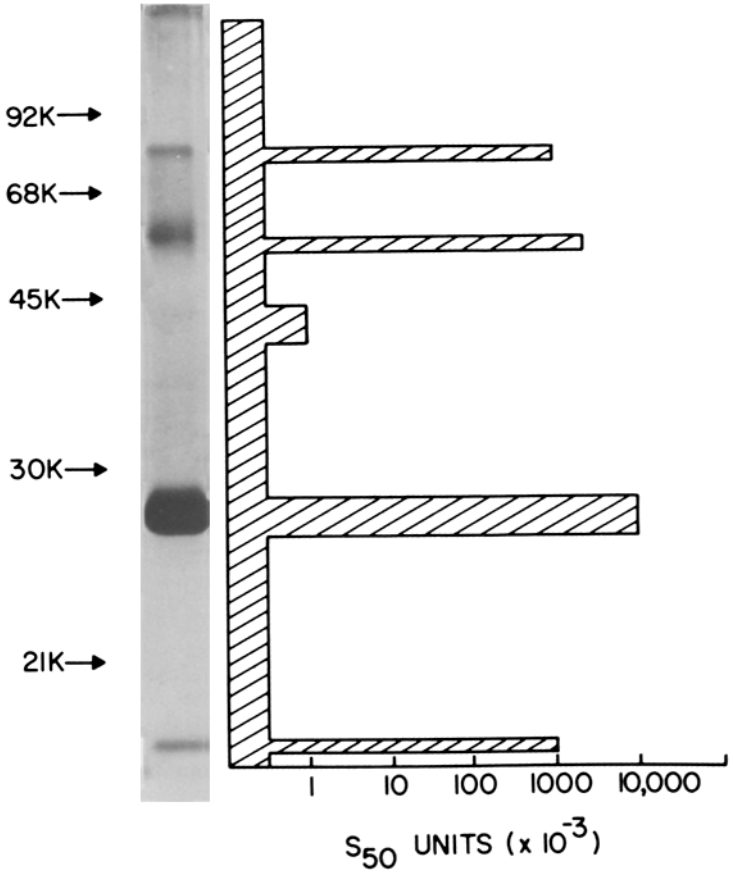

FIGURE 5. SDS-PAGE of GAT-TsF$_R$ purified by HPLC. A sample from one biologically active fraction (panel *C*, Fig. 1; lane *C*, Fig. 4) was applied in duplicate to a 12.5% acrylamide gel and electrophoresed under nonreducing conditions. One lane was stained with silver to visualize protein and the other was divided into 3-mm slices, the protein was eluted from the gel and tested for biological activity at several dilutions. Activity is expressed as S_{50} units; molecular weight standards are indicated by arrows.

constitutively secretes a monoclonal suppressor factor, GAT-TsF$_R$, into the supernatant fluid. GAT-TsF$_R$ is a glycoprotein that bears determinants encoded by the I-J subregion of the H-2 complex, has an antigen-binding site, and determinants cross-reactive with idiotypic determinants of anti-GAT antibody (6) (see Table II). This GAT-TsF$_R$ has been purified to apparent chemical homogeneity by reverse phase HPLC techniques. Starting with 40 l of supernatant fluid with a specific activity of 6.8×10^{-3} S_{50} U/ng protein, the three-step purification process yielded a final specific activity of 28.4×10^{3} S_{50} U/ng protein, or a purification factor of 4.2×10^{6}. Determination of purity is based on analysis by SDS-PAGE, isoelectric focusing, amino acid composition, and the yield of a single amino acid, aspartic acid, following *N*-terminal cleavage.

The purification strategy used is based on that originally reported by Krupen et al. (5), with two important modifications. Partial purification of supernatant material by precipitation with 30% 1-propanol and initial reverse-phase HPLC

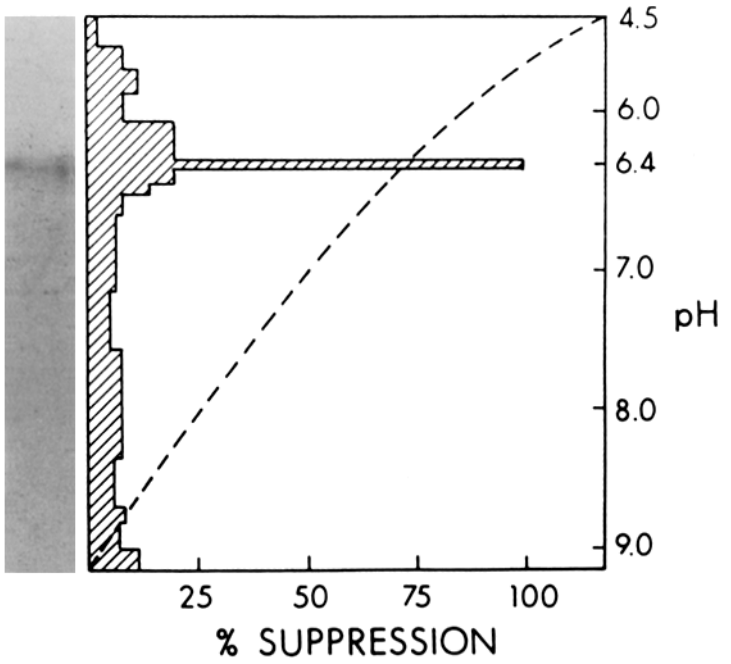

FIGURE 6. Purified 372B3.5 (164 ng/lane) was applied to two adjacent lanes of an isoelectric focusing gel (pH 4–10) and focused at a constant current of 2.5 mA at 4°C for 18 h. Following determination of the pH gradient using a micro-electrode, one lane was visualized by staining with silver and the other was divided into 3-mm slices and eluted into phosphate buffered saline, pH 7, for 24 h at 4°C. Individual fractions were assayed for GAT-specific suppressive activity at a final dilution of 1/1,000. Data are expressed as percent suppression of a control response of 595 IgG GAT PFC/culture.

using a preparative size Lichroprep RP-8 column eliminated the need for the affinity chromatography step and reduced the loss of GAT-TsF$_R$ due to nonspecific adsorption to the affinity matrix. Subsequent analytical reverse phase HPLC was performed using a 10-μm-Lichrosorb RP-8 resin rather than the 5-μm resin previously reported (5), which allows better resolution.

The molecular weight of 372B3.5 GAT-TsF$_R$ has been determined by several independent methods. Chromatography of GAT-TsF$_R$ on Sephacryl S-200 in a physiological buffer yielded a molecular weight of ~46,000–68,000. Chromatography on Sephacryl S-200 in the presence of 0.5 M acetate, pH 5.5, yielded a molecular weight of ~16,000–21,000; an apparent molecular weight of 18,000–21,000 was also obtained by HPLC molecular sieve chromatography using TSK resins. These data suggest that under physiological conditions GAT-TsF$_R$ frequently forms aggregates, which is not surprising since GAT-TsF$_R$ is a relatively hydrophobic protein that elutes in 40% propanol on a reverse phase column.

Analysis of purified GAT-TsF$_R$ by SDS-PAGE shows four distinct protein bands with molecular weights of 18,000, 28,000, 64,000, and 84,000. The possibility that these protein bands are actually multimers or aggregates of a basic subunit is suggested by the comparable amino acid composition of the individual bands. Additional experiments in which the gel was sliced and the

protein eluted confirmed this interpretation by showing that GAT-specific suppressive activity was associated with each of the four protein bands. Data from isoelectric focusing gels also supports the contention that the individual bands seen by SDS-PAGE analysis actually represent aggregated forms of a single protein species. Under these conditions, the GAT-TsF_R focused as a single band with a discrete pI of 6.4. Furthermore, a direct positive correlation was seen between protein bands and specific biological activity. The tendency of GAT-TsF_R to aggregate increases with the degree of purity and is most pronounced when the protein is removed from the 40% 1-propanol buffer used in the HPLC procedures into physiological phosphate buffer. This is best illustrated by the appearance of the multiple biologically active bands on SDS-PAGE, while a portion of the same sample chromatographed on a HPLC molecular sieve TSK column produces only a single peak of biological activity. Unlike SDS-PAGE analysis in which the propanol is removed, the HPLC molecular seive analysis does not require removal of the 40% 1-propanol buffer and is performed under different dissociating conditions, thereby preventing the formation of extensive aggregates. The presence of propanol alone is not sufficient to prevent aggregate or multimer formation; purified GAT-TsF_R stored for prolonged periods of time in 40% propanol buffer will eventually precipitate out of solution, again emphasizing the hydrophobic nature of the protein. Aggregation is virtually irreversible once it has occurred. Analysis by SDS-PAGE under reducing conditions or treatment with thioglycollic acid fails to disrupt the aggregates. Treatment with 10 mM dithiothreitol overnight at 40°C under nitrogen only partially reduces the large molecular weight aggregates (C. Sorensen, unpublished observations).

The sizes of the aggregates or multimers that are formed are also of interest. The smallest biologically functional unit is an 18,000 mol wt protein (glycoprotein) whose precise relationship to the predominant 28,000 mol wt and larger forms is unclear. One interpretation is that the 18,000 mol wt form is actually a breakdown product of the major 28,000 mol wt form that has retained all of the function and serological determinants detected on the larger molecule (6). At this time we cannot rule out this possibility. An alternate interpretation is that the 18,000 mol wt species is actually a "monomeric subunit" of the major biologically active dimeric form (Figs. 4 and 5). One interesting possibility suggested by these observations is that biological activity of GAT-TsF_R requires dimerization to form a functional molecule similar to immunoglobulin molecules.

The discrepancy in molecular weight from what is predicted for dimerization of the 18,000 mol wt species, a ~36,000 mol wt species, to the observed 28,000 mol wt species may be explained by the somewhat anomalous behavior of glycoproteins analyzed by SDS-PAGE or by changes in glycosylation resulting from purification. Any differences in glycosylation or degree of glycosylation presumably do not involve the loss of sialic acid residues since charge heterogeneity is not detected by isoelectric focusing analysis. Analysis of the carbohydrate content and structure(s) on the purified GAT-TsF_R molecule is in progress.

The formation of aggregates provide an explanation for the previous reports of a larger molecular weight for GAT-TsF_R when determination is by molecular sieve analysis with physiological buffers. Other suppressor T cell factors from genetic nonresponders to GAT also exhibit this phenomenon of aggregation to

varying degrees (C. Healy, personal communication), suggesting that this is a characteristic of this class of proteins. Another feature common to all of the hybridoma-derived suppressor T cell factors specific for GAT purified to date is the failure to chromatograph by HPLC as a single sharp peak (see Fig. 1 *C*); instead, these molecules elute as a relatively broad peak of bioactivity and protein but with a highly reproducible retention time in 40% propanol. The reasons for this are unclear, but again may be due to changes in glycosylation induced by the purification process, changes in degree of aggregation, or subtle differences in oxidation/reduction states of the sample applied to the column. Any one of these alterations or differences are capable of being detected by HPLC techniques and would result in broadening of the peak.

An important conclusion from these results and data from purification of the nonresponder GAT-TsF from hybridoma 258C4.4 (5) is that these GAT-specific suppressor T cell factors may represent a family of proteins exhibiting rather unique biochemical characteristics, including similar retention times using reversed phase HPLC (elution from RP-8 resin in 40% propanol), amino acid composition, degree of hydrophobicity and the tendency to form higher molecular weight aggregates. These shared characteristics appear to be independent of antigenic fine specificity; haplotype of origin or responder vs. nonresponder status. GAT-TsF$_R$ from hybridoma 372B3.5 has specificity for GAT and GT, bears an I-J^b determinant, and is derived from an H-2^b responder mouse (6), whereas GAT-TsF from hybridoma 258C4.4 has specificity for GAT and GA bears a putative I-J^q determinant and is derived from an H-2^q nonresponder mouse (4, 5). Independent experiments reported elsewhere have shown that GAT-TsF$_R$ from 372B3.5 and GAT-TsF from 258C4.4 are single polypeptide chains with both I-J-encoded determinants and antigen-binding sites on the same molecule (20).[2] We predict that any significant differences between these molecules will be at the structural level and will be very restricted, reflecting fine antigen specificity or "V_H" region and individual haplotype differences. Further analysis involving determination of repertoire diversity within a haplotype, between haplotypes and between responder and nonresponder strains is now possible for antigen-specific suppressor T cell factors and is in progress.

Summary

A hybridoma-derived, GAT-specific suppressor T cell factor (GAT-TsF$_R$) from responder C57BL/10 mice has been purified to apparent chemical homogeneity using reversed phase HPLC techniques. 40 l of starting material yielded ~880 μg protein with a specific activity of 28.4×10^3 S_{50} U/ng protein representing a purification factor of 4.2×10^6. Purified GAT-TsF$_R$ is a hydrophobic protein with a minimum molecular weight of 18,000 that is capable of forming biologically active aggregates with molecular weights of 28,000, 64,000 and ~84,000 and has a pI of 6.4. GAT-TsF$_R$ is a glycoprotein that binds GAT and GT, but not GA, and bears determinants encoded by the I-J subregion of the H-2 complex. This GAT-TsF$_R$ derived from an H-2^b responder haplotype to GAT is compared

[2] Kapp, J. A., C. M. Sorensen, and C. W. Pierce. 1983. Antigen-specific suppressor T cell interactions. II. Characterization of MHC-restricted factors specific for L-glutamic acid^{50}-L-tyrosine^{50} (GT) and L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) (Submitted for publication).

with GAT-TsF derived from the nonresponder H-2^q haplotype on the basis of biochemical and some serological properties.

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