ANTIGEN-SPECIFIC SUPPRESSION IN GENETIC RESPONDER MICE TO L-GLUTAMIC ACID^{60}-L-ALANINE^{30}-L-TYROSINE^{10} (GAT)

Characterization of Conventional and Hybridoma-derived Factors Produced by Suppressor T Cells from Mice Injected as Neonates with Syngeneic GAT Macrophages*

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The immune response to the synthetic random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)[1] is under the control of an autosomal dominant immune response (Ir) gene(s) that maps to the I-A region of the H-2 complex (1–3). Mice of the H-2^{p,q,s} haplotypes are genetic nonresponders to GAT: these mice fail to develop an antibody response after stimulation with soluble GAT, but develop a population of antigen-specific genetically unrestricted suppressor T cells (Ts cells) that suppress GAT-specific plaque-forming cell (PFC) responses (2, 3). Mice of all other H-2 haplotypes are genetic responders and develop a PFC response after stimulation with soluble GAT (1–3). GAT-specific Ts (GAT-Ts) cells also have been demonstrated in responder mice, but only under certain conditions: (*a*) after stimulation of T cells in vitro with soluble GAT in cultures extensively depleted of macrophages (Mϕ) (4); (*b*) after administration of high concentrations of GAT in vivo (5); and (*c*) after priming with allogeneic GAT-Mϕ in vivo and stimulation with syngeneic GAT-Mϕ in vitro or, conversely, after priming with syngeneic GAT-Mϕ in vivo and stimulation with allogeneic GAT-Mϕ in vitro (6). In the latter situation genetically restricted helper T cell populations that functioned preferentially when stimulated with GAT-Mϕ syngeneic to those Mϕ used for in vivo immunization were also defined (6, 7).

The studies reported here were initiated to determine the effects of injection of

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[1] *Abbreviations used in this paper:* ABA, azobenzene arsonate; BSA, bovine serum albumin; C, complement; cGAT, cross-reactive idiotype of anti-GAT antibodies; GAT, random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-Ts, GAT-specific suppressor T cell; GAT-Ts$_R$, suppressor T cell(s) from responder mice to GAT; GAT-TsF, GAT-specific suppressor T cell factor from nonresponder mice; GAT-TsF$_R$, GAT-specific suppressor T cell factor from responder mice; GA, random polymer of L-glutamic acid^{50}-L-alanine^{50}; GT, random polymer of L-glutamic acid^{50}-L-tyrosine^{50}; Mϕ, macrophages; MBSA, methylated bovine serum albumin; NP, 4-hydroxy-3-nitrophenyl acetyl; OVA, ovalbumin; PFC, plaque-forming cell(s); PNA, peanut agglutinin; SRBC, sheep erythrocytes; Ts cell, suppressor T cell.

neonatal responder mice with syngeneic GAT-Mϕ on the subsequent response patterns of adult T cells, especially the response patterns of the genetically restricted helper T cells. We anticipated that the generation of these helper T cells in adults would be affected by exposure to GAT-Mϕ during the neonatal period. However, we obtained results contrary to our expectations: injection of syngeneic GAT-Mϕ into neonates generated a population of GAT-specific, genetically restricted Ts cells (GAT-Ts_R cells). In this paper we describe the properties of these GAT-Ts_R cells, as well as those of the conventional and hybridoma-derived GAT-specific suppressor factors produced by these cells that mediate their effects. Finally, these GAT-specific Ts cell factors from responder mice (GAT-TsF_R) are compared with GAT-specific suppressor factors derived from nonresponder mice (GAT-TsF) (8–13).

Materials and Methods

Mice. C57Bl/10 (B10), B10.D2, B10.A, B10.BR, BALB/c, and (B10 × B10.D2)F_1 mice were bred in the Animal Resources Facility of The Jewish Hospital of St. Louis, St. Louis, MO. B10.A(5R) and B10.A(4R) mice were provided by Dr. D. Shreffler (Washington University School of Medicine, St. Louis, MO); B10.A(3R) mice were provided by Dr. J. Stimpfling (McLaughlin Research Institute, Great Falls, MT). Mice were maintained on water and laboratory chow *ad libitum* and used at 10–20 wk of age.

Antigens. GAT (~45,000 mol wt; Vega Biochemicals, Tucson, AZ) was prepared as previously described for use as antigen in culture (1), preparation of GAT-Mϕ (6, 7), coupling to sheep erythrocytes (SRBC) for use as indicator cells in the hemolytic plaque assay (1), and for in vivo immunization (1). GAT was complexed to methylated bovine serum albumin (MBSA) as previously described (1). SRBC (Colorado Serum Co., Denver, CO) were prepared for use as antigen in culture and as indicator cells in the hemolytic plaque assay as described previously (14).

Antisera, Monoclonal Antibodies, and Immunoadsorbents. B10.D2 anti-B10 (anti-H-2^b), (B10.A × B10)F_1 anti-B10.D2 (anti-KABJE^d), (B10.A × A)F_1 anti-B10.A(5R) (anti-KAB^b), [A.TL × B10.S(9R)]F_1 anti-B10.HTT (anti-J^s), and B10.A(5R) anti-B10.A(3R) (anti-J^b) alloantisera were provided by Dr. D. Shreffler. (B10.MBR × A/Sn) anti-B10.A(5R) (anti-I-A^b) was provided by Dr. C. David (Mayo Medical Schools, Rochester, MN). The hybridoma-derived monoclonal reagents, WF9.40.5 [B10.A(5R) anti-B10.A(3R)] (anti-J^b) and WF8.C12.8 [B10.A(3R) anti-B10.A(5R)] (anti-J^k) were provided by Dr. C. Waltenbaugh (Northwestern University, Evanston, IL) (15). Monoclonal anti-I-A^d (MKD6) was provided by Dr. P. Marrack (National Jewish Hospital, Denver, CO). The anti-idiotype immunoadsorbent made from guinea pig anti-mouse-anti-GAT antibody (anti-cGAT) was provided by Dr. M. Dorf (Harvard Medical School, Boston, MA) (12). Rabbit anti-mouse IgG and anti-GAT antibodies were prepared as described by Théze et al. (10). The immunoglobulin fractions of the heat-inactivated antisera or the culture supernatant fluids containing monoclonal antibodies were used to prepare Sepharose immunoadsorbents (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions (10, 12). GAT-, L-glutamic acid^{50}-tyrosine^{50}- (GT), L-glutamic acid^{50}-L-alanine^{50}- (GA), and bovine serum albumin- (BSA) Sepharose were provided by Dr. J. Kapp. Peanut agglutinin (PNA) agarose was purchased from E-Y Laboratories, San Mateo, CA.

Monoclonal anti-Thy-1 was derived from the hybridoma T-24/40.7 obtained from the Salk Institute, La Jolla, CA. Monoclonal noncytotoxic anti-Lyt-1 was derived from hybridoma 7.313 obtained from Dr. N. Warner, then at the University of New Mexico, Albuquerque, NM. Arsinilate conjugates of monoclonal anti-Lyt-1 (clone 53.7.3; lot B0302) and anti-Lyt-2 (clone 53.6.7; lot B0301), and rabbit anti-arsinilate (anti-Ars) (lot C0401) were purchased from Becton, Dickinson & Co. (Mountain View, CA). Prescreened rabbit complement (C) was purchased from Cedarlane Laboratories, Div. Accurate Chemical and Scientific Corp., Westbury, NY.

Preparation of Cell Populations. Peritoneal-exudate cells collected from mice injected 4 d previously with 1 ml of 10% proteose peptone were used as Mϕ; Mϕ were pulsed with GAT as previously described (6, 7). T cells were separated from spleen cell suspensions by passage over nylon wool columns (16). Alternatively, spleen cell suspensions at 10×10^6 cells/ml were reacted

in plastic dishes coated with rabbit anti-mouse immunoglobulin (RαMIg) (5 μg/ml); after 60 min at 4°C the nonadherent population was collected by aspiration and used as T cells (17). In one experiment T cells at 10×10^6 cells/ml were reacted with monoclonal anti-Lyt-1 (7.313) at 2 μg/ml for 30 min at 4°C and then fractionated on plastic plates coated with goat anti-rat immunoglobulin (5 μg/ml); nonadherent cells (Lyt-1^-) were collected by aspiration and adherent cells (Lyt-1^+) were recovered by vigorous pipetting. Spleen cells were treated with monoclonal anti-Thy-1 (T-24/40.7; 1:50 dilution) and C diluted 1:12 for 45 min at 37°C and used as T cell-depleted spleen cells. Spleen cells or T cells separated on RαMIg plates at 10×10^6 cells/ml were reacted with the desired anti-I region antibody (1:50 dilution) for 30 min at 4°C, washed, and incubated with C (1:12 dilution) for 45 min at 37°C. Alternatively, these cells were reacted sequentially with the desired arsinilate-conjugated anti-Lyt reagent followed by rabbit anti-Ars antibody. The reagents were diluted according to the manufacturer's instructions; each reaction was for 30 min at 4°C. Cells were then washed, resuspended at 5×10^6 cells/ml in C diluted 1:12 in medium, and incubated for an additional 45 min at 37°C. The remaining cells were washed twice with medium and adjusted to 10×10^6 viable cells/ml before addition to culture.

Immunizations. Neonatal B10 mice received 10×10^6–15×10^6 syngeneic Mϕ, syngeneic GAT-Mϕ that bore 50–100 ng GAT/10^6 cells, or 10 μg soluble GAT via the orbital branch of the anterior facial vein within 18 h of birth (18). The ability of spleen cells from these mice to develop an in vitro PFC response to GAT was analyzed at 10–20 wk of age. Adult B10 mice were immunized by intraperitoneal injection of 10 μg GAT in Maalox-pertussis (1) (Maalox: W. H. Roher, Inc., Ft. Washington, PA.; pertussis: Eli Lilly and Co., Indianapolis, IN.).

Culture System and Hemolytic Plaque Assay. Single cell suspensions of spleen in completely supplemented Eagle's minimum essential medium with 10% fetal calf serum (lot 49502; Reheis Chemical Co., Kankakee, IL) were incubated at 5×10^6 cells in 0.5 ml in 16-mm wells of 24-well tissue culture plates (76-033-05; Linbro Chemical Co., Div. Flow Laboratories, Inc., Hamden, CT) for 5 d at 37°C in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 (1, 6). At the initiation of culture 3×10^4 GAT-Mϕ that bore 5–10 ng GAT/10^5 cells, 2 μg soluble GAT, GAT-MBSA containing 2 μg GAT, or 10^7 SRBC were added to the spleen cells. GAT-specific IgG, and IgM and IgG anti-SRBC PFC responses were assayed on day 5 of culture by the slide modification of the Jerne hemolytic plaque assay (1, 6, 14). Data are expressed as PFC/culture.

Preparation of GAT-TsF_R. Spleen cells from control B10 mice or B10 mice injected as neonates with B10 GAT-Mϕ were incubated at 5×10^6 cells/well with 3×10^4 syngeneic GAT-Mϕ for 24 h under the culture conditions described above. Supernatant fluids were collected, cleared of cellular debris by centrifugation, filtered through 0.20-μm filters (Medical Device Division, Gelman Sciences, Inc., Ann Arbor, MI), assayed for suppressive activity in PFC responses to GAT, and stored at −80°C. Some culture supernatant fluids were concentrated 10-fold by ultrafiltration using a YM10 filter (Amicon Corp., Lexington, MA) and applied to a Sephacryl S-200 column equilibrated with isotonic phosphate buffer, pH 7.2. Appropriate fractions were collected, filtered, and assayed for suppressive activity in PFC responses to GAT.

Generation of T Cell Hybridomas. Spleen cells (5×10^6/well) from B10 mice injected with B10 GAT-Mϕ as neonates were cultured with 3×10^4 B10 GAT-Mϕ for 24 h as described above; these cells containing activated GAT-Ts_R cells were harvested and fused with the hypoxanthine-guanine phosphoribosyl transferase-deficient AKR thymoma, BW5147, using polyethylene glycol (12, 19). The fused cells were cultured in hypoxanthine-aminopterin-thymidine selection medium (12, 19, 20) and scored for growth after 3–6 wk. Cells from primary wells showing growth that yielded supernatant fluids causing >75% specific suppression PFC responses to GAT by B10 spleen cells were cloned by limiting-dilution in soft agar over 3T3 cell feeder layers (12, 19). Individual colonies were expanded, and supernatant fluids were retested for GAT-specific suppressive activity. The hybridomas to be discussed, 372.D6.5 and 372.B3.5, have been maintained in tissue culture for >12 mo without loss of activity, and active hybridoma cells have been recovered after freezing in liquid nitrogen. Supernatant fluids from BW5147 and cells from other wells of the same fusion (e.g., 372.B3.6) were consistently negative for either GAT-TsF_R activity or nonspecific suppressive activity. Supernatant fluids were processed as previously described for conventional GAT-TsF_R.

Results

Immune Responses by Spleen Cells from Neonatally Treated Mice. Spleen cells from B10 mice injected as neonates with B10-Mϕ developed significant responses when stimulated with soluble GAT, GAT-MBSA, and syngeneic B10 or allogeneic B10.D2 and B10.BR GAT-Mϕ (Table I). By contrast, spleen cells from B10 mice injected with B10 GAT-Mϕ as neonates responded to GAT presented on allogeneic B10.D2 and B10.BR Mϕ, but failed to respond to soluble GAT, GAT-MBSA, or syngeneic B10 GAT-Mϕ and had significantly reduced responses to GAT presented on (B10 × B10.D2)F_1 Mϕ. A similar pattern of responses was demonstrated in C57Bl/10 mice injected as neonates with soluble GAT and in BALB/c mice injected as neonates with soluble GAT or BALB/c GAT-Mϕ. In neonatally treated BALB/c mice responses were stimulated by allogeneic B10 and B10.BR GAT-Mϕ, but not by soluble GAT, GAT-MBSA, or BALB/c GAT-Mϕ (data not shown). In all experiments, responses to SRBC by spleen cells from neonatally treated mice were similar in the presence or absence of GAT-Mϕ from the panel of Mϕ used.

Demonstration of Suppressor Cells in Neonatally Treated Mice. Spleen cells from neonatally treated mice responded to allogeneic GAT-Mϕ, suggesting that the GAT-specific B cell populations were intact. The possibility that active suppression was the basis for the inability of spleen cells from neonatally treated mice to respond to soluble GAT or syngeneic GAT-Mϕ was investigated. Spleen cells or nylon wool-nonadherent spleen cells (T cells) from B10 mice injected as neonates with syngeneic B10 Mϕ (control) or B10 GAT-Mϕ (treated) were added to normal B10 spleen cells in cultures stimulated with a panel of GAT-Mϕ (Table II). Responses were unaffected by the addition of spleen cells or T cells from mice injected as neonates with B10 Mϕ. Spleen

TABLE I

Primary In Vitro PFC Responses by Spleen Cells From Neonatally Treated Mice

Antigens and Mϕ in culture*	Day-5 PFC/culture‡	
	Spleen cells	
	Control§	Treated§
GAT	470	28
GAT-MBSA	458	<10
B10 GAT-Mϕ	425	53
B10.D2 GAT-Mϕ	488	478
B10.BR GAT-Mϕ	410	420
(B10 × B10.D2)F_1 GAT-Mϕ	583	190
SRBC	3,450	3,330
SRBC + B10 GAT-Mϕ	4,500	3,150
SRBC + B10.D2 GAT-Mϕ	5,500	3,630

* Spleen cells (5×10^6) were cultured with 2 μg soluble GAT; GAT-MBSA containing 2 μg GAT; 3×10^4 GAT-Mϕ from the indicated strain that bore 5–10 ng GAT/10^5 cells; 10^7 SRBC; or 10^7 SRBC + 3×10^4 GAT-Mϕ from the indicated strain.

‡ IgG GAT-specific PFC/culture: IgM PFC/culture vs. SRBC in those cultures stimulated by SRBC with or without GAT-Mϕ.

§ Control, spleen cells from B10 mice injected with B10 Mϕ as neonates; treated, spleen cells from B10 mice injected with B10 GAT-Mϕ as neonates.

TABLE II

Suppression of PFC Responses by Virgin B10 Spleen Cells by Cells from Neonatally Treated B10 Mice

GAT-Mϕ	Day-5 IgG GAT-specific PFC/culture*				
	B10 spleen cells‡	Control§		Treated§	
		Spleen	T cells	Spleen	T cells
B10	595	580	445	55	36
B10.BR	410	410	450	443	457
B10.D2	580	570	500	565	510
B10 + SRBC	3,750‖	3,330‖	3,630‖	3,450‖	3,530‖

* B10 spleen cells (5×10^6) were cultured with 3×10^4 GAT-Mϕ of the indicated strains bearing 4–5 ng GAT/10^5 cells.

‡ Responses by B10 spleen cells to the GAT-Mϕ in the absence of additional spleen or T cells.

§ Control, B10 mice injected as neonates with B10-Mϕ; treated, B10 mice injected with B10 GAT-Mϕ as neonates. Spleen cells: 10^6/culture; nylon wool-nonadherent spleen cells (T cells): 10^5/culture.

‖ IgM PFC response to SRBC in B10 spleen cell cultures stimulated with B10 GAT-Mϕ and 10^7 SRBC.

cells or T cells from mice injected as neonates with B10 GAT-Mϕ did not alter the responses to B10.D2 or B10.BR GAT-Mϕ, but 10^6 spleen cells or 10^5 T cells significantly suppressed responses to B10 GAT-Mϕ and to GAT or GAT-MBSA (data not shown). Suppression was antigen specific, and no significant suppression of the response to SRBC was observed even when B10 GAT-Mϕ were present in culture.

Characterization of the Suppressor Cells. Suppression of the PFC response to GAT by spleen cells from neonatally treated mice was mediated by a nylon wool-nonadherent cell (Table II) which was also nonadherent to RαMIg-coated plates (Table III). The suppressor cell was sensitive to treatment with anti-Thy-1 + C and 500 rad irradiation. Significant suppression (~50%) of PFC responses by spleen cells stimulated with B10 GAT-Mϕ was observed with as few as 10^4 Ts cells (data not shown).

The Lyt phenotype and expression of Ia antigens by Ts cells were determined by assessing the ability of Ts cells to suppress PFC responses after treatment with either anti-Lyt antisera or anti-I region antisera and C. T cells from B10 mice injected as neonates with B10 Mϕ failed to suppress PFC responses and were not characterized further. Treatment of Ts cells from B10 mice injected as neonates with B10 GAT-Mϕ with arsinilate-conjugated anti-Lyt-1 and anti-Ars + C, anti-Ars + C, anti-I-J^k + C, anti-I-A^b + C, and anti-I-A^d + C failed to abolish the suppressive capacity of these cells (Table III). Treatment with arsinilate-conjugated anti-Lyt-2 and anti-Ars + C or anti-I-J^b + C destroyed the ability of these Ts cells to suppress PFC responses. Lastly, Ts cells, collected as nonadherent cells from RαMIg plates, were subsequently reacted with noncytotoxic anti-Lyt-1 (7.313) and separated on the basis of adherence to plates coated with goat anti-rat immunoglobulin; as few as 10^4 nonadherent (Lyt-1^-) cells suppressed GAT-specific PFC responses stimulated by B10 GAT-Mϕ, whereas adherent (Lyt-1^+) cells failed to suppress these responses (Table III, experiment 3). Collectively, these results demonstrated the Ts cells were Lyt-1^-2^+, I-J^+,I-A^-.

The kinetics of suppression was examined by adding Ts cells activated by incubation with B10 GAT-Mϕ or B10.BR GAT-Mϕ for 24 h to virgin B10 spleen cells stimulated with 2 μg GAT or SRBC. The Ts cells activated by B10 GAT-Mϕ, but not B10.BR GAT-Mϕ, must be present during the first 24 h of culture for significant suppression of responses to GAT (Table IV). Activated Ts cells had no significant effect on PFC

TABLE III

Phenotypic Analysis of GAT-TsF$_R$ Cells

Experiment	Treatment*	Added T cells‡	Day-5 IgG GAT-specific PFC/culture§	
			B10 spleen cells	
			B10 GAT-Mϕ	B10.BR GAT-Mϕ
1	None	Control	537	387
	None	Treated	67	360
	Anti-Lyt-1 + C	Treated	<10	350
	Anti-Lyt-2 + C	Treated	507	322
	Anti-Ars + C	Treated	<10	315
2	None	Control	498	340
	None	Treated	20	303
	Anti-I-J^k + C	Treated	<10	363
	Anti-I-J^b + C	Treated	508	320
	Anti-I-A^d	Treated	<10	288
	Anti-I-A^b	Treated	20	328
3	None	Control	678	508
	None	Treated	53	443
	Lyt-1$^-$	Treated	58	515
	Lyt-1$^+$	Treated	545	550

* Nonadherent T cells recovered from RαMIg plates were treated with arsinilate-conjugated anti-Lyt-1 or anti-Lyt-2 antibody followed by anti-Ars + C, anti-Ars + C, or anti-I-J or anti-I-A antibodies + C (Materials and Methods) before addition of 10^5 cells to cultures (experiments 1 and 2). T cells nonadherent to RαMIg plates on the basis of adherence to plates coated with goat anti-rat immunoglobulin; 10^5 cells nonadherent cells (Lyt-1$^-$) and adherent cells (Lyt-1$^+$) were added to culture (experiment 3).

‡ Control, T cells from B10 mice injected as neonates with B10-Mϕ; treated, T cells from B10 mice injected with B10 GAT-Mϕ as neonates.

§ B10 spleen cells (5×10^6) were cultured with 3×10^4 GAT-Mϕ that bore 4–5 ng GAT/10^5 cells from the indicated strains.

responses to SRBC. The activated Ts cells, which had no effect when added at 36 and 48 h (Table IV), suppressed PFC responses to GAT when added at the initiation of independent cultures.

GAT-Ts$_R$ were unable to suppress secondary PFC responses to GAT under conditions where significant suppression of primary GAT-specific PFC responses was demonstrated. The addition of GAT-Ts$_R$ to cultures of spleen cells from mice immunized 14 d previously with 10 μg GAT failed to suppress responses stimulated with B10 GAT-Mϕ, whereas these cells suppressed responses by virgin B10 spleen cells in a restricted manner (Table V). Spleen cells from mice injected 14 d previously with GAT were considered to be primed, as evidenced by their preferential restimulation by B10 GAT-Mϕ and absence of significant responses to GAT presented on allogeneic B10.BR Mϕ, as has previously been described (7).

Genetic Restrictions Regulating GAT-specific Suppression. Genetic restrictions in the

TABLE IV

Kinetics of Suppression by GAT-Ts$_R$ Cells

Time of addition of GAT-Ts cells	GAT-Mϕ	Day-5 IgG PFC/culture	
		SRBC	GAT
h			
0	B10.BR	3,390	383
0	B10	3,525	<10
24	B10.BR	3,300	387
24	B10	3,445	<10
36	B10.BR	3,450	386
36	B10	3,420	290
48	B10.BR	3,440	428
48	B10	3,420	400

T cells from neonatally treated B10 mice were incubated with 3×10^4 B10 or B10.BR GAT-Mϕ for 24 h and 10^5 cells were added at the indicated times to cultures of 5×10^6 virgin B10 spleen cells stimulated with 2 μg GAT or SRBC.

TABLE V

GAT-Ts$_R$ Cells Are Unable to Suppress Secondary Responses to GAT

Responder spleen cells*	T cells‡	Day-5 PFC/culture§		
		B10 GAT-Mϕ	B10.BR GAT-Mϕ	SRBC
Control	Control	580	410	4,883
	Treated	50	440	5,003
GAT-immune	Control	420	18	4,133
	Treated	515	30	4,195

* Control, virgin B10 spleen cells; GAT-immune, spleen cells from B10 mice immunized 14 d earlier with 10 μg GAT in Maalox-pertussis.

‡ Nylon wool-nonadherent T cells from control B10 mice injected with B10-Mϕ as neonates or treated B10 mice injected with B10 GAT-Mϕ as neonates.

§ B10 spleen cells (5×10^6) were cultured with 10^5 control or treated B10 T cells and 3×10^4 of the indicated GAT-Mϕ that bore 3–4 ng GAT/10^5 cells.

activation of GAT-Ts$_R$ cells from B10 mice treated as neonates with B10 GAT-Mϕ were examined. Significant suppression was observed after activation of GAT-Ts$_R$ with B10 GAT-Mϕ and B10.A(5R) GAT-Mϕ, but not after stimulation with B10.A(4R) GAT-Mϕ (Table VI). Thus, activation of GAT-Ts$_R$ cells required stimulation with GAT presented on Mϕ syngeneic with the Ts cell at the K,I-A subregions of H-2. GAT-Ts$_R$ cells from neonatally treated B10 mice suppressed primary GAT-specific PFC responses by B10, B10.A(3R), and B10.A(4R) spleen cells, but not responses by B10.D2 or B10.A(5R) spleen cells (Table VII). These results indicated that syngeneity at the I-J subregion between the GAT-Ts$_R$ cell and its target cell was necessary and sufficient for suppression. Furthermore, the ability to suppress apparently did not require homozygosity in the I-J subregion: responses by (B10.D2 × B10)F$_1$ spleen cells were effectively suppressed by B10 GAT-Ts$_R$ cells (Table VII).

Demonstration of GAT-TsF$_R$ in Responder Mice. Supernatant fluids from spleen cells of B10 mice neonatally injected with B10 GAT-Mϕ, when cultured with 3×10^4 B10 GAT-Mϕ for 24 h, profoundly suppressed primary PFC responses by B10 spleen cells

TABLE VI

Genetic Requirements for Activation of GAT-Ts_R Cells

GAT-Mϕ*	Shared H-2 regions‡	Day-5 IgG GAT-specific PFC/culture§	
		T cells‖	
		Control	Treated
B10	KABJECSGD	605	<10
B10.A(5R)	KAB	583	90
B10.A(4R)	BJECSGD	568	522
B10.D2	None	505	515

* 3×10^4 GAT-Mϕ/culture bearing ~3–4 ng GAT/10^5 cells.

‡ H-2 regions shared between stimulating GAT-Mϕ and the Ts cell.

§ B10 spleen cells (5×10^6) were cultured with the indicated GAT-Mϕ and 10^5 control or neonatally treated B10 T cells.

‖ Control, B10 mice injected with B10 Mϕ as neonates; treated, B10 mice injected with B10 GAT-Mϕ as neonates.

TABLE VII

Genetic Restrictions on GAT-Ts_R Cell Activity

Responder spleen cells	Shared H-2 regions*	Day-5 PFC/culture‡		
		T cells§		
		Control	Treated	SRBC
B10	KABJECSGD	533	28	2,110
B10.A(3R)	KABJ	465	40	3,910
B10.A(4R)	BJECSGD	418	60	3,400
B10.A(5R)	KAB	493	433	2,310
B10.D2	None	440	453	2,800
(B10.D2 × B10)F_1	KABJECSGD	572	100	2,970

* H-2 regions shared between the GAT-Ts_R cells and the responder spleen cells.

‡ Responder spleen cells (5×10^6) from the indicated strain were cultured with 3×10^4 B10 GAT-Mϕ and 10^5 control or treated B10 T cells; IgG GAT-specific PFC/culture were assayed on day 5. In control cultures, IgM PFC responses to SRBC were assayed on day 5 in cultures of responder spleen cells and 10^5 treated T cells stimulated with 3×10^4 B10 GAT-Mϕ and 10^7 SRBC.

§ Control, B10 mice injected with B10 Mϕ as neonates; treated, B10 mice injected with B10 GAT-Mϕ as neonates.

to GAT, but not to SRBC (Fig. 1; Table VIII). Significant activity (~60% suppression) was detected at final dilutions of 1:2,000 in culture; the suppression observed varied slightly with preparations of conventional GAT-TsF_R. Supernatant fluids from neonatally treated B10 cells, cultured with B10.D2 GAT-Mϕ (Fig. 1), or from control B10 cells, cultured with either B10 or B10.D2 GAT-Mϕ, failed to suppress responses by B10 spleen cells at all concentrations tested (data not shown). No differences in cell numbers or cell viability was observed in cultures containing control or active suppressor factors.

T cell hybridomas that produce GAT-TsF_R on a constitutive basis were constructed to allow purification and biochemical analysis and comparison with nonresponder GAT-TsF (13). Activated GAT-Ts_R cells were fused with the AKR thymoma BW5147; supernatant fluids from two cloned hybridomas, 372.B3.5 and 372.D6.5, suppressed

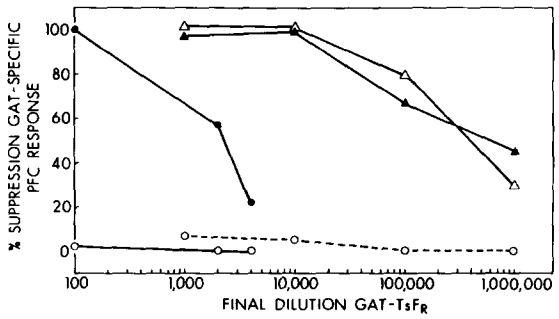

FIG. 1. Effects of GAT-TsF_R on primary GAT-specific PFC responses in vitro. Conventional (●), hybridoma 372.B3.5 (▲) and 372.D6.5 (△) GAT-TsF_R, control supernatant fluid (activated by B10.D2 GAT-Mϕ) (○), and fluid from a negative fusion well 372.B3.6 (○--○) were tested at various dilutions for their ability to suppress PFC responses by normal B10 spleen cells (5×10^6) stimulated with 2 μg GAT. IgG-GAT-specific PFC/culture were determined on day 5. GAT-specific response in absence of factor was 490 PFC/culture.

TABLE VIII

Effects of GAT-TsF_R on In Vitro PFC Responses

Experiment	B10 Spleen cells	Source of GAT-TsF_R	Day 5-PFC responses		
			GAT		SRBC
1	Virgin	372.B3.6 (control)	373		1,595
	Virgin	Conventional	53		1,613
	Virgin	372.D6.5	<10		1,885
	Virgin	372.B3.5	38		1,830
	GAT-immune	372.B3.6 (control)	525		1,860
	GAT-immune	Conventional	495		1,875
	GAT-immune	372.D6.5	560		1,725
	GAT-immune	372.B3.5	535		1,613
			GAT	GAT-MBSA	SRBC
2	Virgin	372.B3.6 (control)	385	368	2,500
	Virgin	Conventional	33	35	2,750
	Virgin	372.D6.5	23	25	2,800
	Virgin	372.B3.5	35	83	3,780

Spleen cells (5×10^6) from virgin B10 mice or B10 mice immunized 3 wk previously with 10 μg GAT in Maalox-pertussis were cultured with either 2 μg GAT, GAT-MBSA containing 2 μg GAT, or 10^7 SRBC and conventional GAT-TsF_R (1:500 final dilution), hybridoma 372.D6.5 or 372.B3.5 GAT-TsF_R (1:20,000 final dilution), or control hybridoma fluid 372.B3.6 (1:10 final dilution). Data are expressed as IgG GAT-specific PFC in cultures stimulated with GAT or GAT-MBSA and IgM PFC vs. SRBC. GAT-specific response in the absence of factor was 390 PFC/culture for virgin spleen cells and 500 PFC/culture for immune spleen cells.

primary GAT-specific PFC responses by B10 spleen cells by 50% at final dilutions of 1:2,000,000 to 1:6,000,000 (Fig. 1). Supernatant fluids from BW5147 and other hybridomas (e.g., 372.B3.6) from the same fusion were negative for GAT-TsF_R activity and failed to suppress at final dilutions of 1:1000—the lowest dilution tested. All hybridoma culture supernatant fluids failed to suppress responses to SRBC (Table VIII).

Parameters of GAT-TsF_R-mediated Suppression. Conventional and hybridoma-derived

GAT-TsF$_R$ suppressed primary in vitro GAT-specific PFC responses to GAT and GAT-MBSA, but not PFC responses to SRBC by B10 spleen cells (Table VIII). Secondary responses to GAT were not suppressed by either hybridoma-derived or conventional GAT-TsF$_R$. Kinetic analysis of GAT-TsF$_R$-mediated suppression demonstrated that hybridoma-derived and conventional GAT-TsF$_R$ had to be present during the first 24 h of culture for significant suppression to occur (Fig. 2A). GAT-TsF$_R$ blocked initiation of the GAT-specific PFC response rather than causing a delay in the response, because responses on days 6 and 7 were also completely suppressed (Fig. 2B).

Genetic Restrictions on GAT-TsF$_R$ Activity. Production of conventional GAT-TsF$_R$ required compatibility at the K,I-A subregions of H-2 between the GAT-Mϕ and GAT-Ts$_R$ cells, as previously shown for activation of the GAT-Ts$_R$ cells (Table VI). Genetic restrictions on the activity of GAT-TsF$_R$ at the level of interaction with target cells was examined using B10 congenic mice sharing portions of the H-2 complex with the cell producing GAT-TsF$_R$ (Table IX). Conventional GAT-TsF$_R$ and hybridoma 372.D6.5 GAT-TsF$_R$ suppressed GAT-specific PFC responses by B10 and B10.A(3R) spleen cells, but failed to suppress responses by B10.D2 or B10.A(5R) spleen cells. These results demonstrate a restriction mapping to the I-J subregion of H-2 to obtain suppression with hybridoma 372.D6.5 or conventional GAT-TsF$_R$. Analysis of suppression by hybridoma 372.B3.5 GAT-TsF$_R$ demonstrated no genetic restrictions in activity, as the responses to GAT by all tested spleen cells were suppressed to comparable degrees. Lastly, the responses of (B10 × B10.D2)F$_1$ spleen cells were suppressed by all three sources of GAT-TsF$_R$, and responses to SRBC were not significantly affected.

Initial Characterization of Conventional B10 and Hybridoma-derived GAT-TsF$_R$. Apparent molecular weights of conventional and hybridoma-derived GAT-TsF$_R$ were determined by gel filtration on a Sephacryl S-200 column equilibrated with isotonic phosphate buffer, pH 7.2. The specific suppressive activity of conventional and hybridoma 372.B3.5 and 372.D6.5 GAT-TsF$_R$ eluted between the marker proteins

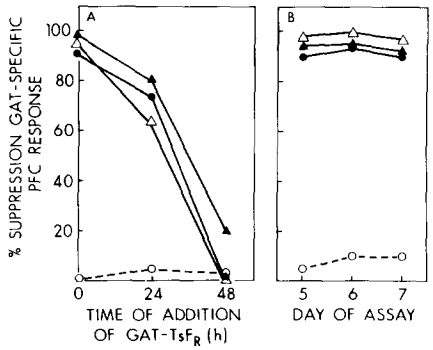

FIG. 2. Kinetics of GAT-TsF$_R$-mediated suppression. B10 spleen cells (5 × 10^6) were cultured with 2 μg GAT with conventional GAT-TsF$_R$ (●) (1:500 final dilution), hybridoma 372.D6.5 (△) or 372.B3.5 (▲) GAT-TsF$_R$ (1:20,000 final dilution), or control hybridoma 372.B3.6 supernatant fluids (○--○) (1:10, final dilution). GAT-TsF$_R$ were added at the indicated times; IgG GAT-specific PFC/culture were determined on day 5 (A). IgG GAT-specific PFC/culture were determined on the indicated days in cultures receiving GAT-TsF$_R$ on day 0 (B). GAT-specific response in the absence of factor was 510 PFC/culture.

TABLE IX

Genetic Restrictions on GAT-TsF$_R$ Activity

Spleen cells	Source of GAT-TsF$_R$	Shared H-2 regions	Day-5 PFC/culture	
			GAT	SRBC
B10	None	All	435	1,550
B10	Conventional	All	28	1,975
B10	372.D6.5	All	63	2,110
B10	372.B3.5	All	38	2,120
B10.A(3R)	None	KABJ	455	1,910
B10.A(3R)	Conventional	KABJ	30	1,720
B10.A(3R)	372.D6.5	KABJ	33	2,980
B10.A(3R)	372.B3.5	KABJ	50	2,310
B10.A(5R)	None	KAB	390	1,810
B10.A(5R)	Conventional	KAB	403	1,755
B10.A(5R)	372.D6.5	KAB	385	2,010
B10.A(5R)	372.B3.5	KAB	13	2,970
B10.D2	None	None	425	2,000
B10.D2	Conventional	None	418	1,950
B10.D2	372.D6.5	None	488	2,800
B10.D2	372.B3.5	None	45	2,610
(B10 × B10.D2)F$_1$	None	All	525	1,430
(B10 × B10.D2)F$_1$	Conventional	All	100	1,650
(B10 × B10.D2)F$_1$	372.D6.5	All	<10	1,815
(B10 × B10.D2)F$_1$	372.B3.5	All	<10	1,980

Spleen cells (5×10^6) from the indicated strains of mice were cultured with 2 μg GAT or 10^7 SRBC and conventional GAT-TsF$_R$ (1:500 final dilution) or hybridoma 372.D6.5- or 372.B3.5-derived GAT-TsF$_R$ (1:20,000 final dilution). IgG GAT-specific and IgM vs. SRBC PFC/culture were determined on day 5. The H-2 regions shared between the cells producing GAT-TsF$_R$ and the responder spleen cells are indicated.

BSA and ovalbumin (OVA), thus placing the apparent molecular weight between 48,000 and 63,000 (Fig. 3). Activity of all three GAT-TsF$_R$ was totally destroyed by exposure to trypsin and to a pH of 2.0 or 3.0 and was partially destroyed by heating to 50°C for 30 min (data not shown).

Conventional and hybridoma-derived GAT-TsF$_R$ from B10 mice were absorbed by immunoadsorbents composed of B10.D2 anti-B10 (anti-H-2^b), B10.A(5R) anti-B10.A(3R) (anti-J^b) alloantisera, and the monoclonal WF9.40.5 (anti-J^b) reagent, but not by immunoadsorbents composed of (B10.A × A) anti-B10.A(5R) (anti-KAB^b), (B10.A × B10) anti-B10.D2 (anti-KABJE)^d, [A.TL × B10.S(9R)] anti-B10.HTT (anti-J^s) alloantisera, or the monoclonal WF8.C12.8 (anti-J^k) reagent (Table X). In all cases activity bound to the immunoadsorbents could be eluted quantitatively with 2.0 M KCl (data not shown). These findings demonstrate the presence of I-J^b determinants on the three GAT-TsF$_R$. RαMIg or rabbit anti-GAT immunoabsorbents failed to remove significant suppressive activity from GAT-TsF$_R$.

The three GAT-TsF$_R$ were also analyzed for antigen-binding capacity and fine specificity by their ability to bind to immunoadsorbents composed of different proteins or polypeptides related to GAT. Suppressive activity was retained by immunoadsorb-

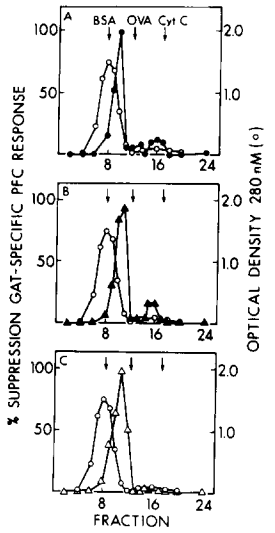

FIG. 3. Molecular weight of GAT-TsF$_R$ by gel filtration. Conventional GAT-TsF$_R$ (●) (A), hybridoma 372.B3.5 (▲) (B), and 372.D6.5 (△) (C) GAT-TsF$_R$ after chromatography on a Sephacryl S-200 column with phosphate buffer were assayed for ability to suppress a primary response to GAT by B10 spleen cells. Data are expressed as the percentage of suppression of GAT-specific PFC response, which was 525 PFC/culture in control cultures. Arrows indicate the fractions where the marker proteins BSA, OVA, and cytochrome *c* (Cyt C) eluted; OD at 280 nm (○).

ents composed of GAT and the related polymer, GT, but not by immunoadsorbents composed of GA or BSA (Table X). Suppressive activity could be eluted from GAT and GT immunoadsorbents with 2.0 M KCl. The GAT-TsF$_R$ were analyzed for the presence of idiotype determinants shared with murine anti-GAT antibodies (21). The anti-idiotype immunoadsorbent anti-cGAT quantitatively removed suppressor activity (Table X), which could be recovered after elution with 2.0 M KCl. Finally, suppressive activity was also absorbed by PNA agarose, showing the presence of carbohydrate moieties on the three species of GAT-TsF$_R$.

Discussion

Adult spleen cells from B10 mice injected intravenously with syngeneic GAT-Mϕ within 18 h of birth failed to develop significant GAT-specific PFC responses after stimulation with soluble GAT, GAT-MBSA, or syngeneic GAT-Mϕ; responses to allogeneic GAT-Mϕ and an irrelevant antigen, SRBC, were intact. The response to GAT presented on allogeneic Mϕ suggested that the B cell pool capable of generating anti-GAT antibody responses was intact and the selective inability to respond to GAT was not a result of a B cell defect. The cell responsible for the unresponsiveness to syngeneic GAT-Mϕ was identified as Ts cell by showing that T cells from neonatally treated B10 mice could suppress responses to GAT by virgin B10 spleen cells stimulated by B10 GAT-Mϕ. The GAT-specific Ts cell from responder mice (GAT-Ts$_R$ cell) was sensitive to 500 rad irradiation, treatment with anti-Thy-1 + C, anti-Lyt-2 + C, and anti-I-J^b + C, but was unaffected by treatment with anti-Lyt-1 + C or anti I-A^b + C. Therefore, the phenotype of the GAT-Ts$_R$ cell was Lyt-1$^-$2$^+$, I-J$^+$. GAT-Ts$_R$ cells had to be present within the first 24 h of culture for significant suppression to occur. Suppression was restricted to primary GAT-specific PFC re-

TABLE X

Immunochemical Characterization of Conventional and Hybridoma-derived GAT-TsF$_R$

Experiment	Immunoadsorbent	Control response		
		Conventional GAT-TsF$_R$	372.D6.5 GAT-TsF$_R$	372.B3.5 GAT-TsF$_R$
			%	
1	None	9	4	8
	Anti-H-2^b	85	86	83
	Anti-J^b (alloantiserum)	92	88	94
	Anti-J^b (monoclonal)	82	91	87
	Anti-(KAB)^b	11	5	11
	Anti-(KABJE)^d	13	6	18
	Anti-J^s (alloantiserum)	3	5	3
	Anti-J^k (monoclonal)	5	3	5
	Anti-Ig	18	12	12
	Anti-GAT	15	10	10
2	None	10	5	9
	GAT	99	112	112
	GT	88	119	128
	GA	9	11	6
	BSA	8	8	5
	Anti-cGAT	91	89	84
	PNA agarose	100	101	108

Conventional and hybridoma-derived GAT-TsF$_R$ were absorbed with the indicated immunoadsorbent before addition to culture at final dilutions of 1:500 (conventional GAT-TsF$_R$) or 1:20,000 (hybridoma GAT-TsF$_R$). B10 spleen cells (5×10^6) were cultured with 2 μg GAT and GAT-TsF$_R$ absorbed with the various immunoadsorbents; data are presented as percent control PFC response, which was 525 IgG GAT-specific PFC/culture in experiment 1 and 625 IgG GAT-specific PFC/culture in experiment 2.

sponses; secondary PFC responses to GAT were resistant to GAT-Ts$_R$, as were responses to an irrelevant antigen, SRBC. These GAT-Ts$_R$ cells were activated by GAT presented on Mϕ syngeneic at K,I-A with the Ts cell; once activated, the action of GAT-Ts$_R$ cells was restricted to target cells syngeneic at I-J with the Ts cell.

Restimulation of these cells with B10 GAT-Mϕ resulted in the production and secretion of a suppressor factor that functionally substituted for the GAT-Ts$_R$ cells into the supernatant fluid. The suppressor factor, GAT-TsF$_R$, exhibited the same genetic restrictions and kinetics of action, and affected the same range of responses as the GAT-Ts$_R$ cells. T cell hybridomas producing GAT-TsF$_R$ were generated to facilitate characterization and purification. Initial characterization of conventional and two hybridoma-derived GAT-TsF$_R$ showed that the GAT-TsF$_R$ were glycoproteins with an apparent molecular weight between 48,000 and 63,000 whose activity was destroyed by trypsin, a pH of 3.0, and by heating to 50°C for 30 min, suggesting that tertiary structure is required for activity. The three GAT-TsF$_R$ lacked detectable Ig-constant-region determinants, but had determinants recognized by antisera directed against the common idiotype on anti-GAT antibodies (the cGAT idiotype) and had determinants encoded by the I-J subregion of the H-2 complex. The GAT-TsF$_R$ also were specifically absorbed by GAT and GT immunoadsorbents, but not by

the closely related polymer GA. Of the two T cell hybridomas that secrete GAT-TsF_R on a constitutive basis, hybridoma 372.D6.5 GAT-TsF_R was identical to conventional GAT-TsF_R in activities and properties, whereas hybridoma 372.B3.5 GAT-TsF_R differs from hybridoma 372.D6.5 and conventional GAT-TsF_R in genetic restrictions on interactions with target cells (see below).

The ability to generate GAT-Ts cells in responder mice has been previously demonstrated, but only under certain circumstances (4–6). The activation of GAT-specific, but genetically unrestricted, Ts cells has been suggested as the mechanism responsible for the pattern of genetic restrictions observed in secondary responses to GAT by spleen cells from immune responder and (responder × nonresponder)F_1 mice (6, 7, 22). GAT-specific Ts cells may also be generated in vitro by exposure of responder T cells to GAT in the absence of Mϕ; this result has been interpreted to suggest that Mϕ-bound antigen stimulates helper T cells, whereas soluble antigen preferentially stimulates Ts cells (4). The induction of GAT-Ts_R cells by neonatal injection of syngeneic GAT-Mϕ is, to our knowledge, the first demonstration of induction of Ts cells by this mechanism and appears to contradict the previous findings of Pierres and Germain (4). The precise relationships among the various GAT-specific Ts cells induced in responder mice by these different regimens are not yet clear, but are currently under investigation.

It is also important to note that studies similar to these have been reported by Debré (5) where mice were given an intraperitoneal injection of GAT in Maalox-pertussis at birth and were examined for suppressor activity later in neonatal life rather than as adults; Ts cells could be induced in mice injected at birth, but not on day 8 or after. The Ts cells described here are induced within 24 h of birth, are phenotypically stable into the adult life and may be of the same lineage as those described by Debré.

GAT-Ts cells from responder mice injected as neonates with syngeneic GAT-Mϕ and nonresponder mice injected as adults with soluble GAT have identical biological activity: both Ts cell populations suppress primary, but not secondary, PFC responses to GAT by spleen cells from the appropriate mouse strain(s) in an antigen-specific manner and with identical kinetics. Important subtle differences, however, do exist between these two populations: GAT-Ts_R cells are induced by antigen-pulsed syngeneic Mϕ or soluble antigen; nonresponder GAT-Ts cells are induced after injection of soluble antigen only. GAT-Ts_R cells are also initially stimulated in neonatal animals and require reactivation to function, whereas nonresponder GAT-Ts cells are stimulated directly in adult animals and do not exhibit memory (2, 3). GAT-Ts_R cells are genetically restricted and require syngenicity at the I-J subregion between the Ts cell and target cell to achieve suppression; similar experiments with nonresponder GAT-Ts cells have not been carried out to determine if these cells require syngenicity at the I-J subregion to suppress. The ability of GAT-Ts cells, but not nonresponder GAT-Ts cells, to suppress responses by appropriate F_1 spleen cells may suggest different mechanisms of suppression in responder and nonresponder mice to GAT. This would predict a minimum of two similar (although unique and probably parallel) mechanisms for regulating a specific immune response. An alternative possibility is that the two GAT-Ts cells represent different cells along a hypothetical common suppressor pathway similar to that suggested by Germain and Benacerraf (23), implying that although the mechanism of suppression may be the same in responder and nonre-

sponder mice, the point of entry into the suppressor pathway is different.

These GAT-Ts_R cells mediate suppression via a soluble suppressor factor, GAT-TsF_R, which can substitute for the Ts cell. Antigen-specific suppressor factors derived from Ts cells have been demonstrated in a number of different systems (23–25) and those from nonresponder Ts cells have been well-characterized in the GAT system (8–13). The nonresponder GAT-specific suppressor factor GAT-TsF is a glycoprotein, originally extracted from Ts cells from nonresponder mice immunized with soluble GAT, and more recently produced by T cell hybridomas on a constitutive basis; the hybridoma-derived product is indistinguishable from the conventionally extracted GAT-TsF (12, 13). GAT-TsF from hybridoma 258.C4.4 has a binding site for GAT, shares determinants from the Ig-variable region with anti-GAT antibodies (i.e., bears the cGAT idiotype) and bears determinants encoded by the I-J subregion of the H-2 complex. The glycoprotein has a molecular weight of 24,000 and has been extensively characterized in terms of its immunochemical properties and range of activities (8–13).

The conventional and hybridoma-derived GAT-TsF_R described here share many characteristics with nonresponder GAT-TsF. For example, determinants encoded by the I-J subregion of the strain producing the suppressor factors have been demonstrated on GAT-TsF and the three species of GAT-TsF_R; relatively crude preparations of GAT-TsF and GAT-TsF_R have similar molecular weights when determined by gel filtration methods using physiologic buffers. When the molecular weight of GAT-TsF from hybridoma 258.C4.4 was initially determined by Sephacryl S-200 chromatography, it was in the 48,000–63,000 range. However, the 24,000 mol wt was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after extensive purification on high-pressure liquid chromatography resins in high-ionic-strength buffers (13). Preliminary purification of GAT-TsF_R from hybridomas 372.B3.5 and 372.D6.5 in isotonic buffers indicated molecular weights in the 48,000–63,000 range. Purification in high-ionic-strength buffers (0.5 M acetic acid; 1 M pyridine) by high-performance liquid chromatographic procedures indicates the molecular weight of GAT-TsF_R from hybridoma 372.B3.5 is 24,000, whereas GAT-TsF_R from hybridoma 372.D6.5 has a 60,000 mol wt (26; and C. M. Sorensen, Pierce, C. W., and D. R. Webb, manuscript in preparation). GAT-TsF and GAT-TsF_R bind to GAT and bear the cGAT idiotypic determinants; as determined by the ability of the anti-cGAT idiotypic reagent, produced in guinea pigs immunized with affinity-purified serum anti-GAT antibodies (21), to absorb nonresponder GAT-TsF (27) and the conventional and the two species of hybridoma responder GAT-TsF_R. Thus, the various GAT-specific suppressor factors could bear the identical idiotypic determinant expressed on antibody to GAT, or at least bear a determinant that is cross-reactive. The purification of hybridoma-derived GAT-TsF and GAT-TsF_R and a detailed comparison with monoclonal anti-GAT antibodies should resolve this issue. Lastly, GAT-TsF and GAT-TsF_R suppress primary GAT-specific PFC responses in an antigen-specific manner and also suppress GAT-specific T cell-proliferative responses (11; and C. M. Sorensen and C. W. Pierce, unpublished observations).

Although the GAT-TsF_R and nonresponder GAT-TsF have characteristics in common, important differences exist. The differences in molecular weights after purification in high-ionic-strength buffers noted above and in genetic restrictions at the level of interactions of suppressor factor with target cells are the major differences

currently noted between the two hybridoma-derived GAT-TsF_R and conventional or hybridoma-derived nonresponder GAT-TsF. Conventional GAT-TsF derived from one nonresponder strain (DBA/1, H-2^q) suppressed PFC and T cell-proliferative responses to GAT by other nonresponder strains in an antigen-specific manner (9, 11, 28). The hybridoma 258.C4.4 GAT-TsF, derived from DBA/1 Ts cells, also suppresses PFC and proliferative responses to GAT by other nonresponder strains (12). This same pattern of unrestricted suppression has been observed with hybridoma 372.B3.5 GAT-TsF_R, which suppressed PFC responses by spleen cells from all H-2 haplotypes tested in an antigen-specific manner. Recent preliminary experiments demonstrated that hybridoma 258.C4.4 GAT-TsF and hybridoma 372.B3.5 GAT-TsF_R cross-suppressed GAT-specific PFC responses of nonresponder spleen cells to GAT-MBSA and responder spleen cells to GAT (C. W. Pierce, J. A. Kapp, and C. M. Sorensen, unpublished observations). Thus, these two species of GAT-specific suppressor factors, 258.C4.4 GAT-TsF and 372.B3.5 GAT-TsF_R, appear to represent one class of factors that suppressd PFC and/or proliferative responses in a genetically unrestricted manner and have a molecular weight of 24,000 after purification by high-ionic-strength buffers and high-pressure liquid chromatography (13, 26; and C. M. Sorensen, C. W. Pierce, and D. R. Webb, manuscript in preparation).

By contrast, conventional GAT-TsF_R and hybridoma 372.D6.5 GAT-TsF_R only suppress PFC responses by spleen cells from mice bearing the I-J^b haplotype. These GAT-TsF_R appear to represent a second class of factors that suppress responses in a genetically restricted manner. Because the conventional GAT-TsF_R and hybridoma 372.D6.5 GAT-TsF_R are identical in all respects tested to date, it is reasonable to conclude that the GAT-Ts_R cell producing conventional GAT-TsF_R has been immortalized in hybridoma 372.D6.5. Thus far we have not detected activity comparable to hybridoma 372.B3.5 GAT-TsF_R in conventional GAT-TsF_R preparations. Although syngenicity at the I-J subregion is necessary and sufficient for suppression by these two species of GAT-TsF_R, homozygosity at the I-J subregion is not required, because responses by spleen cells from (B10 × B10.D2)F_1 mice, which are (I-J^b × I-J^d)F_1 at I-J, are suppressed to a degree comparable to responses by spleen cells from a I-J^b homozygote such as B10. The ability to suppress responses by F_1 mice has not been demonstrated for nonresponder GAT-TsF unless both parents in the F_1 are nonresponder haplotypes (9).

The two hybridoma-derived GAT-TsF_R described were derived from the same population of GAT-Ts_R cells, yet may represent fusions of unique populations of GAT-Ts_R cells. Hybridoma 372.D6.5 GAT-TsF_R is genetically restricted in action by the I-J subregion, whereas hybridoma 372.B3.5 GAT-TsF_R is genetically unrestricted, which is analogous to nonresponder GAT-TsF. These two hybridoma-derived GAT-TsF_R are, in many respects, similar to other Ts cell factors that may be divided into two broad categories: one type of TsF is I-J^+ and acts in an unrestricted manner; a second type of TsF is also I-J^+ but acts in a genetically restricted manner (15, 24, 25, 29).

Suppressor factors with properties of the first group, referred to as TsF_1 in the GAT system (26), have been detected after injection of syngeneic cells derivatized with haptens, such as azobenzene arsonate (ABA) (30) and 4-hydroxy-3-nitrophenyl acetyl (NP) (31), injection of nonresponder mice with GAT (8–13), or injection of mice with the copolymer GT (29). Hybridoma 372.B3.5 GAT-TsF_R and hybridoma 258.C4.4

are representative of TsF_1. These TsF_1 factors are products of Ts cells that appear to be activated early in the suppressor pathway (23–25, 29); these Ts cells and their TsF_1 products are essential for the induction of a second-order of Ts cells that produce suppressor factors, termed TsF_2 in the GAT-system. Hybridoma 372.D6.5 GAT-TsF_R is representative of a TsF_2 in the GAT-system. TsF_2 are distinguished from TsF_1 primarily by the genetic restrictions on their activity, although differences in molecular weights and number of polypeptide chains are beginning to emerge as these molecules are further characterized (26; C. M. Sorensen, C. W. Pierce, and D. R. Webb, manuscript in preparation). The TsF to be detected in the NP (31, 32) and ABA (25, 30, 33) systems (and called TsF_2 in these systems) are in fact anti-idiotype in reactivity, and are idiotype negative, non-antigen binding, and are induced in the absence of antigen. In the GT system, however, TsF_1 alone does not induce a species of TsF_2, but TsF_1 plus GT induces TsF_2, which has antigen-binding specificity for GT (29). The GT-TsF_2 and hybridoma 372.D6.5 GAT-TsF_R have many similarities with the carrier-specific, I-J^+-region-restricted suppressor factors described by Tada and Okumura (24) and Taniguchi et al. (34). One possible and intriguing interpretation of our data is that the two hybridomas, 372.B3.5 and 372.D6.5, represent sequential cells along the GAT-suppressor pathway. It is not known how these two distinct GAT-Ts_R cells were activated by injection of GAT-Mϕ into neonates. The precise location of these two GAT-TsF_R along the GAT suppressor pathway, the biochemical and functional relationships between responder and nonresponder GAT-TsF species, and the analysis of the mechanism(s) of suppression are currently being investigated.

Summary

Spleen cells from C57Bl/10 mice injected with syngeneic B10 L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$ (GAT)-pulsed macrophages (GAT-Mϕ) within 18 h of birth were unable to respond to soluble GAT, GAT-methylated bovine serum albumin, or B10 GAT-Mϕ as adults. Spleen cells from these neonatally treated mice responded at control levels to GAT presented in allogeneic Mϕ and to sheep erythrocytes. Partially purified T cells from these neonatally treated mice suppressed responses by syngeneic virgin, but not primed, spleen cells in an antigen-specific manner and acted during the early phases of the response. These responder GAT-specific suppressor T cells (GAT-Ts_R) were sensitive to anti-Thy-1 + C and 500-rad irradiation and have the phenotype Ly-1^-2^+, I-J^+; GAT-Ts_R cells can only suppress responses by spleen cells syngeneic with the GAT-Ts_R cells at the I-J subregion of H-2. Restimulation of these Ts cells with syngeneic GAT-Mϕ induces an antigen-specific suppressor factor within the supernatant fluid. The factor, GAT-TsF_R, is a glycoprotein with a molecular weight between 48,000 and 63,000, as determined by gel filtration chromatography using isotonic buffers; it bears serologically detectable determinants encoded by the I-J subregion of the H-2 complex, has an antigen-binding site for GAT and L-glutamic $acid^{50}$-L-$tyrosine^{50}$, and shares idiotypic determinants with anti-GAT antibodies. The presence of GAT-TsF_R in the first 36 h of in vitro culture is required for significant suppression. Furthermore, only responses by spleen cell syngeneic with the cells producing GAT-TsF_R at the I-J subregion are suppressed. The fusion of GAT-TsF_R-producing cells with BW5147 resulted in generation of two hybridomas with properties and characteristics identical to those of the conventional GAT-TsF_R with one exception: conventional and hybridoma 372.D6.5 GAT-TsF_R only suppress responses by

spleen cells of the I-J^b haplotype, whereas suppression mediated by the second hybridoma GAT-TsF_R (372.B3.5) is genetically unrestricted. These hybridoma GAT-TsF_R are compared with nonresponder GAT-Ts factor (GAT-TsF) and these responder and nonresponder GAT-TsF are considered in the context of suppressor pathways.

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