IMMUNOSUPPRESSIVE FACTOR(S) SPECIFIC FOR L-GLUTAMIC ACID^{50}-L-TYROSINE^{50} (GT)
III. Generation of Suppressor T Cells by a Suppressive Extract Derived from GT-Primed Lymphoid Cells*

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Studies from our laboratory have demonstrated that the terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)[1] stimulates the development of T cells capable of specifically suppressing the in vivo and in vitro antibody responses of nonresponder mice (*H-2*^{p,q,s} haplotypes) to GAT complexed with the immunogenic carrier, methylated bovine serum albumin (MBSA) (1, 2). These studies were extended to another synthetic antigen, the copolymer of L-glutamic acid^{50}-L-tyrosine^{50} (GT) (3). GT is not immunogenic in any of the 20 inbred mouse strains tested. Preimmunization with GT has a suppressive effect on antibody responses to GT-MBSA in mouse strains bearing *H-2*^{d,f,k,s} haplotypes, but not in strains with *H-2*^{a,b,q} haplotypes. Spleen cells from GT-primed nonresponder BALB/c mice (*H-2*^{d}) specifically inhibit the GT-specific plaque-forming cell (PFC) responses to GT-MBSA of normal syngeneic mice (3). These suppressor cells are T cells as demonstrated by their sensitivity to anti-Thy-1 and C (C. Waltenbaugh, unpublished observations). Furthermore, GT-specific suppression was shown to be controlled by two complementing, *H-2*-linked, immune suppressor genes (4, 5).

Tada and associates have described a cell-free antigen-specific T-cell factor extracted by sonication from spleen and thymus cells of immunized mice (6, 7). Kapp et al. (8) have demonstrated the preparation of a similar T-cell suppressive factor extracted from lymphoid cells of GAT-primed nonresponder mice. More recently, we have described the preparation of an active GT-specific suppressive

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[1] *Abbreviations used in this paper:* ECDI, 3-dimethylamino propyl carbodiimide; GAT, terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-T_sF, GAT-specific suppressor T-cell factor; GT, copolymer of L-glutamic acid^{50}-L-tyrosine^{50}; GT-T_sF, GT-specific suppressor T-cell factor; MBSA, methylated bovine serum albumin; *P* value, statistical significance was determined by comparing data groups by using Student's *t* test; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

material extracted from spleen and/or thymus cells of BALB/c ($H\text{-}2^d$) and B10.BR ($H\text{-}2^k$) strains (9). The GT-suppressive extracts are very similar to GAT-suppressive factor, moreover, their activity in allogeneic strains has been investigated (9). Thus, BALB/c GT-suppressive extract was shown to suppress GT-MBSA responses in the GT-nonsuppressor A/J ($H\text{-}2^a$) strain, which can neither be suppressed by GT preimmunization nor produce a GT-suppressive factor (GT-T_sF) (9). In addition, we have shown that pretreatment of BALB/c mice with cyclophosphamide abolishes their ability to develop GT-specific suppression; under these conditions injection of GT did not inhibit GT-MBSA responses (10). However, administration of BALB/c GT-suppressive extract to cyclophosphamide-treated BALB/c mice suppressed their GT-MBSA responses (10). These are two examples of suppression by the appropriate specific suppressive extract in mice unable to be specifically suppressed by antigen. Collectively, these results indicate that specific suppressor factor can stimulate the development of suppressor T cells and suggest a two-step model for the induction of antigen-specific suppression (9, 11). The first step is initiated by antigen, while the second is factor-mediated. According to this hypothesis: (*a*) cyclophosphamide treatment of suppressor haplotype mice abolishes the first without affecting the second step; (*b*) A/J mice have a genetic defect at the antigen-initiated step, illustrated by their inability to produce suppressive factor. Like the cyclophosphamide-treated animals, however, A/J mice can be suppressed by the appropriate suppressive extract.

We have investigated further the stimulation of specific suppressor T cells by GT- and GAT-suppressor extracts. In this paper, we shall report (*a*) the suppression of the GT-MBSA response of BALB/c and GAT-MBSA response DBA/1 mice by the appropriate extracts administered up to 5 wk before antigenic challenge; (*b*) the considerably greater suppressive activity of the BALB/c GT-suppressive extract when injected 1 wk before immunization with GT-MBSA compared to administration of extract on the day of immunization; (*c*) the in vivo adoptive transfer of GT-specific suppression to normal, syngeneic recipient mice with spleen cells from suppressor or antigen-nonsuppressor mice injected with GT-suppressive extract; (*d*) the in vitro suppression of GAT-MBSA responses of DBA/1 spleen cells by syngeneic spleen cells from mice primed with GAT-factor (GAT-T_sF). These findings have led to the conclusion that both GT- and GAT-suppressive extracts stimulate the production of antigen-specific suppressor T cells and lend supportive evidence for the two-step model of suppression.

Materials and Methods

Mice. BALB/c mice were purchased from Health Research Laboratories, Inc., West Seneca, N. Y. A/J and DBA/1 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Mice used in these experiments were 2–4 mo old and were maintained on laboratory chow and acidified, chlorinated drinking water ad lib.

Antigens. A preparation of GT with molar amino acid ratio $Glu^{50}Tyr^{50}$ and average mol wt of 133,000 was used for immunizations. A preparation of GAT with molar amino acid ratio $Glu^{60}Ala^{30}Tyr^{10}$ and average mol wt of 38,000 was used both for immunizations and sensitizing sheep erythrocytes (SRBC). Both polymers were purchased from Miles Laboratories, Inc., Miles Research Products, Elkhardt, Ind. MBSA was purchased from Worthington Biochemical Corp., Freehold, N. J. GT and GAT solutions and GT-MBSA complexes were prepared as previously described (3).

Preparation of Cell-Free Immunosuppressive Extracts. Cell-free extracts were prepared as described previously (8, 9). Briefly, BALB/c or DBA/1 mice were injected i.p. with 100 μg of GT in a mixture of aluminum-magnesium hydroxide gel (Maalox, W. H. Rorer, Inc., Fort Washington, Pa.) or with 10 μg GAT in Maalox, respectively, or with Maalox alone (control). 3 days later, the mice were sacrificed and spleens and thymuses removed. The tissues were teased, pooled, washed twice in Hanks' balanced salt solution, and resuspended to a final concentration of 6×10^8 cells/ml in a medium consisting of Eagles' minimum essential medium supplemented with 4 mM HEPES buffer, 2 mM L-glutamine, and 50 U each of penicillin and streptomycin (Microbiological Associates, Bethesda, Md.). Cells were disrupted by a Sonifier Cell Disruptor, model W-140-E equipped with a standard microtip (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) by applying 50 W for 5 min to 3-8-ml samples. The sonicate was centrifuged for 1 h at 40,000 *g*. The resulting supernate was collected and stored at −80°C until use. The extracts were assayed either in vivo or in vitro at concentrations indicated in the tables or figure.

Determination of Antigen Present in the Extracts. GT was labeled with [^{14}C] methylamine as follows: (*a*) GT (1 mg/ml final) was dissolved in a N/5 NaOH solution and was adjusted to pH 7.5 with HCl, (*b*) 1-ethyl-3 (3-dimethylamino propyl) carbodiimide (ECDI) (30 mg/ml final) was dissolved in water and adjusted to pH 7.5 with NaOH, (*c*) the ethanol of a solution containing 250 μCi of [^{14}C] methylamine (New England Nuclear, Boston, Mass.) was evaporated, (*d*) 1 mg of GT and 3 mg of ECDI were mixed with the radioactive material and the mixture was reacted overnight at room temperature. The mixture was then dialyzed three times against 500 ml of phosphate-buffered saline (PBS). GT was labeled such that 1 μg of antigen corresponded to 6×10^3 cpm.

Mice were injected with 10 and 100 μg of [^{14}C]labeled GT in Maalox and 3 days later extracts were prepared as described in the previous section. The extracts were treated with Protosol (New England Nuclear) and bleached with H_2O_2. Quenching was determined by adding a known amount of radiolabeled GT to control extracts. Based on the radioreactivity of the thymus and spleen extracts, we calculated that 1 ml of extract prepared from 6×10^8 thymic and spleen cells contains 0.035 μg of GT if the animals were injected with 100 μg of GT, and about 0.003 μg of GT if they were injected with 10 μg of GT.

Preparation and use of Immunoadsorbent Columns. Anti-*H-2D*^{d} and anti-*H-2I*^{d} sera were prepared by Dr. Martin Dorf. Anti-*D*^{d} was produced in (B10 × LP.RIII)F_1 mice immunized with 18R lymphoid cells; anti-*I*^{d} was from (C3H × LG/ckc)F_1 mice immunized with C3H.OH lymphoid cells. All sera were collected after six or more immunizations; mice were bled individually, and the high-titered sera were pooled. Before use, the sera were adsorbed for 1 h at 4°C with thymocytes (10^8 cells/ml) from mice of the strain used to produce the antiserum. Anti-*H-2D*^{d} and anti-*H-2I*^{d} alloantisera were heat-inactivated (56°C for 30 min) before coupling the corresponding globulin fractions to CNBR-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (12). The immunoadsorbents were stored at 4°C in PBS-containing 0.02% sodium azide. Before use, 2 ml anti-*H-2D*^{d}-Sepharose or anti-*H-2I*^{d}-Sepharose were packed in 5-ml columns and extensively washed in PBS. 2-ml samples of the crude extract diluted $^1/_{10}$ (equivalent to 6×10^7 lymphoid cells/ml) were adsorbed on the gels for 3 h at 4°C. Various dilutions of the unbound material were immediately injected i.v. into BALB/c mice 7 days after injection with column eluates, the mice were immunized with GT-MBSA.

Immunizations. Mice were injected i.v. with 0.5 ml of the appropriate dilution of extract or antigen as indicated in the tables and figure. At the appropriate time interval, after antigen or extract administration, mice were immunized with 10 μg GT as GT-MBSA or 10 μg GAT as GAT-MBSA in complete Freund's adjuvant (CFA) or in Maalox and 2×10^9 killed Bordetella pertussis organisms (Eli Lilly and Company, Indianapolis, Ind.). In certain instances, mice were injected with 0.5 ml of 1% (vol/vol) SRBC suspension in saline.

In Vivo and In Vitro Cell Transfer. Mice used as spleen cell donors were injected i.v. 7 days before transfer with 0.5 ml BALB/c Maalox- or GT extracts equivalent to 15×10^7 lymphoid cells. Single cell suspensions in Hank's balanced salt solution were prepared, washed, and 20×10^6 lymphoid cells were adoptively transferred i.v. into recipient mice. In certain experiments, Thy-1-bearing cells were depleted from normal spleen cell suspensions by treatment with appropriate concentrations of AKR anti-Thy-1 C3H and a 1:3 dilution of guinea pig serum as a source of C. Immediately after cell transfer, the mice were immunized with 10 μg GT as GT-MBSA in either complete Freund's adjuvant or Maalox and *B. pertussis*.

For in vitro studies, DBA/1 mice were injected i.v. with 0.5 ml of an extract prepared from a pool of thymus and spleen cells from either Maalox- or GAT-primed mice. 6 days later, replicate 1-ml cultures containing 8×10^6 normal DBA/1 spleen cells were established according to the modifications of the Mishell-Dutton system used in our laboratory (1, 2), $2\text{–}8 \times 10^6$ spleen cells from normal, Maalox- or GAT-suppressive extract-primed DBA/1 mice were added at culture initiation. The IgG plaque-forming cell (PFC) responses were measured 5 days later.

Hemolytic Plaque Assay. 7 days after injection of antigen or 5 days after culture initiation, IgG PFC responses were determined by using GAT-SRBC as indicator cells as described previously (1). As in earlier studies, GT-MBSA responses were assayed on SRBC coupled with the cross-reacting polymer GAT, (GAT-SRBC) (3). GT- or GAT-specific IgG plaques were determined by subtracting the number of PFC detected in the presence of a suitable dilution of GAT from the number of plaques detected on GAT-SRBC in the absence of the specific inhibitor. All assays were performed in duplicate and the number of IgG PFC's per spleen recorded.

Results

Effect of Time of Administration of Suppressive Extracts on the PFC Responses of BALB/c and DBA/1 Mice. In experiments reported previously (8, 9), suppressive extracts were administered on the day of GT-MBSA or GAT-MBSA immunization. We investigated whether BALB/c GT-suppressive extract can inhibit BALB/c GT-MBSA PFC responses when administered 1 or more wk before GT-MBSA challenge. Injection of BALB/c GT-suppressive extracts at time intervals up to 3 wk before antigenic challenge results in near total suppression of the BALB/c GT-MBSA responses in vivo (Table I). Extracts from Maalox-primed control mice, on the other hand, do not suppress the GT-MBSA response when injected the same day as antigen or 7 days before antigen (see Table IX). Similarly, extracts prepared from thymus and spleen cells of GAT-primed DBA/1 mice were administered at various time intervals before GAT-MBSA immunization, (Table I). Likewise, administration of DBA/1 GAT-T_sF up to 34 days before antigenic challenge suppresses the GAT-MBSA PFC responses of DBA/1 mice.

Increased Inhibitory Activity of BALB/c GT-Suppressive Extract when Administered 1 wk before GT-MBSA. The effectiveness of BALB/c GT-suppressive extract in inhibiting GT-MBSA responses was compared when serial dilutions of the extract were administered on the day of or 1 wk before immunization with GT-MBSA (Table II). The BALB/c GT-suppressive extract was effective at an 8–10 times lower concentration when administered 1 wk before immunization with GT-MBSA than on the day of immunization.

Free GT Cannot Explain the Suppressive Activity of the Extracts. A serious concern in the above experiments is the possibility that small quantities of soluble antigen may be carried over in the suppressive extracts from the primed animals and the characteristics attributed to a suppressive factor may be due to trace amounts of antigenic contamination. We have, therefore, investigated (*a*) the minimal amout of free antigen required to induce antigen-specific suppression when administered 1 wk before GT-MBSA immunization and, (*b*) the amount of antigen present in the extract. Fig. 1 shows the effects of injection of different doses of GAT or GT intravenously into DBA/1 and BALB/c mice, respectively, either on the day of immunization or 1 wk before antigenic challenge. BALB/c mice are suppressed by i.v. injection of 1.0 μg GT on the day of GT-MBSA immunization. However, the i.v. administration of 0.1 μg GAT into

TABLE I

Effect of Time of Administration of BALB/c GT-T_sF or DBA/1 GAT-T_sF on the Respective GT-MBSA or GAT-MBSA PFC Responses of BALB/c or DBA/1 Mice

Extract* administered	Time interval‡ between extract injection and immunization	BALB/c GT-specific IgG PFC responses per spleen§	Suppression‖	DBA/1 GAT-specific IgG PFC responses per spleen§	Suppression
	days	*Arithmetic mean ± SE*	*%*	*Arithmetic mean ± SE*	*%*
None	0	7,000 ± 862		6,051 ± 638	
Maalox	0	6,810 ± 1,169		4,593 ± 404¶	
T_sF	0	1,480 ± 479	79	363 ± 55	94
T_sF	3–4	735 ± 357	90	536 ± 223	91
T_sF	7	1,266 ± 556	82	334 ± 76	94
T_sF	14	208 ± 42	97	ND**	
T_sF	21	1,462 ± 79	79	ND	
T_sF	34	ND		457 ± 136	92

* Extracts prepared from a pool of spleen and thymus cells from either Maalox- or GT-primed BALB/c mice or Maalox- or GAT-primed DBA/1 mice were injected i.v. into BALB/c or DBA/1 mice, respectively; extracts equivalent to 15×10^7 cells were administered.

‡ BALB/c mice were immunized i.p. with 10 μg GT as GT-MBSA in CFA and DBA/1 mice were immunized i.p. with 10 μg GAT as GAT-MBSA in Maalox and *B. pertussis* as adjuvant. 7 days later, the number of antigen-specific PFC per spleen was determined.

§ Numbers represent the arithmetic mean of antigen-specific PFC per spleen ± SE for 6–15 animals per data group.

‖ Percent suppression is expressed for those groups that are statistically different from control response (no extract) group. In all cases, $P < 0.001$ as determined by Student's t test.

¶ This group is not statistically different ($P = 0.113$) from the control GAT-MBSA response group.

** Not determined.

DBA/1 or 0.1 μg GT into BALB/c 1 wk before immunization significantly suppresses the GAT-MBSA or GT-MBSA responses, respectively. Therefore, GT-specific suppression can be easily induced with 10 times less antigen or suppressive extract when injected 1 wk before GT-MBSA immunization. Although it is unlikely that the extract contains suppressive doses of antigen, it is very important to eliminate the possibility that the suppressive extract is contaminated with suppressive quantities of free copolymer. Therefore, we have radiolabeled GT with [^{14}C]methylamine and quantitated the amount of GT present in the extracts prepared in the usual manner (see Materials and Methods). By this method, we have determined that the amount of radioactivity recovered after injection of 100 μg GT/mouse corresponds to 0.035 μg GT/ml of undiluted extract. Consequently, 0.5 ml of suppressive extract diluted $^1/_2$ to $^1/_4$ (which induces near total suppression when injected on the day of immunization with GT-MBSA) contains 0.004–0.009 μg GT which is at least 100 times less than the minimum i.v. dose of GT which gives detectable suppression under these conditions (Fig. 1). Similarly, injection of 0.5-ml extracts 7 days before GT-MBSA yields near total suppression at dilutions of at least $^1/_{64}$ (Table II) which is equivalent to 0.0003 μg GT which is at least 100 times less than the minimum dose of antigen required for significant suppression (Fig. 1). We may then

TABLE II

Titration of BALB/c GT-Suppressive Extract Injected on the Day of or 7 Days before GT-MBSA Immunization of BALB/c Mice

Interval between* injection of extract and GT-MBSA immunization	BALB/c GT-T_sF‡	GT-specific IgG PFC per spleen§	*P* Value
		Arithmetic mean ± SE	
None	None	9,291 ± 440	–
	1:2	<200	<0.001
	1:4	675 ± 284	<0.001
	1:8	2,884 ± 1,442	0.007
	1:16	9,218 ± 1,538	0.957
7 days	None	4,600 ± 605	–
	1:4	212 ± 12	0.004
	1:8	212 ± 12	0.004
	1:16	212 ± 12	0.004
	1:32	<200	0.003
	1:64	600 ± 400	0.001
	1:128	5,600 ± 1,987	0.582
	1:256	3,550 ± 1,354	0.446

* BALB/c mice were immunized i.p. with 10 μg GT as GT-MBSA in complete Freund's adjuvant either immediately or 7 days after injection of extracts.

‡ GT-suppressive extract was prepared from a pool of thymocytes and spleen cells from GT-primed BALB/c mice. 1/2 ml of extract, initially equal to 3×10^8 cells, was administered i.v. at the appropriate dilution as indicated

§ 7 days after GT-MBSA immunization, antigen-specific IgG PFC were determined. Numbers represent the arithmetic mean ± SE for six mice per control groups, those receiving no factor, and four mice per group, those receiving factor.

conclude that the amount of GT present in the extracts cannot account by itself for their suppressive activity.

Adsorption of the GT-Suppressive Activity by Alloantisera Directed against the I Subregion of the H-2 Complex. We have determined that GT-T_sF extracted from GT-primed BALB/c mice is an *I* region product;[2] more precisely the GT-T_sF extracted from B10.BR mice bears determinants of the *I-J* subregion (13). These results were obtained in vitro by the addition of alloantisera adsorbed extract at culture initiation. It remained to be established whether the suppressive activity of the BALB/c GT-suppressive extract administered 7 days before antigenic challenge is also an *H-2* product. Table III demonstrates that the suppressive activity is totally removed by alloantisera directed against $H\text{-}2I^d$ when the extract is assayed at 1/80 dilution and partially removed when assayed at 1/20 dilution. When the suppressive extracts are incubated with an immunoadsorbent made with alloantisera directed against $H\text{-}2D^d$, no loss of suppressive activity is seen at any dilution tested.

[2] Thèze, J., C. Waltenbaugh, R. Germain, and B. Benacerraf. 1977. Immunosuppressive factor(s) specific for L-glutamic acid^{50}-L-tyrosine^{50} (GT). IV. *In vitro* activity and immunochemical properties of the GT-specific suppressive factor. *Eur. J. Immunol.* In press.

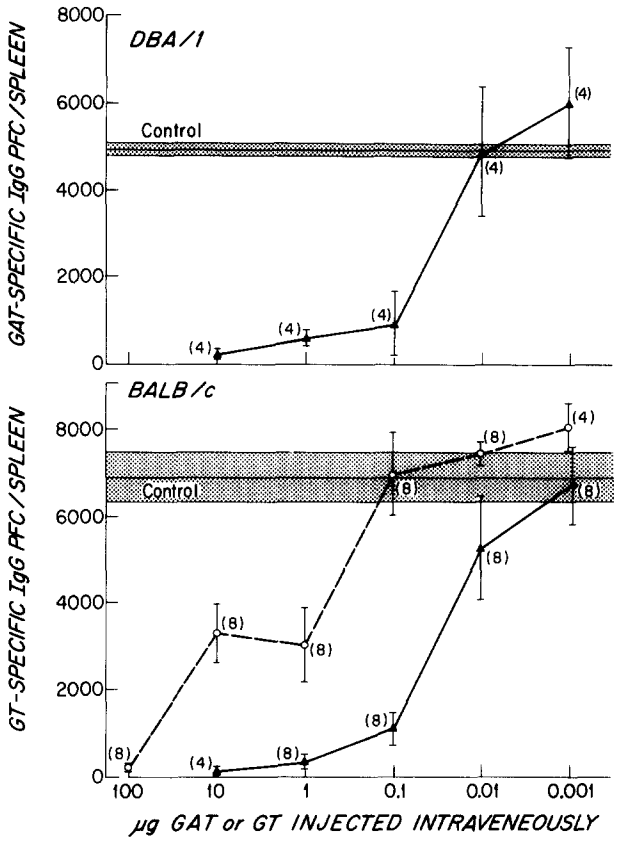

FIG. 1. Effect of intravenous injection of GAT or GT on the respective GAT-MBSA or GT-MBSA PFC responses of DBA/1 or BALB/c mice. DBA/1 mice (upper panel) injected with the appropriate amounts of GAT were immunized 7 days later with GAT-MBSA; 1 wk later the numbers of GAT-specific PFC per spleen were determined. Statistical comparisons of control GAT-MBSA PFC responses (dotted bar) with those groups receiving preimmunization with GAT gave the following results; 10, 1, 0.1 μg GAT yielded $P < 0.002$ in all cases, while 0.01 and 0.001 μg GAT yielded $P > 0.470$.

BALB/c mice (lower panel) were injected intravenously with the appropriate amount of GT on the same day as GT-MBSA immunization (○-----○) or 7 days before GT-MBSA immunization (▲——▲). 1 wk after GT-MBSA immunization the numbers of GT-specific PFC per spleen were determined. Statistical comparisons of control GT-MBSA PFC responses (dotted bar) with those groups receiving GT preimmunization gave the following results: GT injected the same day as GT-MBSA; 100, 10, μg GT yielded $P \leq 0.002$ in all cases, while 0.1, 0.01, 0.001 μg GT yielded $P > 0.530$. GT injected 7 days before GT-MBSA 10, 1, 0.1 μg GT yielded $P < 0.001$, whereas, 0.01 and 0.001 μg GT yielded $P > 0.2$.

Transfer of Suppression with Spleen Cells from Mice Treated with Suppressive Extract. The hypothesis that BALB/c GT-suppressive extract inhibits GT-MBSA PFC responses by stimulating the production of GT-suppressor T cells was tested by the adoptive transfer of spleen cells from BALB/c mice injected 1 wk earlier with BALB/c GT-suppressive extracts into normal BALB/c recipients. Table IV illustrates such an experiment, demonstrating that 20×10^6 spleen cells from BALB/c mice injected with BALB/c GT-suppressive extract 1 wk earlier suppress the PFC responses of normal BALB/c recipients to GT-

TABLE III

Effect of Administration of Alloantisera-Adsorbed BALB/c GT-Suppressive Extract 7 Days before GT-MBSA Immunization of BALB/c Mice

		Extract treatment*	Number of mice per group	GT-specific IgG PFC per spleen‡			
				No extract	Final dilution of extract		
					1/20	1/40	1/80
				Arithmetic mean ± SE			
Experiment I							
	Control response	–	6	15,050 ± 1,711			
		Untreated	4	–	458 ± 208	1,031 ± 452	2,000 ± 954
		Anti-H-2D^d	4	–	344 ± 94	344 ± 44	1,906 ± 1,494
		Anti-H-2I^d	4	–	7,469 ± 1,287	8,594 ± 801	12,350 ± 4,256
Experiment II							
	Control response	–	4	7,362 ± 1,372	–	–	–
		Untreated	4	–	1,375 ± 428	ND§	ND
		Anti H-2D^d	4	–	937 ± 308	ND	ND
		Anti-H-2I^d	4	–	4,050 ± 2,019	ND	ND

* Extracts were prepared from a pool of spleen and thymus cells from GT-primed BALB/c mice. 2-ml samples of crude extract diluted 1/10 (equivalent to 6 × 10^7 lymphoid cells) were adsorbed with Sepharose-bound alloantisera directed against H-2D^d and H-2I^d. The unbound material was injected (0.5 ml) immediately i.v. into BALB/c mice at dilutions indicated above.

‡ Mice were immunized i.p. with 10 μg GT as GT-MBSA in *B. pertussis* and Maalox as adjuvant 7 days after administration of extracts i.v. 7 days after immunization, the number of antigen-specific PFC per spleen was determined.

§ Not determined.

TABLE IV

BALB/c GT-Suppressive Extract Stimulates the Development of Suppressor Cells in BALB/c Mice

Group*	GT-specific IgG‡ PFC per spleen	Suppression	*P* value
	Arithmetic mean ± SE	%	
I	5,881 ± 1,504		
II	<200	97	0.002
III	7,137 ± 2,195		
IV	262 ± 62	96	0.007
V	8,540 ± 1,800		
VI	838 ± 419	90	0.007

* **Groups I and II.** Normal BALB/c mice received i.v. injection of BALB/c Maalox extract (group I) or BALB/c GT-suppressive extract (group II), equivalent to 15 × 10^7 lymphoid cells. 7 days later, these mice were immunized intraperitoneally with 10 μg GT as GT-MBSA in CFA, i.p.
Groups III and IV. Normal BALB/c mice received 20 × 10^6 spleen cells, i.v., from BALB/c mice injected 7 days earlier with BALB/c Maalox extract (group III) or BALB/c GT-suppressive extract (group IV), equivalent to 15 × 10^7 lymphoid cells. Immediately after adoptive transfer, the recipient mice were immunized with 10 μg GT as GT-MBSA in CFA, i.p.
Groups V and VI. Normal mice received 20 × 10^6 spleen cells, i.v., from BALB/c mice injected 7 days earlier with BALB/c GT-suppressive extract adsorbed on an anti-*H-2I^d* immunoadsorbent (group V) or on an anti-*H-2D^d* immunoadsorbent (group VI). Immediately after adoptive transfer, mice were injected with 10 μg GT as GT-MBSA in CFA, i.p.

‡ 7 days after GT-MBSA immunization the number of antigen-specific PFC per spleen was determined. Numbers represent the arithmetic mean ± SE for eight mice per group.

MBSA. The degree of suppression achieved by the transfer of spleen cells from suppressive extract-treated mice (group IV) is as efficient as the inhibition caused by injecting suppressive extract 1 wk before GT-MBSA immunization (group II). On the other hand, spleen cells from Maalox extract-treated animals (group III) had no suppressive effect compared to mice receiving Maalox extract 1 wk before GT-MBSA injection (group I). Furthermore, the factor responsible for the generation of suppressor cells bears determinants of the *I* subregion of the *H-2* complex. This was demonstrated by the inability of cells from mice previously injected with extracts adsorbed with alloantisera directed against $H\text{-}2I^d$ to transfer suppression (group V). However, suppressive extract passed over an anti-$H\text{-}2D^d$ immunoadsorbent was still able to generate suppressor cells (group VI). Table V shows that the cells that transfer suppression from BALB/c GT-suppressive extract-primed donors are sensitive to treatment with anti-Thy-1 antiserum and C, thus demonstrating that this suppression is mediated by T cells.

In Vitro Suppression of GAT-MBSA Response by Suppressor Cells Induced In Vivo by Suppressive Extracts. DBA/1 mice were treated with DBA/1 GAT-suppressive extract 6 days before their cells were studied in culture (Table VI). Spleen cells from normal DBA/1 mice (A) and from DBA/1 mice that received control extracts (B) develop PFC responses to GAT-MBSA, whereas spleen cells from DBA/1 mice that received GAT-extracts (C) do not. In addition, 2×10^6 spleen cells from suppressive extract-treated mice were added to 8×10^6 normal DBA/1 spleen cells in vitro. The addition of spleen cells from suppressive extract-treated mice caused a marked suppression of GAT-MBSA PFC responses (F) as compared to cultures receiving spleen cells from Maalox extract-treated mice (E) or control cultures (D).

Stimulation of Suppressor Cells in A/J Mice by BALB/c GT-Suppressive Extract. The GT-MBSA responses of A/J mice were shown to be unaffected by the previous i.p. (5) or i.v. injection of GT (14). Table VII shows that i.v. injection of 100 μg GT has no inhibitory effect on the GT-MBSA PFC responses of A/J mice. On the other hand, BALB/c GT-suppressive extract was shown to suppress the GT-MBSA responses of A/J mice although A/J mice do not produce a GT-T_sF (9). These results suggested that A/J mice were genetically defective in cells capable of producing a T_sF but did possess cells that could respond to the appropriate suppressive extract. In a preliminary series of experiments (not shown) we determined that BALB/c GT-suppressive extract administered 1 wk before antigenic challenge suppresses the GT-MBSA responses of A/J mice. Table VIII presents evidence that suppression can be transferred to normal A/J mice with spleen cells from A/J mice treated 1 wk earlier with BALB/c GT-suppressive extract. This experiment can also be considered evidence that the generation of effector suppressor cells is stimulated by BALB/c GT-T_sF and not the result of antigen alone present in the extract, since GT administered i.v. is not suppressive in A/J mice (Table VII).

Specificity of Suppression Induced by Suppressive Extracts. We have demonstrated above that BALB/c GT-suppressive extracts generate cells capable of suppressing the GT-MBSA PFC responses of both BALB/c and A/J mice. We have investigated the specificity of the extract-induced suppression. Table IX

TABLE V

Effect of Depletion of Thy-1-Positive Spleen Cells from BALB/c Mice Primed with GT-Suppressive Extract on the GT-MBSA PFC Responses of Normal Syngeneic Recipients

Donor BALB/c mice*		GT-specific IgG‡ PFC per spleen	Suppression	*P* value
Extract administered	Treatment of 20×10^6 spleen cells transferred			
Day 4	Day 0	*Arithmetic mean ± SE*	%	
None	No transfer	3,720 ± 968		
BALB/c Maalox	–	4,866 ± 2,081	0	0.652
BALB/c GT	–	<200	95	0.007
BALB/c GT	Anti-Thy 1+ C′	3,960 ± 790	0	0.852
BALB/c GT	C′	683 ± 234	82	0.009

* Normal BALB/c mice were injected i.v. with BALB/c Maalox- or GT-suppressive extracts equivalent to 15×10^7 lymphoid cells. 4 days later, 20×10^6 spleen cells treated with anti-Thy-1 and C, C or untreated were transferred into normal syngeneic recipients i.v. Immediately after cell transfer, mice were immunized with 10 μg GT as GT-MBSA in CFA, i.p.

‡ 7 days after GT-MBSA immunization, the number of GT-specific IgG PFC were determined. Numbers represent the arithmetic mean ± SE for six mice per data group.

TABLE VI

Induction of Suppressor Cells in the Spleens of DBA/1 Mice Injected with Lymphoid Cell Extracts from GAT-Primed DBA/1 Mice

Group	Normal spleen cells	Mice injected with: spleen cells* from		GAT-specific IgG responses *in vitro* PFC/10^6 viable cells‡	Percent control response§
		Maalox extract	GAT-extract		
A	8×10^6	–	–	664	
B	–	8×10^6	–	900	135
C	–	–	8×10^6	60	9
D	10×10^6	–	–	891	
E	8×10^6	2×10^6	–	683	76
F	8×10^6	–	2×10^6	118	17

* Spleen cells from normal DBA/1 mice or DBA/1 mice that received 0.5 ml extract from 6×10^8 Maalox-primed or GAT-primed DBA/1 lymphoid cells 6 days earlier.

‡ Day 5 GAT-specific PFC responses stimulated by GAT-MBSA.

§ Percent control responses were calculated by comparing groups B and C with group A and groups E and F with group D.

demonstrates that injection of GT-suppressive extract 7 days before antigenic challenge suppresses the GT-MBSA responses of BALB/c mice without diminishing SRBC responses. Maalox extract shows no suppressive activity for either GT-MBSA or SRBC. In addition, the BALB/c GT-suppressive extract does not inhibit the SRBC response even in the presence of GT-MBSA, thus excluding that this extract in the presence of antigen acts by causing the release of

TABLE VII
Effect of GT Administered Intravenously on the GT-MBSA PFC Responses of A/J Mice

GT*	GT-specific IgG§ PFC per spleen	*P* value
μg	*Arithmetic mean ± SE*	
None	8,720 ± 1,270	–
100	8,180 ± 983	0.745
10	10,680 ±846	0.235
1	11,620 ± 1,815	0.227
0.1	7,108 ±1,749	0.477
0.01	10, 625 ± 633	0.257

* A/J Mice were injected intravenously with the indicated amount of soluble GT in 0.5 ml Hank's balanced salt solution. Immediately thereafter, mice were immunized with 10 μg GT as GT-MBSA by using Maalox and *B. pertussis* as adjuvant, i.p.

‡ 7 days after GT-MBSA injection, the number of antigen-specific PFC per spleen were determined. Numbers represent the arithmetic mean ± SE for eight mice per group.

TABLE VIII
Effect of BALB/c GT/Suppressive Extract on the Stimulation of Suppressor Cells in A/J Mice

A/J spleen cells transferred*	GT-specific IgG PFC‡ per spleen	Suppression	*P* value
(20×10^6)	*Arithmetic mean ± SE*	%	
No transfer	5,512 ± 685		
BALB/c Maalox extract	6,793 ± 903	0	0.270
BALB/c GT extract	375 ± 138	93	<0.001

* Normal A/J mice were injected i.v. with BALB/c Maalox extract or BALB/c GT-suppressive extract, equivalent to 15×10^7 lymphoid cells. 7 days later 20×10^6 spleen cells were adoptively transferred into normal A/J mice. Immediately after adoptive transfer, the recipient mice were immunized with 10 μg GT as GT-MBSA in Maalox and *B. pertussis* as adjuvant, i.p.

‡ 7 days after GT-MBSA immunization, the number of antigen-specific PFC per spleen were determined. Numbers represent the arithmetic mean ± SE for 12 mice per group.

nonspecific-suppressive mediators. An enhancement of the SRBC responses of those mice receiving both GT-MBSA and SRBC is observed and this response is further enhanced by injection of the GT-suppressive extract. Table X illustrates that even the adoptive transfer of spleen cells from normal BALB/c mice primed with BALB/c GT-T_sF has no suppressive effect upon the SRBC PFC responses of recipient mice. Thus, evidence is provided that the suppressor cells generated by BALB/c GT-T_sF are antigen specific.

Discussion

We have previously described specific suppressor factors prepared from the spleen and thymuses of GAT- and GT-primed nonresponder mice (8, 9). Al-

TABLE IX

Specificity of Suppression Induced by Extracts of Lymphoid Cells from GT-Primed BALB/c Mice

BALB/c extract*	Antigen‡	Indicator cells	GT-specific IgG PFC per spleen	Suppression	Anti-SRBC PFC per spleen	
					IgM	IgG
			Arithmetic mean ± SE	%		*Arithmetic mean ± SE*
None	GT-MBSA	GAT-SRBC	8,650 ± 2,685			
	SRBC	SRBC			20,580 ± 5,388	9,060 ± 3,925
	GT-MBSA + SRBC	SRBC			42,900 ± 8,475	45,120 ± 13,528
Maalox	GT-MBSA	GAT-SRBC	9,700 ± 2,788	0		
	SRBC	SRBC			32,160 ± 4,442	10,080 ± 409
	GT-MBSA + SRBC	SRBC			49,680 ± 5,207	48,360 ± 16,852
GT	GT-MBSA	GAT-SRBC	1,150 ± 460	87		
	SRBC	SRBC			34,440 ± 5,648	23,160 ± 5,697
	GT-MBSA + SRBC	SRBC			67,740 ± 10,530	136,440 ± 21,673

* Extracts prepared from a pool of spleen and thymus cells from either Maalox- or GT-primed BALB/c mice were injected i.v.; extract equivalent to 15×10^6 cells (a 1/20 dilution) were administered.

‡ 7 days after administration of extracts, mice were immunized i.p. with 10 μg GT as GT-MBSA in Maalox and *B. pertussis* and with 10^8 SRBC in 0.5 ml saline. 7 days later the number of antigen-specific PFC per spleen was determined. Numbers represent the arithmetic mean ± SE for five mice per data group.

TABLE X

Specificity of Suppressor Cells Induced by Extracts of Lymphoid Cells from GT-Primed BALB/c Mice

BALB/c extract*	Antigen	Indicator cells	GT-specific IgG‡ PFC per spleen	Suppression	Anti-SRBC PFC per spleen‡	
					IgM	IgG
			Arithmetic mean ± SE	%	*Arithmetic mean ± SE*	
None	GT-MBSA	GAT-SRBC	4,845 ± 1,698			
	SRBC	SRBC			22,833 ± 3,349	30,500 ± 6,018
Maalox	GT-MBSA	GAT-SRBC	4,880± 935	0		
	SRBC	SRBC			39,725 ± 5,881	41,775 ± 9,199
GT-T,F	GT-MBSA	GAT-SRBC	400± 200	91		
	SRBC	SRBC			28,700 ± 3,975	24,975 ± 4,123

* Extracts prepared from a pool of spleen and thymus cells from either Maalox- or GT-primed BALB/c mice were injected i.v.; extract equivalent to 15×10^6 cells (a 1/20 dilution) was administered. 7 days after injection of extract, 20×10^6 spleen cells were adoptively transferred into normal BALB/c mice. Immediately after transfer, recipient mice were immunized i.p. with 10 μg GT as GT-MBSA in Maalox pertussis or with 10^8 SRBC in 0.5 ml saline. 7 days later the number of anti-SRBC PFC per spleen were determined.

‡ Numbers represent arithmetic mean ± SE for five mice per data group.

though much has been learned concerning the immunochemical properties of suppressor factors, little is known of their mode of action. In this report, we propose a mechanism of action of specific suppressive factors.

We have shown that injection of suppressive extracts even several weeks preceding antigenic challenge results in complete specific suppression. This has been established for both GT- and GAT-suppressive extracts in BALB/c and DBA/1 mice, respectively. Furthermore, the quantity of suppressive extract required for the induction of suppression 1 wk before antigenic challenge is approximately 10 times less than the amount required when the extract is injected the same day as antigen. This suggested a cell-mediated amplification mechanism. We have, indeed, demonstrated the adoptive transfer of suppres-

sion induced by both GAT-T_sF and GT-T_sF. DBA/1 GAT-suppressive extract was injected into DBA/1 mice and the presence of suppressor cells was demonstrated in vitro by the suppression of the GAT-MBSA PFC responses of normal DBA/1 spleen cells. BALB/c GT-suppressive extracts were injected into normal BALB/c and A/J mice; 7 days later their spleen cells were transferred into normal syngeneic recipients, and the suppressive effects upon the normal GT-MBSA PFC responses were demonstrated. In BALB/c mice we demonstrated that the factor-induced suppression is mediated by T cells, as the transfer of suppression was abolished by treatment of the cell population with anti-Thy-1 and C. In addition, the factor-generated suppressor cells are specific, and do not inhibit the responses to an irrelevant antigen. SRBC.

We have shown in previous reports (12, 13) that the active suppressive moiety of GAT- and GT-suppressive extracts are products of the *I* region and more specifically of the *I-J* subregion of the *H-2* complex (14), in the case of B10.BR GT suppressor factor. In addition, they display affinity for antigen. We excluded the possibility that the suppression induced by suppressive extracts was due to low doses of free antigen. Several lines of evidence have been presented to this effect: (*a*) the amount of free antigen present in the suppressive extracts is insufficient to stimulate suppressor cells. We estimated, with radiolabeled GT, that the amount of antigen present in the suppressive extracts is at least 100 times less than the minimum suppressive dose. (*b*) BALB/c GT-suppressive extract generates effector suppressor cells in A/J mice, while preimmunization of A/J mice with a wide dose range of GT has no suppressive effect. This result definitely excludes the possibility that the activity of BALB/c GT-suppressive extract is due to free antigen itself. After excluding the presence of suppressive quantities of free antigen in suppressive extract, we have established that the factor responsible for the generation of suppressor T cells bears determinants controlled by the *I* region of the *H-2* complex. It remains to be resolved, however, whether the factor responsible for the generation of suppressor T cells bears the same *H-2* subregion determinants as the GT-suppressive factor already described (14). To approach this question, we are currently investigating the possibility that alloantisera directed against $I\text{-}J^k$ will remove the B10.BR GT factor responsble for the generation of suppressor cells.

We have recently proposed a two-step model for the induction of antigen-specific suppression (9). The first step is antigen-mediated, resulting in the production of antigen-specific T_sF; the second step being factor-mediated. We postulate two T-cell populations for this model; one population produces T_sF in response to antigen and this factor acts upon a second distinct subset of cells which then become the effector suppressor T cells. There are two lines of evidence in support of this hypothesis: (*a*) we have shown previously (10) that the GT-MBSA PFC responses of cyclophosphamide-treated BALB/c mice are not suppressed by GT but are suppressed by BALB/c GT-suppressive extract. We propose that cyclophosphamide acts by the elimination of the T_sF-producing cells, while the second cell subset remains unaffected and can be activated by the appropriate suppressor factor. (*b*) Similarly, according to our hypothesis, A/J mice are genetically defective in cells capable of producing GT-T_sF. Accordingly, A/J mice are not suppressed by GT and cannot produce T_sF, but can be

suppressed by BALB/c GT-T_sF. The results presented in this paper are in agreement with this hypothesis and demonstrate that suppressive factors act by inducing effector suppressor T cells. This has been established both in BALB/c and A/J mice for BALB/c GT-suppressive extracts and in DBA/1 for GAT-suppressive extracts.

Further characterization of these two suppressor T-cell populations is required. Their relative radiosensitivities and cyclophosphamide sensitivities as well as their Ly phenotypes remain to be determined. Since we know that B10.BR GT-T_sF bears I-J determinants (13), it may be possible to distinguish one or the other population by the presence of cell surface markers controlled by the *I-J* subregion.

Previous studies from our laboratory (12) demonstrated that the DBA/1 GAT suppressor activity in the crude extract was specifically retained by an anti-GAT immunoadsorbent indicating that the GAT-T_sF in the extract is bound to antigen or antigenic fragments. Since this is precisely the type of material which has been shown in the experiments to stimulate the production of suppressor T cells, the possibility must be considered whether the material which stimulates the production of suppressor T cells is a very active complex of specific suppressor factor and antigen at a concentration of antigen which is not suppressive by itself. Experiments are in progress to resolve this important point.[3]

Biologically, a two-step mechanism for specific immune suppression has certain advantages. Activation of a limited number of factor-producing cells would result in the generation of a greater number of effector suppressor T cells. Although it remains to be established whether the suppressor factor is a secretory product, its secretion would allow the widespread distribution of suppression throughout the organism. In addition, a limited number of cells producing factors over an extended period of time may be responsible for a powerful, long-lasting suppression. Thus, the mediation of specific immune suppression by two distinct populations of suppressor T cells will allow for the amplification and maintenance of suppression after antigenic challenge.

Summary

Injection of mice with L-glutamic acid^{50}-L-tyrosine^{50} (GT)- or L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)-specific suppressor T-cell factor (GT-T_sF or GAT-T_sF) up to 5 wk before antigenic challenge suppresses GT-methylated bovine serum albumin (MBSA) and GAT-MBSA plaque-forming cells responses. T suppressor cells are responsible for the suppression induced by the suppressive extract as demonstrated by adoptive transfer and sensitivity to anti-Thy-1 and complement treatment. We conclude that suppressive extract induces specific suppressor T cells. The material responsible for generation of suppressor T cells is a product of the *I* subregion of the *H-2* complex. We have excluded that suppressive quantities of antigens are present in the extract.

A/J mice, which can neither be suppressed by GT nor make GT-T_sF can be suppressed by BALB/c GT-T_sF. Spleen cells from BALB/c GT T_sF-primed A/J

[3] Germain, R. N., J. Thèze, J. A. Kapp, and B. Benacerraf. Manuscript in preparation.

mice can adoptively transfer suppression to normal syngeneic recipients. A/J mice appear to be genetically defective in cells involved in factor production. These results are discussed in the light of a two-step model for induction of antigen-specific suppressor cells.

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