IMMUNOSUPPRESSIVE FACTOR(S) EXTRACTED FROM LYMPHOID CELLS OF NONRESPONDER MICE PRIMED WITH L-GLUTAMIC ACID^{60}-L-ALANINE^{30}-L-TYROSINE^{10} (GAT)

III. Immunochemical Properties of the GAT-Specific Suppressive Factor*

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Immunization of mice bearing the nonresponder haplotypes, *H-2*^{p,q,s}, with the terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)[1] stimulates suppressor T cells (T_S) that specifically inhibit GAT-specific IgG plaque-forming cell (PFC) responses by syngeneic mice to GAT complexed to the immunogenic carrier methylated bovine serum albumin (GAT-MBSA) in vitro and in vivo (1, 2). Analogous to the results of Tada and Taraguchi with other systems (3, 4), previous studies from our laboratory have shown that cell-free extracts of spleens or thymuses from GAT-primed, nonresponder DBA/1 (*H-2*^{q}) or A.SW (*H-2*^{s}) mice suppress the PFC responses to GAT-MBSA, but not to sheep red blood cells (SRBC) in the strains of origin (5). In the previous paper of this series, we have described and evaluated the in vitro assay for the activity of GAT-specific suppressive factor, defined the activity of S_{50} units/ml as the inverse of the dilution of the extracts that cause 50% inhibition of PFC responses, verified the reproducibility of the suppression observed with different extracts, and demonstrated that the suppressive factor(s) extracted from lymphoid cells of GAT-primed nonresponder mice is obtained from T cells (6). These techniques have permitted an initial characterization of the properties of the GAT-specific suppressor T-cell factor (GAT-T_SF) in the crude extracts of lymphoid cells from GAT-primed DBA/1 mice that is described in this communication. Two main issues were then addressed. First, the fine specificity of GAT-T_SF was

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[1] *Abbreviations used in this paper*: CFA, complete Freund's adjuvant; GAT, random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-MBSA, GAT complexed to methylated bovine serum albumin; GAT-SRBC, GAT coupled to sheep red blood cells; GAT-T_SF, GAT-specific suppressor T-cell factor; PBS, phosphate-buffered saline; PFC, plaque-forming cell(s); S_{50} units/ml, inverse of dilution of extract that causes 50% suppression of a PFC response; T_S, suppressor T cell(s).

determined, and its avidity for the antigen was compared with the avidity of anti-GAT antibodies produced in the same murine strain after immunization with GAT-MBSA. Second, the optimal conditions for the elution of GAT-T_sF from GAT-Sepharose columns to which it has been specifically bound were defined, and the properties of this partially purified factor were compared with those of crude GAT-T_sF.

Materials and Methods

Mice. DBA/1 (*H-2^q*) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c (*H-2^d*) mice were obtained from the Health Research Laboratories, Buffalo, N. Y. Mice used in these studies were from 2 to 8-mo old and were maintained on acidified-chlorinated drinking water and laboratory chow ad libitum.

Antigens. The polymers of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT), L-glutamic acid^{50}-L-tyrosine^{50} (GT), and L-glutamic acid^{60}-L-alanine^{40} (GA) were purchased from Miles Laboratories, Miles Research Division, Elkhart, Ind. Preparation of GAT-MBSA (1) and GT-MBSA (9) has been described.

Preparation of GAT Suppressive Extract. DBA/1 mice received 10 μg of GAT in Maalox (William H. Rorer, Inc., Fort Washington, Pa.) i.p. 3 days before sacrifice. Single cell suspensions were prepared from spleen and thymus, adjusted to 6×10^8 cells/ml, and sonicated as described previously (5, 6). Control extracts were prepared from the spleen and thymus of mice injected with Maalox alone. For the preparation of the GT factor, BALB/c mice were immunized i.p. with 100 μg of GT in Maalos (William H. Rorer, Inc.). 3 days later the cell-free extract was prepared from spleen and thymus (7).

Assay of the GAT-T_sF. For the in vivo assay of GAT-T_sF, DBA/1 mice were injected i.v. with 0.5 ml of various dilutions (1:2, 1:4, and 1:8) of the cell-free extract. The same day, each animal received 10 μg of GAT as GAT-MBSA in Maalox and pertussis vaccine (Eli Lilly Company, Indianapolis, Ind.) i.p. 7 days later, the splenic GAT-specific IgG PFC responses were determined using GAT-SRBC as indicator cells in a modified Jerne hemolytic plaque assay (1, 5).

For the in vitro assay of GAT-T_sF, replicate 1-ml cultures containing 8×10^6 spleen cells were established according to the modifications of the Mishell-Dutton system used in our laboratory (8), and dilutions of GAT-T_sF and 10 μg GAT as GAT-MBSA or SRBC were added at initiation. The IgG PRC responses were measured 5 days later. As shown by the cell recovery, the cell-free extracts were toxic and suppressed both GAT-MBSA and SRBC response at dilutions below (1/200) (5). Therefore, higher dilutions of the extracts were routinely tested and this activity expressed as S_{50} units/ml as previously described (6). The GT suppressive factor was assayed only in vivo in BALB/c mice. The animals were injected i.v. with 0.5 ml of various dilutions of the cell-free extract. On the same day, the animals received 10 μg of GT as GT-MBSA emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.) i.p. 7 days later the IgG PFC responses were determined using GAT-SRBC as indicator cells (9).

Antisera. The different anti-H-2^q sera were prepared by Dr. Martin Dorf. Anti-H-2^q serum was from D1.LP mice immunized with DBA/1 lymphoid cells; anti-D^q serum was from (C3H × B10.D2) F_1 mice immunized with B10.AKM cells. Anti-K^q + I^q was from (DBA/2 × B10.BR)F_1 mice immunized with B10.T (6R) cells, and an anti-K^q serum was prepared in (C57BL/10SN × A/WY)F_1 mice by immunization with B10.AQR 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 absorbed for 1 h at 4°C with thymocytes (10^8 cells/ml) from mice of the strain used to produce the antiserum.

Anti-mouse Ig serum was obtained from rabbits that received multiple subcutaneous injections of 1 to 2 mg of purified mouse Ig emulsified in CFA (Difco laboratories). The hyperimmune antiserum used was analyzed for precipitating antibodies by double gel diffusion against purified myeloma proteins. This serum contained antibodies specific for μ, γ_1, γ_{2a}, γ_{2b}, and α-heavy chains and κ-light chain. The antibodies were purified by adsorption to CnBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) to which mouse Ig has been coupled, according to the manufacturer's directions. The adsorbed antibody was eluted with glycine-HCl, pH 2.8, concentrated by ultrafiltration and stored at −20°C.

Rabbit anti-GAT serum was obtained from rabbits 7 days after the second subcutaneous immunization with 1 mg GAT in CFA. DBA/1 anti-GAT serum was prepared by i.p. injection of male DBA/1 mice with 10 μg GAT as GAT-MBSA in CFA four times at 2-wk intervals. 10 wk later, the mice received a single i.p. injection of GAT-MBSA in Maalox and were bled after 2 wk. The anti-GAT titer of these antisera was measured by hemagglutination using GAT-SRBC as indicator cells.

Preparation of the Immunoadsorbents. All sera were heat-inactivated at 56°C for 30 min, and the globulin fractions of the different sera were prepared by precipitation with either 50% ammonium sulfate or 18% sodium sulfate. The precipitates were dissolved in and extensively dialyzed against 0.1 M $NaHCO_3$ containing 0.5 M NaCl (coupling buffer), and putative aggregates were removed by centrifugation at 15,000 *g* for 30 min.

The globulin fractions of rabbit anti-mouse Ig, rabbit anti-GAT, and the four alloantisera against different subregions of the H-2^q complex were coupled to Cnbr-activated Sepharose 4B by mixing the activated Sepharose beads with the dialyzed globulin fraction in coupling buffer for 2 hr. Unbound material was removed by washing with coupling buffer, and the remaining active groups were inactivated by overnight reaction at 4°C with 1 M ethanolamine (pH 8). Noncovalently adsorbed protein was removed by three washing cycles of 0.1 M acetate buffer containing 1 M NaCl (pH 4) followed by 0.1 M borate buffer containing 1 M NaCl (pH 8). The coupled beads were stored at 4°C in phosphate-buffer saline (PBS) containing 0.02% NaN_3.

The efficiency of the coupling was determined by measuring the optical density at 280 nm of the original globulin fraction and the wash fluids. A coupling efficiency of 80–95% was usually obtained. The amount of globulin attached to the Sepharose was calculated using an extinction coefficient of $E_{1cm}^{1\%} = 15$, and the coupling conditions were adjusted to obtain 2 mg of protein per ml of packed Sepharose.

Radioactive Labeling of the Antigens. GAT, GT, and BSA were labeled with ^{125}I (New England Nuclear, Boston, Mass.) using the chloramine T method (10). Since GA does not contain tyrosine, it was labeled with [^{14}C]methylamine as follows: 2 mg of GA, 0.31 mg of cold methylamine, 20 μCi of [^{14}C]methylamine (New England Nuclear), and 3 mg of 1-ethyl-3 (3-dimethylamino propyl) carbodiimide (ECDI) were dissolved in 1 ml of water. The pH was adjusted to 5.5, and the mixture was reacted at room temperature for 4 h with stirring. The free methylamine was separated from the GA-bound radioactivity by passing the mixture through a Sephadex G-25 column and then dialyzing the excluded radioactivated material against PBS. 100% of the recovered radioactivity was precipitable by 20% trichloroacetic acid, and approximately 25% of the glutamic acid residues in the GA molecule were conjugated.

Coupling of GAT, GT, GA, and BSA to Sepharsoe 4B. GAT, GT, GA, and BSA were coupled individually to either amino ethyl-Sepharose 4B or to amino hexyl-Sepharose 4B (Pharmacia Fine Chemicals). The amino ethyl-Sepharose was prepared according to the method of Cuatrecasas (11). The amino-hexyl-Sepharose (commercial name AH-Sepharose) was obtained from Pharmacia Fine Chemicals. The antigens were dissolved in water and then coupled to the beads using ECDI. The pH was carefully maintained at 7.5 in mixtures containing GAT and GT to avoid precipitation of these polymers at acid pH. The mixtures were rotated end-over-end for 18 h at room temperature. The antigens employed had been trace-labeled (see above), and after the coupling procedure, the beads were washed extensively with PBS until no radioactivity could be detected in the eluates.

The amount of antigen bound was estimated by the amount of radioactivity bound to the beads. The conditions of the coupling were empirically adjusted such that 1–2 mg of antigen were coupled per ml of packed Sepharose. The specific activity of the radiolabeled antigens was such that the leakage of 0.1 μg of antigen could have been detected except for GA-Sepharose where leakage of a minimum of 0.5 μg would have been detectable.

Use of Immunoadsorbent Columns. The immunoadsorbent columns were prepared by packing required quantities of beads into barrels of 3- or 5-ml syringes. The beads were washed with 20 ml PBS, then with 20 ml PBS containing 10% fetal calf serum, and finally the excess fetal calf serum was removed with a 10 ml PBS wash. The quantities of wash solutions given are for a column of 1 ml of packed beads. The cell extract appropriately diluted in PBS, was applied to the column, reacted at 4°C for different periods of time (1–3 h depending on the experiment), and then eluted with PBS. Various dilutions of the eluates were then assayed in vivo or in vitro on the GAT-MBSA response of DBA/1 mice.

Treatment with DNase, RNase, and Pronase. DNase (2,700 U/mg, RNase free) was obtained from Worthington Biochemical Corp., Freehold, N. J., RNase (90 U/mg) from Sigma Chemical Co., St. Louis, Mo., and pronase (45,000 PUK) from Calbiochem, La Jolla, Calif. 1-ml portions of GAT-T_sF were incubated with 200 μg DNase, RNase, or pronase for 1 or 3 h at room temperature. The treated GAT-T_sF were assayed immediately for suppressive activity on the GAT-MBSA responses by DBA/1 mice in vivo.

Purification of the GAT-T_sF. GAT-Sepharose was prewashed with 3 M KCl in half strength PBS (PBS/2). Repeated testing revealed no detectable "leakage" of GAT from the column during both washing and actual elution of the factor.

GAT-T_sF was diluted 1/5 in PBS/2 and passed 3–4 times very slowly (1 ml/h) through the GAT-Sepharose. The column was then washed with 10 ml of PBS/2 per ml of packed beads. For the one-step elution, 2 M KCl in PBS was then applied to the beads, and 3–5 times the volume of the column was used to elute the suppressive material. Elution by a continuous linear KCl gradient was performed as follows: the gradient was prepared using 10 ml each of two solutions of PBS containing 0.25 M or 2 M KCl. During the elution the flow rate was 5 ml/h, and 7-ml fractions were collected. The KCl concentration of each fraction was determined by measuring the refractive index, and the KCl was removed by gel filtration on Sephadex G-25 using cytochrome (1 mg/ml) as a marker. The purified GAT-T_sF was assayed in vitro on the response by DBA/1 spleen cells to GAT-MBSA (5, 6).

Elution of Rabbit and DBA/1 Anti-GAT Antibodies from GAT-Sepharose. The GAT-Sepharose columns were prepared as above and anti-GAT antiserum (0.5 ml) was reacted for 1 h at 4°C with 2 ml of packed beads. The antibodies were then eluted with the linear KCl gradient. The KCl concentration of each fraction was measured, and then the KCl was removed by gel filtration on a Sephadex G-25 column using cytochrome *c* (1 mg/ml) as a marker. The titer of the anti-GAT antibodies in the original sera and in each fraction was determined by hemagglutination using GAT-SRBC as indicator cells.

Gel Filtration on Sephadex G-100. The molecular weight of the purified GAT-T_sF was estimated by gel filtration on a Sephadex G-100 column (diameter 1.5 cm, length 90 cm) equilibrated at 4°C in PBS. A constant flow rate of 6 ml/h was used. Before use, the column was calibrated with the following protein markers: bovine gamma globulin (mol wt 150,000), bovine serum albumin (mol wt 65,000), ovalbumin (mol wt 45,000), bovine pancreas chymotrypsinogen A (mol wt 25,000), and horse heart cytochrome *c* (mol wt 12,500). GAT-T_sF was purified by one-step elution from the GAT-Sepharose column with 2 M KCl, and 0.5 ml of eluate was mixed with 0.5 ml of a control extract from nonimmunized DBA/1 mice. This mixture was applied to the Sephadex G-100 column and eluted with PBS. Fractions were pooled according to the desired molecular weight ranges determined by the previous calibration. The fractions were tested in vitro without concentration. The volumes of the fractions were measured and, for comparative purposes, the dilutions of the suppressive factor in the various fractions were arbitrarily calculated as if all the original activity had eluted in that fraction.

Results

GAT-T_sF Activity is Destroyed by Pronase. GAT-T_sF was treated with DNase, RNase, or pronase and injected into mice that were immunized with 10 μg of GAT as GAT-MBSA in Maalox-pertussis on the same day. Two representative experiments are shown in Table I. Incubation with DNase and RNase for 1 h did not affect significantly the suppressive activity of GAT-T_sF, whereas incubation with pronase reduced it considerably. The reduction of activity was a time-dependent phenomenon, as shown when the extract was incubated for 3 h with pronase (Exp. 2). The GAT-T_sF activity was completely destroyed under these conditions.

GAT-T_sF has Affinity for GAT. Using an in vivo assay, we have previously demonstrated that GAT-T_sF binds to GAT-Sepharose, but not BSA-Sepharose (5). We have repeated this experiment and quantitated the suppressive activity in vitro. The activity (S_{50} units/ml) of a GAT-T_sF was determined before and

TABLE I
Effect of DNase, RNase, and Pronase on GAT-T_sF

	Groups*	Enzyme	Time of treatment	IgG PFC/Spleen (arith. mean ± SE)	Percent inhibition‡	*P* value§
					%	
Exp. 1	A	–	–	9,700 ± 1,500		
	B	None	1 h	700 ± 700	93	
	C	DNase	1 h	1,300 ± 510	87	0.474
	D	RNase	1 h	2,100 ± 790	78	0.240
	E	Pronase	1 h	4,500 ± 1,300	54	0.043
Exp. 2	F	–		5,200 ± 960		
	G	None	3 h	1,300 ± 1,050	75	
	H	Pronase	3 h	5,200 ± 580	0	0.01

* DBA/1 mice received 0.5 ml of 1/2 dilution of GAT-T_sF intravenously either untreated or treated with enzymes as indicated. The mice were immunized with GAT-MBSA in Maalox-pertussis, and 7 days later the GAT-specific IgG PFC/spleen was determined. All groups contained four animals except group G which contained three animals. Group A and F did not receive extracts and served as controls.

‡ Percent inhibition $= 1 - \left[\frac{\text{PFC/spleen for experimental}}{\text{PFC/spleen for control}}\right] \times 100.$

§ *P* value determined by Student's *t* test comparing all experimental groups to suppression by untreated extract.

after passage over BSA- or GAT-Sepharose column. 1 ml of extract from 6×10^8 cells was applied to columns containing 2 ml of either packed GAT- or BSA-Sepharose. These mixtures were reacted for 1 h at 4°C, the extracts were eluted with PBS, and the eluates assayed in vitro. As shown in Fig. 1, BSA-Sepharose did not remove any GAT-T_sF activity. In contrast, GAT-Sepharose removed all detectable suppressive activity. These results confirm that GAT-T_sF has affinity for GAT.

Fine Specificity of the GAT-T_sF. The avidity of GAT-T_sF for antigens that cross-react serologically with GAT [the copolymers of L-glutamic acid-L-alanine (GA) and L-glutamic acid-L-tyrosine (GT)] was determined to provide an indication of the fine specificity of the GAT-T_sF.

1-ml samples of crude extract from spleen and thymus of GAT-primed DBA/1 mice were reacted for 1 h at 4°C with 3 ml of GAT-Sepharose, GT-Sepharose, GA-Sepharose, or BSA-Sepharose. The material eluted by PBS was tested for suppressive activity in vivo by injecting DBA/1 mice with 0.5 ml of the eluates, i.v., a dose that was equivalent to a 1/2 dilution of the original material. The same day, mice were injected i.p. with 10 μg of GAT as GAT-MBSA in Maalox-pertussis, and 7 days later the splenic GAT-specific IgG PFC responses were measured.

The untreated extract suppressed the response to GAT-MBSA in vivo in a dose-dependent manner, a 1/8 dilution of the extract suppressed the GAT-MBSA response by only 29% (Fig. 2). All detectable suppressive activity was absorbed by GAT-Sepharose (group E). The activity of the extract was reduced, but not totally absorbed by GT-Sepharose (group F) or GA-Sepharose (group G). Comparison of the suppressive activity in the eluate from GAT-Sepharose with

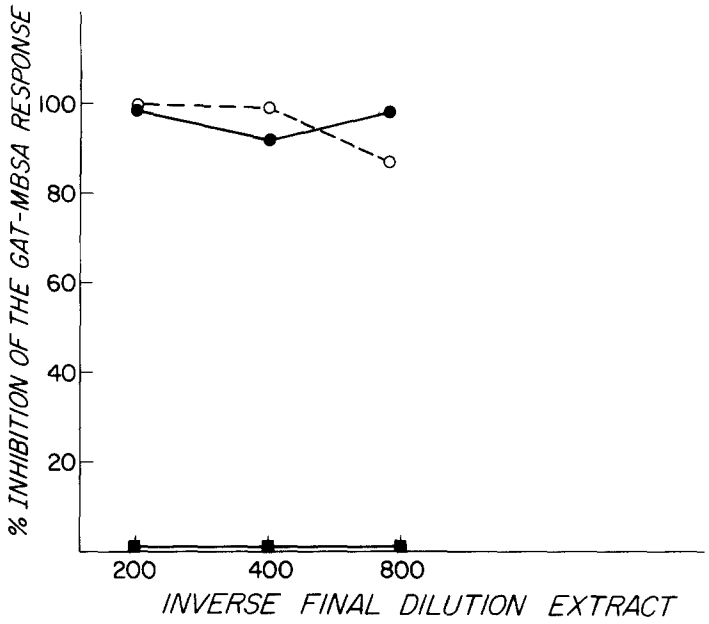

FIG. 1. Absorption of GAT-T_sF with GAT- or BSA-Sepharose. 1 ml of GAT-T_sF was incubated 1 h at 4°C with 2 ml of GAT-Sepharose or BSA-Sepharose. The results are expressed as percent inhibition of the control IgG PFC response to GAT-MBSA in vitro by different dilutions of the eluates and of the original extract. (○—○) Untreated extract; (●—●) extract adsorbed on a BSA-Sepharose column; (■—■) extract adsorbed on a GAT-Sepharose column.

GROUP	EXTRACT		Specific IgG PFC/Spleen	% INHIBITION GAT-MBSA RESPONSE 0 20 40 60 80 100
	Treatment	Dilution		
A	no extract	–	6860 ± 1470	
B	none	1/2	780 ± 200	
C	none	1/4	2030±430	
D	none	1/8	4800±670	
E	GAT-seph	1/2	7000±1050	
F	GT-seph	1/2	4540±530	
G	GA-seph	1/2	4400±550	
H	BSA-seph	1/2	1630±650	

FIG. 2. Specificity of GAT-T_sF for GAT-, GT-, and GA-Sepharose. The data are expressed as the arithmetic mean ± SE of the PFC response of eight mice per group. P values determined by Student's t test are: F:E = 0.051; G:E = 0.042; F:G = 0.865.

eluates from GT-Sepharose and GA-Sepharose indicates that these differences are statistically significant (P = 0.051 and 0.042, respectively).

Since the observed absorption patterns might be explained by differences in the inherent efficiency of the antigen-coated beads, we tested the absorbing capacity of these gels with the suppressive factor obtained from spleen and

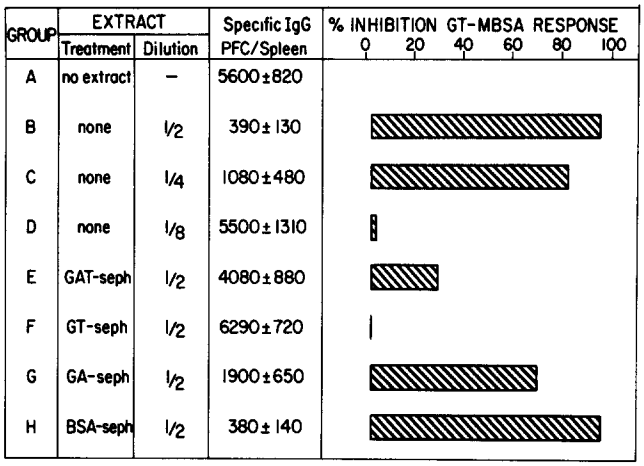

FIG. 3. Specificity of the GT suppressive factor for GT-, GAT, and GA-Sepharose. The data are expressed as the arithmetic mean ± SE of the PFC response of nine mice/group. P values determined by Students' t test are: E:F = 0.071; G:F < 0.001; G:E = 0.067.

thymus of nonresponder BALB/c mice immunized with GT. This factor was shown in previous studies to suppress the GT-MBSA response of BALB/c mice, and to behave like GAT-T_sF (7). The GT factor was passed through the various immunoadsorbents and the eluates assayed on the GT-MBSA response by BALB/c mice. GT-Sepharose removed all detectable suppressive activity, whereas GAT-Sepharose and GA-Sepharose removed only a fraction of this activity (Fig. 3). However, the difference of absorptive efficiency between the two cross-reacting immunoadsorbents, GAT-Sepharose and GA-Sepharose, was appreciable (P = 0.067). Clearly, differential absorption of an extract by the homologous antigen-Sepharose complex is not due to variation in efficiency of the immunoadsorbents.

GAT-T_sF is not Bound by an Anti-Ig Immunoadsorbent. Inasmuch as GAT-T_sF has binding sites for the GAT, it is important to investigate the relationship of this factor to mouse Ig. The rabbit anti-mouse Ig serum used for this purpose contained antibodies against μ, γ_1, γ_{2a}, γ_{2b}, α-heavy chains and κ-light chains. We have not tested the antiserum for anti-λ or δ-chain activity. The globulin fraction of the rabbit anti-mouse Ig serum was coupled to Sepharose 4B, and the capacity of this immunoadsorbent was measured with an anti-GAT serum obtained from DBA/1 mice immunized with GAT-MBSA. 1 ml of a 1/25 dilution of this serum was passed over 1 ml of anti-mouse Ig-Sepharose, and the titers of anti-GAT antibodies in the serum and the eluate were assessed by hemagglutination of GAT-SRBC (Table II). Anti-GAT antibodies were removed by the anti-mouse Ig column. 1 ml of a 1/10 dilution of GAT-T_sF from DBA/1 mice was also passed over 1 ml of anti-mouse Ig-Sepharose, and the activity of the GAT-T_sF in the original preparation and the eluate was titrated in vitro (Table II). Instead of a reduction in the S_{50} units/ml, a slight increase in suppressive activity in the eluate was observed in this and several other experiments. In spite of its specificity for GAT, GAT-T_sF does not bear determinants of the constant regions of the μ, γ, or α-heavy chains or κ-light chain of mouse Ig and, therefore, is not a conventional immunoglobulin.

TABLE II
Absorbtion of Anti-GAT Antibodies and GAT-T_sF by a Specific Rabbit Anti-Mouse Ig Column

Treatment	GAT-T_sF – S_{50} units/ml*			Anti-GAT antibody – HA units‡		
	SRBC	GAT-MBSA	Percent recovery	SRBC	GAT-SRBC	Percent recovery
			%			%
Unabsorbed	<200	860	–	5	400	–
Absorbed on normal rabbit Ig-Sepharose column	<200	1,200	>100	ND§	400	100
Absorbed on rabbit anti-mouse Ig-Sepharose column¶	<200	1,200	>100	ND	10	2.5

* S_{50} units/ml: inverse of dilution causing 50% suppression of the immune response to GAT-MBSA in vitro.

‡ HA units: inverse of the highest dilution causing hemagglutination of GAT-SRBC.

§ ND, not done.

¶ Rabbit anti-Ig antiserum contained precipitating antibodies specific for mouse μ, γ, γ_{2a}, γ_{2b}, and α-heavy chain and κ-light chain as determined by double gel diffusion.

GAT-T_SF has Determinants Encoded by the I-Region of the H-2 Complex. T-cell factors with specific helper or suppressor activity for antibody responses have been shown to bear determinants encoded by the *I* region of the murine *H-2* complex (3, 4, 12, 13). We, therefore, investigated whether GAT-T_sF obtained from DBA/1 mice bears determinants encoded by the *I* region of *H-2*^{q}. GAT-T_sF (0.75 ml of a 1/10 dilution) was reacted for 3 h at 4°C with 1 ml of packed Sepharose to which the globulin fraction of alloantisera against products of the subregions of the *H-2*^{q} complex had been coupled. The various eluates were assayed in vitro for GAT-T_sF activity (Fig. 4). The suppressive factor was completely removed by an anti-*H-2*^{q} immunoadsorbent produced in D1.LP mice against DBA/1 cells, which differ only at the *H-2* complex. This experiment, therefore, demonstrates that GAT-T_sF bears determinants encoded by the *H-2* complex. Further mapping of the GAT-T_sF determinants has been achieved using alloantisera against the *K*, *D*, and *K* + *I* subregions of the *H-2*^{q} complex. The activity of the DBA/1 GAT-T_sF was also removed by immunoadsorbents specific for determinants of *K* + *I* regions of the *H-2*^{q} complex, but not by immunoadsorbents specific for only the *K* or *D* determinants. We may, therefore, conclude that the GAT-T_sF from DBA/1 mice bears specificities encoded by the *I* region of *H-2*^{q}.

Association of Antigen with the GAT-T_SF. Since GAT-T_sF has affinity for GAT (5) and is extracted from lymphoid cells of mice recently injected with GAT (6), it would not be surprising if GAT determinants were associated with the active moiety of GAT-T_sF. To test this possibility, the capacity of a rabbit anti-GAT immunoadsorbent column was first measured to verify that anti-GAT antibodies had not been altered by the coupling process; 1 ml of packed anti-GAT Sepharose was able to bind 1 μg of GAT. Then, 1 ml of a 1/10 dilution of GAT-T_sF

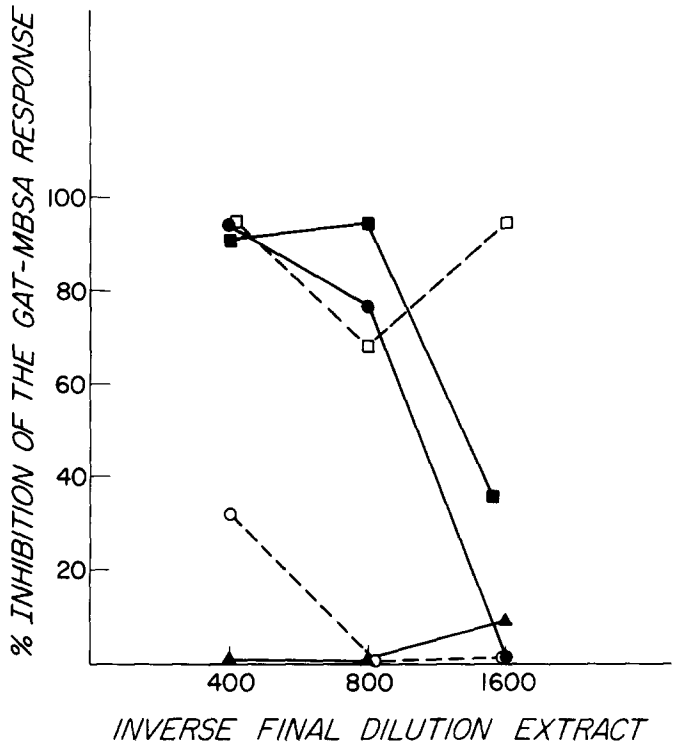

FIG. 4. Absorption of the GAT-T_sF on immunoadsorbents specific for products of subregions of the *H-2* complex. GAT-T_sF (0.75 ml of a 1/10 dilution) was incubated 3 h at 4°C with 1 ml of packed Sepharose to which the globulin fraction of the following alloantisera had been coupled: (▲—▲) anti-H-2^q (D1.LP anti-DBA/1J); (□- - -□) anti-D^q (C3H × B10.D2)F_1 anti-B10.AKM; (○- - -○) anti-K^q + I^q (DBA/2J × B10.BR)F_1 anti-B10.T (6R); (■—■) anti-K^q (C57BL/10SN × A/WY)F_1 anti-B10.AQR; (●—●) unadsorbed extract. The results are expressed as percent inhibition of the control IgG PFC response to GAT-MBSA in vitro by different dilutions of the eluates and of the original extract.

was applied to rabbit anti-GAT-Sepharose or normal rabbit serum-Sepharose column, incubated for 1 h at 4°C, and eluted with PBS. In three typical experiments, (Fig. 5 and Table III), the S_{50} units/ml of the GAT-T_sF was significantly decreased by passage through the rabbit anti-GAT column, indicating that GAT is associated with the active suppressive factor.

Elution of the GAT-T_sF from GAT-Sepharose. In a preliminary experiment (data not shown), GAT-T_sF was eluted from GAT-Sepharose by PBS containing 3 M KCl. The critical concentration of KCl required to elute GAT-T_sF was then determined using a gradient of 0.25 M to 2 M KCl. A crude extract containing the GAT-T_sF was applied to a GAT-Sepharose column. The column was repeatedly washed (see Materials and Methods) until no material was detectable at 280 nm, the KCl gradient was applied, and fractions of equal volumes were collected. The titer of GAT-T_sF (S_{50} units/ml) and the KCl concentration of each fraction were determined (Fig. 6). The GAT-T_sF eluted as a single peak at 0.4 M to 0.6 M KCl. The crude extract enhanced the response to SRBC, but none of the fractions (I–V) displayed this activity (data not shown). None of the fractions had detectable optical density at 280 nm.

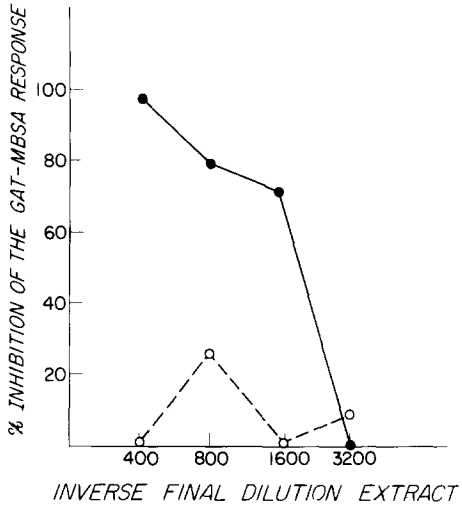

FIG. 5. Absorption of the GAT-T_sF on rabbit anti-GAT immunoadsorbent. 1 ml of a 1/10 dilution of GAT-T_sF was incubated 1 h with 2 ml of the rabbit anti-GAT-Sepharose. The results are expressed as percent inhibition of the control IgG PFC response to GAT-MBA in vitro by different dilutions of the eluates and of the original extract. (●—●) untreated extract; (○—○) extract absorbed on rabbit anti-GAT-Sepharose column.

TABLE III

Absorption of the GAT-T_sF on a Rabbit Anti-GAT-Sepharose Column

Immunoadsorbents	Experiment II S_{50} units/ml		Experiment III S_{50} units/ml	
	SRBC	GAT-MBSA	SRBC	GAT-MBSA
Unabsorbed	<400	1,500	<400	>2,000
Normal rabbit Ig-Sepharose	<400	1,800	<400	1,300
Rabbit anti-GAT Ig-Sepharose*	<400	570	<400	600

* Rabbit anti-GAT was obtained 7 days after the second immunization with 1 mg of GAT in CFA. The immunoadsorbent was able to bind 1 μg of GAT per ml of packed beads.

For comparative purposes, DBA/1 anti-GAT-MBSA and rabbit anti-GAT antibodies were absorbed to GAT-Sepharose and eluted with a KCl gradient. Most of the antibodies in the serum of DBA/1 mice immunized with GAT-MBSA eluted at a concentration of 0.5 M KCl (Fig. 7). In a parallel experiment (not shown), rabbit anti-GAT antibodies eluted at 1.2 M KCl, indicating that this method distinguished differences in avidity.

These experiments demonstrate several important points: (*a*) The total activity of the purified GAT-T_sF was considerably higher than the activity of the crude extract that was applied to the colum; (*b*) GAT/T_sF eluted by KCl is very highly purified, since a crude extract containing 10 mg protein per ml after elution yields a fraction with considerably greater suppressive activity than in initial preparation and yet no detectable optical density at 280 nm; and (*c*) if the KCl concentration required for elution of the DBA/1 anti-GAT antibodies and

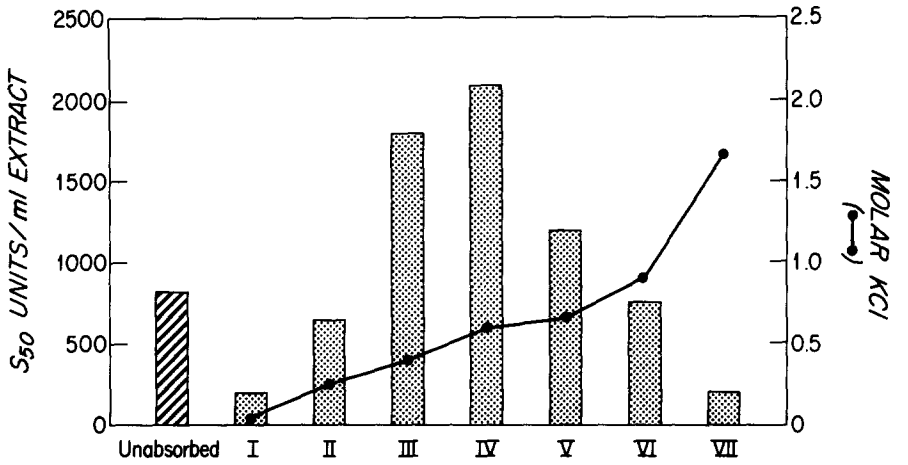

FIG. 6. Elution of GAT-T_sF from GAT-Sepharose by a KCl gradient. Fractions were assayed for suppressive activity on IgG PFC responses to GAT-MBSA and SRBC in DBA/1 spleen cultures. None of the fractions suppressed PFC responses to SRBC (not shown). The suppressive activity is expressed in S_{50} units/ml. The concentration of KCl in each fraction was determined by refractometry.

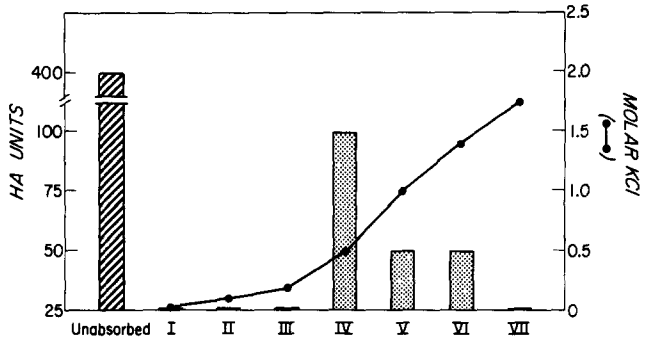

FIG. 7. Elution of DBA/1 anti-GAT-MBSA antibodies from GAT-Sepharose by a KCl gradient. The serum was diluted 1/25 by the elution procedure, and this was the lowest dilution tested by hemagglutination on SRBC. The concentration of KCl in each fraction was determined by refractometry.

the GAT-T_sF from the GAT-Sepharose immunoadsorbent accurately reflects the strength of interaction between these molecules and GAT, then GAT-T_sF and anti-GAT antibodies have comparable avidities for the antigen.

For routine purification of GAT-T_sF a one-step elution with 2 M KCl was used. Under these conditions, the degree of purification is comparable to the one obtained by continuous gradient elution since no optical density is detected. However, the increased activity obtained by the one-step elution procedure was smaller and less reproducible than that observed using the linear gradient. The factor obtained by the one-step procedure will be referred to as "purified GAT-T_sF."

Elution of a Control Extract from GAT-Sepharose Does Not Generate Suppressive Activity. This experiment was designed to verify that GAT is not

TABLE IV
Elution of a Control Extract From GAT-Sepharose Does Not Generate Suppressive Activity

	S_{50} units/ml*	
	SRBC	GAT-MBSA
GAT-T_sF	<400	1,200
Extracts eluted from GAT-Sepharose (2 M KCl)		
GAT-T_sF	<400	1,200
Maalox extract	<400	<400
GAT + Maalox extract‡	<400	<400

* S_{50} units/ml = inverse of dilution causing 50% suppression of the immune response in vitro.

‡ Maalox extract to which 10 μg GAT was added per ml undiluted extract and incubated 1 h at 4°C, before application to a GAT-Sepharose column.

responsible for the suppressive activity of purified GAT-T_sF. It had been previously demonstrated that addition of GAT (25 μg/ml) to a crude extract obtained from Maalox-primed mice (Maalox extract) does not generate suppressive activity at dilutions of GAT-T_sF which are active in vitro or in vivo (6). The experiment in Table IV established that a Maalox extract does not become suppressive when processed by the protocol used for the purification of GAT-T_sF from GAT-Sepharose. Since no leakage of GAT from GAT-Sepharose was detected by monitoring the radioactivity of the eluates, we conclude that the suppressive activity in purified GAT-T_sF cannot be attributed to the introduction of free GAT from the absorbent. Furthermore, addition of 10 μg GAT/ml of Maalox extract before purification does not generate suppressive activity in the eluate demonstrating, again, that the suppressive activity of GAT-T_sF is not due to native GAT.

The Purified GAT-T_sF Bears H-2 Determinants. Purified GAT-T_sF was applied to an anti-H-2q immunoadsorbent column. After reaction for 2 h at 4°C and elution with PBS, the recovered material was assayed for suppressive capacity in vitro (Fig. 8). The suppressive material, purified on the basis of avidity for GAT-Sepharose, was removed by an anti-H-2^q immunoadsorbent. The purified GAT-T_sF was titrated before and after passage through the anti-H-2^q column in two additional experiments. In both experiments, the S_{50} units/ml in the purified extract were significantly reduced after passage over the anti-H-2^q immunoadsorbent.

Purified GAT-T_sF Is Not Associated with GAT. We have observed that GAT-T_sF was absorbed by rabbit anti-GAT Sepharose, demonstrating that the active moiety in the crude extract was bound to GAT or a serologically reactive fragment of GAT. The possibility that the suppressive activity in GAT-T_sF can be attributed to native GAT has been eliminated (6). However, the possibility that GAT together with an antigen-specific *I*-region gene product plays a crucial role in the suppression of responses to GAT-MBSA must be considered.

Therefore the next experiments determined whether GAT was also associated

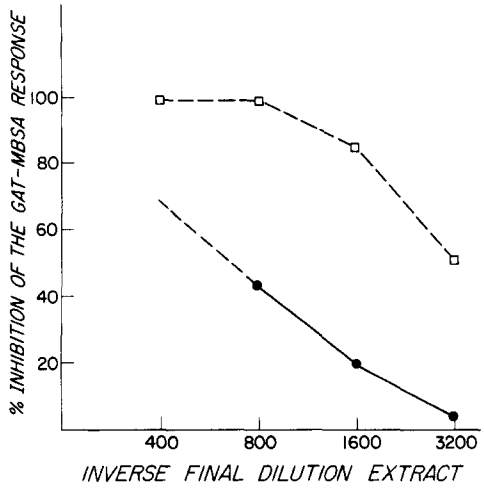

FIG. 8. Adsorption of the purified GAT-T_sF on an anti-H-2^r immunoabsorbent. The KCl eluate from the GAT-Sepharose column was passed over a Sephadex G-25 column, a portion of this material was assayed for suppressor activity (□- - -□), and 0.75 ml of that material (diluted 1/20 by the purification) was applied to 1 ml of the immunoabsorbent. After a 2-h incubation, the material was eluted with PBS and assayed for suppressor activity in vitro (●—●).

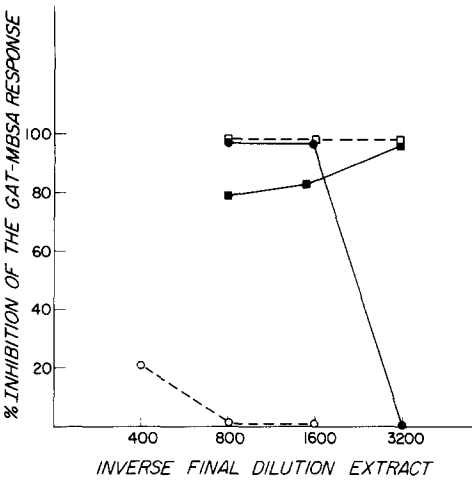

FIG. 9. Binding of the GAT-T_sF by an anti-GAT immunoadsorbent before and after purification on GAT-Sepharose. The crude extract (0.75 ml of a 1/20 dilution) and purified GAT-T_sF (0.75 ml of a 1/20 dilution) were incubated 2 h with 1 ml of the anti-GAT-Sepharose. The columns were eluted with PBS and suppressive activity assayed in vitro. (●—●) Crude extract; (○- - -○) crude extract absorbed by rabbit anti-GAT-Sepharose; (□- - -□) purified GAT-T_sF; (■—■) purified GAT-T_sF absorbed by rabbit anti-GAT-Sepharose. Rabbit anti-GAT was obtained 7 days after secondary immunization with 1 mg of GAT in CFA. The immunoadsorbent was able to bind 1 μg of GAT per ml of packed gel.

with the purified GAT-T_sF. Purified GAT-T_sF and a portion of the crude extract were applied to rabbit anti-GAT-Sepharose columns, and the eluates were tested for suppressive activity in vitro (Fig. 9). As previously observed, the titer GAT-T_sF in the crude material is reduced significantly by passage over the anti-

TABLE V

Purified GAT-T_sF Is Not Associated with Serologically Detectable GAT Determinants

Treatment of extract	Experiment II S_{50} units/ml		Experiment III S_{50} units/ml	
	SRBC	GAT-MBSA	SRBC	GAT-MBSA
Crude GAT-T_sF untreated	<400	1,300	500	2,000
Crude GAT-T_sF absorbed by rabbit anti-GAT-Sepharose*	400	800	<400	<400
Purified GAT-T_sF	<400	3,300	<400	1,100
Purified GAT-T_sF absorbed by anti-GAT-Sepharose*	<400	>3,200	<400	900

* Rabbit anti-GAT was obtained 7 days after the second immunization with 1 mg of GAT in CFA. The immunoadsorbent was able to bind 1 μg of GAT per ml of packed gel.

GAT column. By contrast, the titer of the purified GAT-T_sF was not detectably reduced by passage over rabbit anti-GAT-Sepharose. Two additional confirmatory experiments in which the results are expressed in S_{50} units/ml are in Table V. Thus, GAT is not associated with purified GAT-T_sF, and the GAT that was associated with GAT-T_sF in the crude extract was displaced in the purification procedure.

Estimation of Molecular Weight of the Purified GAT Suppressor Factor. The molecular weight of the GAT-T_sF activity in the crude extract has been estimated to be approximately 45,000, both by gel filtration on Sephadex G-100 and ultrafiltration through Amicon membranes (5). However, for unknown reasons, the suppressive activity eluted in a broad band from Sephadex. Therefore, purified GAT-T_sF was fractionated on a Sephadex G-100 column. The majority of the suppressive activity was in fraction III (mol wt 40,000–55,000) (Table VI). Fractions IV and V had much smaller, but detectable, amounts of activity; fraction I, which corresponds to the void volume of the column (mol wt >80,000), had no detectable activity, and fraction II (mol wt 55,000–80,000) had very little activity.

Discussion

Some of the properties of GAT-T_sF, an extract of lymphoid cells from GAT-primed nonresponder DBA/1 mice, have been analyzed. GAT-T_sF is a protein that has affinity for GAT. Despite its affinity for the stimulating antigen, the active factor does not bear μ, γ_1, γ_{2a}, γ_{2b}, or α-heavy chain or κ-light chain constant region determinants.

The fine specificities of GAT-T_sF and of a factor extracted from GT-primed BALB/c mice have been characterized by comparing the absorption of suppressive activity by columns containing homologous or chemically related and serologically cross-reactive insolubilized copolymers. The results demonstrated that these factors bind preferentially to the antigen that stimulated their production, but that binding to cross-reactive antigens is comparable to that of

TABLE VI
Estimate of the Molecular Weight of Purified GAT-T_sF by Gel Filtration on Sephadex G-100

	S_{50} units/ml	
	SRBC	GAT-MBSA
Crude GAT-T_sF	<400	1,200
Purified GAT-T_sF	<400	>1,600
Purified GAT-T_sF eluted from Sephadex G-100*		
Fraction I	<400	<400
Fraction II	<400	400
Fraction III	<400	>1,600
Fraction IV	<400	800
Fraction V	<400	600

* The size of the molecules eluted in the different fractions is: Fraction I, >80,000; Fraction II, 55,000–80,000; Fraction III, 40,000–55,000; Fraction IV, 20,000–40,000; Fraction V, <20,000. The column was calibrated with bovine gamma globulin (150,000), bovine serum albumin (65,000), ovalbumin (45,000), bovine pancreas chymotrypsinogen A (25,000), and horse heart cytochrome *c* (12,500).

antibodies produced in the same strain of mice. Based on the assumption that the concentration of KCl required to dissociate a factor bound to an insolubilized antigen is an estimate of avidity, the relative avidities of GAT-T_sF and anti-GAT antibodies produced in DBA/1 mice were compared. Both GAT-T_sF and anti-GAT antibodies from DBA/1 mice were eluted with approximately 0.5 M KCl, whereas rabbit anti-GAT antibodies were eluted with 1.2 M KCl. Therefore, on the basis of specificity and avidity, GAT-T_sF resembles the anti-GAT antibodies produced by the same strain. However, GAT-T_sF is much smaller than immunoglobulin (5) and does not bear constant region determinants of heavy or light chains. The interesting possibility that GAT-T_sF shares idiotypic determinants with anti-GAT antibodies is currently under investigation.

When GAT-T_sF was eluted from GAT-Sepharose by a KCl gradient, the total suppressive activity obtained was much greater than that in the crude extract. Three observations must be considered to explain this difference. First, extracts of lymphoid cells from control mice routinely enhance the PFC responses in cultures stimulated with either GAT-MBSA or SRBC. Second, extracts from GAT-primed mice enhance responses to SRBC in vitro. Finally, this nonspecific enhancing activity was not detected in the fractions of GAT-T_sF eluted from GAT-Sepharose by KCl gradients. Therefore, GAT-T_sF activity in the crude extracts appears to be partially masked by the nonspecific enhancing factors. The nature of these enhancing factors is unknown, but since these extracts do not enhance immune responses in vivo, they may act to improve the culture conditions in a nonimmunological manner.

Similar to the suppressor factor described by Tada and Tanaguchi (3, 4), GAT-T_sF possesses determinants encoded by the *I* region of the *H-2* complex. Furthermore, purified GAT-T_sF was absorbed by insolubilized anti-H-2^q sera (Fig. 8), conclusively demonstrating that the same molecule (or molecular complex) possesses a combining site(s) for GAT as well as determinants encoded by the *I*

region of the *H-2* complex. The KLH-specific suppressor factor of Tada and associates has been reported to bear determinants controlled by the *I-J* subregion of the *H-2* complex (14). Further serologic and genetic analysis of GAT-T_sF from DBA/1 mice is currently prevented by the lack of appropriate recombinant strains.

The GAT-T_sF activity in the crude extracts could be absorbed by an immunoadsorbent containing anti-GAT antibodies, indicating that at least a fragment of antigen is associated with this suppressive factor. This finding is not surprising since GAT-T_sF has binding sites for the antigen, and the mice from which the factor is extracted received 10 μg of GAT only 3 days before the preparation of the cell-free extract. However, the presence of antigen in the KLH suppressor factor has not been reported by Tada and associates (3, 4, 14).

The demonstration of GAT associated with the active suppressive factor and the ease with which suppressor T cells are stimulated by GAT in nonresponder DBA/1 mice (1, 2) raised the issue of whether sufficient native antigen is present in the crude lymphoid cell extracts from GAT-primed mice to cause suppression itself. To test this possibility, an amount of GAT equivalent to that injected into donor mice was added to extracts from Maalox-primed animals. These control extracts containing GAT were tested in vivo and in vitro, and no significant suppression was detected at dilutions where GAT-T_sF was suppressive (6). Therefore, it is clear that the amount of GAT associated with the active suppressor extract is by itself insufficient to cause the suppression. In addition, the demonstration that the active factor bears *Ia* determinants and has antigen-binding specificity for GAT further indicates that it is not simply native or unprocessed GAT. Nevertheless, the possibility must be considered that GAT together with an antigen-specific *I*-region product plays a crucial role in suppression of the responses to GAT-MBSA. If this were the case, associations of an *I*-region product with native or processed GAT must result in a highly suppressive form of GAT. In contrast to the suppressive activity of crude extracts, purified GAT-T_sF was not absorbed by an anti-GAT-Sepharose column. This suggests that the antigen-GAT-T_sF complex in the crude extract was dissociated during the purification. The observation that the purified GAT-T_sF is not associated with GAT does not establish that antigen is unnecessary for its suppressive activity since the assay for activity requires the presence of GAT-MBSA. The factor could readily bind to GAT and, in fact, may have to bind to antigen in order to exert is specific suppressive effect. The availability of antigen-free purified GAT-T_sF should allow the determination of the role of antigen-factor interactions in the inhibition of responses to GAT-MBSA. Furthermore, the methodology described in these papers should also permit the preparation and isolation of GAT-T_sF from primed T cells in amounts sufficient for molecular characterization by classical biochemical techniques.

The experiments reported in this paper establish that GAT-T_sF belongs to the same class of immunosuppressive molecules described by Tada et al. (3, 4, 14) and Herzenberg et al. (15). These mediators resemble a helper T-cell replacing factor described by Mozes (13) and Taussig et al. (16). Thus, it appears that a new family of regulatory proteins have been described that are physically and immunologically similar, antigen-specific, but of opposing activities.

Summary

The GAT-specific suppressor T-cell factor (GAT-T_sF) extracted from lymphoid cells from GAT-primed, nonresponder DBA/1 mice has been partially characterized. It is a protein that has affinity for GAT and determinants encoded by the *I* region of the *H-2* complex.

On the basis of specificity and avidity, GAT-T_sF resembles anti-GAT-MBSA antibodies produced by DBA/1 mice in spite of the fact that it is too small to be classical antibody and has no constant-region determinants of heavy or light chains. Further, GAT or a fragment of GAT is associated with the GAT-T_sF.

GAT-T_sF has been partially purified from the crude extract by absorption to GAT-Sepharose and elution with 0.4 to 0.6 KCl. GAT-T_sF purified on the basis of its affinity for GAT bears *I*-region determinants but not detectable GAT or GAT fragment.

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