

IMMUNOSUPPRESSIVE FACTOR(S) EXTRACTED FROM
LYMPHOID CELLS OF NONRESPONDER MICE
PRIMED WITH L-GLUTAMIC

ACID⁶⁰-L-ALANINE³⁰-L-TYROSINE¹⁰ (GAT)

II. Cellular Source and Effect on Responder
and Nonresponder Mice*

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The immune response by inbred strains of mice to the synthetic terpolymer L-glutamic acid⁶⁰-L-alanine³⁰-L-tyrosine¹⁰ (GAT)¹ is controlled by an *H-2*-linked immune response (*Ir*) gene(s) (1, 2). Both responder (*H-2^{a, b, d, k}*) and nonresponder (*H-2^{p, q, s}*) mice develop primary and secondary GAT-specific IgG plaque-forming cell (PFC) responses to GAT complexed to methylated bovine serum albumin (GAT-MBSA) in vivo and in vitro (3, 4). In responder, but not nonresponder, mice GAT stimulates development of: (a) primary and secondary GAT-specific IgG PFC responses in vivo and in vitro (3); (b) radioresistant GAT-specific helper T cells (5); and (c) specific T-cell proliferative responses in cultures containing peritoneal exudate T lymphocytes from immunized mice (6). Nevertheless, GAT does interact with the immune system of nonresponder mice causing specific inhibition of the development of GAT-specific PFC responses to a subsequent injection of GAT-MBSA (7). This unresponsiveness is mediated by GAT-specific suppressor T cells (T_s) (8).

The mechanisms by which these and other T_s regulate the immune response are currently the subject of intense investigation. Although some T_s release soluble mediators (9-11), no suppressive activity has been detected in supernatant fluids of cultured GAT-specific T_s (Kapp, unpublished observations). However, a suppressive factor has been extracted from thymus, spleen, and lymph node cells from GAT-primed nonresponder mice (12). The suppression mediated by these extracts is dose dependent, demonstrable in vivo and in vitro, and specific for GAT (12). The active component in these extracts has a binding site(s) for GAT and an estimated mol wt of 45,000 (12).

The correlation of: (a) suppression by GAT; (b) GAT-induced T_s and; (c) the suppressive activity of extracts from lymphoid cells of GAT-primed mice with genetic nonresponsiveness strongly suggests that the study of the soluble sup-

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¹ Abbreviations used in this paper: GAT, random terpolymer of L-glutamic acid⁶⁰-L-alanine³⁰-L-tyrosine¹⁰; GAT-MBSA, GAT complexed to methylated bovine serum albumin; GAT-SRBC, GAT coupled to sheep red blood cells; PFC, plaque-forming cell(s); T_s, suppressor T cell(s).

pressor substances will result in further clarification of the mechanism(s) by which *Ir* genes regulate antibody responses. The experiments reported here establish the reproducibility of the *in vitro* assay for suppressive activity, the time after GAT-priming when the suppressive factor can be extracted, the cellular source of suppressive factor, and the effect(s) of these factors on the immune responses of allogeneic and semisyngeneic mice. In addition, these experiments provide the foundation for the molecular characterization of this factor that will be described in subsequent papers.

Materials and Methods

Mice. C57BL/6 (*H-2^b*), DBA/1 (*H-2^d*), SJL (*H-2^s*), and A.SW (*H-2^a*) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. (C57BL/6 × DBA/1)*F*₁ (*H-2^{b/d}*) and (SJL × DBA/1)*F*₁ (*H-2^{s/d}*) mice were bred in our animal facilities. Mice were used at 2–8 mo of age and were maintained on laboratory chow and acidified-chlorinated water *ad libitum*.

Antigens. GAT, mol wt 32,000, was purchased from Miles Laboratories, Inc., Elkhart, Ind. MBSA, sheep red blood cells (SRBC), GAT, and GAT-MBSA were prepared as described previously (3).

Preparation of Cell-Free Extracts. Cell-free extracts were prepared as described previously (12). Briefly, DBA/1 mice were injected *i.p.* with 10 μg GAT in Maalox (William H. Rorer, Inc., Fort Washington, Pa.) or with Maalox alone (control). Unless otherwise stated, mice were sacrificed 3 days later, and single cell suspensions of spleen and thymus were prepared and pooled, washed twice in Hanks' balanced salt solution, and resuspended to 6 × 10⁸ cells/ml in Eagle's minimum essential medium supplemented with 4 mM HEPES, 2 mM L-glutamine, and 50 U/ml each penicillin and streptomycin (Microbiological Associates, Bethesda, Md.). The cells were sonicated in a Sonifier Cell Disruptor, Model W-140-E (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) using a standard microtip. From 50 to 60 watts were delivered for 5 min to 3–8-ml samples maintained at 7°C in a continuous flow-cooling cell. The lysate was centrifuged at 40,000 *g* at 4°C for 1 h, and the supernate was collected and stored at –80°C until use.

Elimination of Thymic Lymph Nodes. Peri- and intra-thymic lymph nodes were labeled by injecting 0.2 ml India ink *i.p.* into donor mice 24 h before sacrifice (13). The black lymph nodes were removed before preparation of single cell suspensions of thymus.

Cell Separation Techniques. Adherent cells (mostly macrophages) were depleted from suspensions of pooled thymus and spleen cells by serial passage over plastic Petri dishes as described previously (5). The remaining nonadherent cells were fractionated into T-cell and B-cell populations by passage over an immunoadsorbent column containing rabbit anti-mouse Ig coupled to Sephadex G-200 (8). Briefly, nonadherent cells were resuspended at 20 × 10⁶ cells/ml in Medium 199 (Grand Island Biological Company, Grand Island, N. Y.) containing 5% fetal calf serum, 2.5 mM EDTA, and 50 U/ml each penicillin-streptomycin and applied in 10-ml vol to the immunoadsorbent column containing 10 ml Sephadex at 4°C. The T cells were eluted by addition of the EDTA-containing medium until the effluent was cell-free. The bound cells (B cells) were eluted with medium containing 10% mouse serum as a source of gamma globulin. This method of separation routinely yields T cells contaminated with only 0–7% Ig-bearing cells and B cells that are 85–96% Ig positive.

Immunization of Mice. Mice were immunized *i.p.* with 10 μg GAT as GAT-MBSA in Maalox and pertussis vaccine (Eli Lilly Co., Indianapolis, Ind.). Extracts, 0.5 ml, were injected *i.v.* within 2 h of antigen.

Spleen Cell Cultures and PFC Assay. Suspensions of single spleen cells containing 8 × 10⁶ nucleated cells/ml in completely supplemented culture medium were stimulated with antigen and incubated according to the method of Mishell and Dutton (14) with modifications previously described (15). PFC responses were assayed 7 days after *in vivo* immunization or 5 days after culture initiation using SRBC or GAT coupled to SRBC (GAT-SRBC) as indicator cells (3).

Results

Characterization of Suppressive Activity *In Vitro*. We have previously demonstrated that lymphoid cell extracts from control DBA/1 mice (Maalox primed)

were not cytotoxic and did not suppress the in vitro responses by DBA/1 spleen cells to SRBC or GAT-MBSA at dilutions $\geq 1/200$ (equivalent to 3×10^6 lymphoid cells/ml) (12). In fact, dilutions of control extracts from 1/200 to 1/20,000 routinely enhanced the PFC responses to both SRBC and GAT-MBSA by normal DBA/1 spleen cells (12). To standardize the in vitro assay of suppressive activity, extracts from GAT-primed DBA/1 mice were titrated on responses to SRBC and GAT-MBSA by normal DBA/1 spleen cells. The results of a single representative experiment (Fig. 1, panel A) demonstrate that an extract from GAT-primed DBA/1 mice inhibited the GAT-specific PFC response to GAT-MBSA, while the PFC response to SRBC was enhanced at all dilutions tested. To compare results of several experiments, the data have been standardized by expressing the suppressive activity of dilutions of an extract as the percent inhibition of the response to antigen developed by cultures containing no extract (Fig. 1, panel B). Since the response to SRBC is enhanced in culture containing these extracts, expression of these control data as percent inhibition yields negative numbers; therefore the SRBC controls were performed in each experiment, but not plotted. As shown in Fig. 1, panel B, repeated titrations of individual extracts yielded remarkably consistent data, and different batches of extracts showed similar suppressive activity. In each experiment, therefore, the extracts were titrated, the data plotted as percent inhibition, and the 50% end points determined. The suppressive activity of each extract can then be expressed in S_{50} units as the inverse of the dilution giving 50% inhibition of the response (Fig. 1, panel C). If the 50% end point is not reached, but the data can be extrapolated, it is expressed as the approximate S_{50} units (see no. 35, panel C). If all tested dilutions of the extract give 100% inhibition, the data are expressed as \geq the last dilution tested and if no dilution tested is inhibitory the S_{50} units are expressed as $<$ the lowest dilution tested.

Soluble Suppressive Component is Not Native GAT. The original observation that the GAT specifically inhibited the development of GAT-specific PFC responses to GAT-MBSA by nonresponder mice in vivo and in vitro (7) raises the possibility that the immunosuppressive material extracted from lymphoid cells of GAT-primed nonresponder mice is, in fact, native GAT. If all the GAT injected into the donor mice were recovered in the spleen and thymus, the concentration of GAT would be 20–30 $\mu\text{g/ml}$ of extract. Therefore, GAT was added to an extract prepared from lymphoid cells of control DBA/1 mice to give a GAT concentration of 25 $\mu\text{g/ml}$ (Table I, Groups C, D, E, F). The suppressive activity of these GAT-containing control extracts was compared to that of extracts prepared from GAT-primed DBA/1 mice (Groups G, H, I). The responses to GAT-MBSA in the absence of any factor (Group A) or in the presence of control extract (Group B) demonstrated the lack of effect of the control extract alone. Significant suppression occurred only in mice that received 1:5 and 1:10 dilutions of the extract from GAT-primed mice (Groups G and H).

The effect of these three types of extracts on the responses to GAT-MBSA in DBA/1 spleen cell cultures was also examined (Fig. 2). Only the extract prepared from GAT-primed DBA/1 mice was suppressive. We have previously demonstrated that inhibition of the GAT-specific PFC responses to GAT-MBSA by nonresponder mice requires injection of $\geq 1.0 \mu\text{g}$ of GAT in Maalox i.p. or

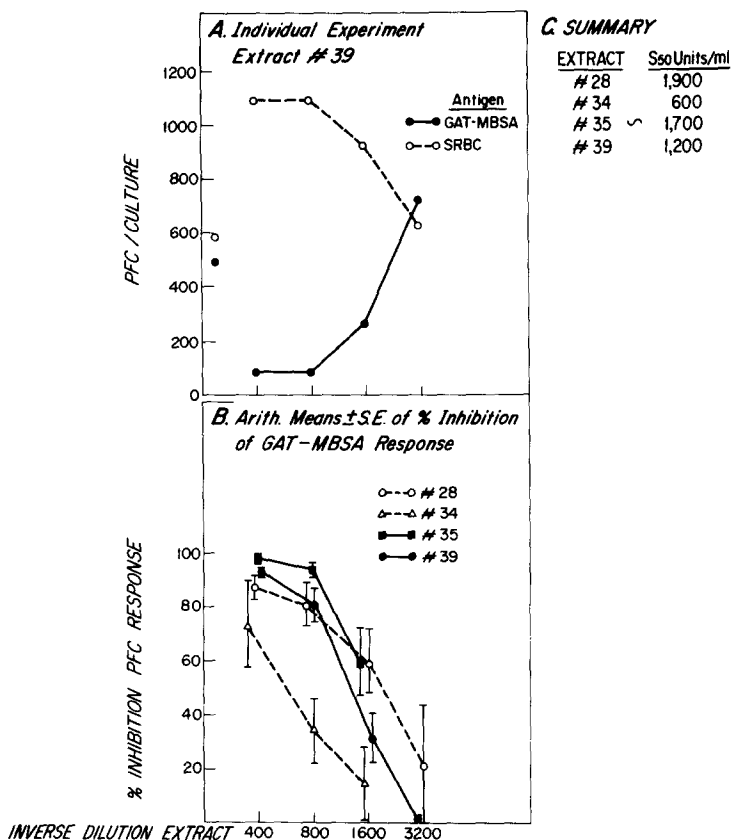


FIG. 1. In vitro analysis of lymphoid cell extracts from GAT-primed DBA/1 mice. All extracts were prepared from pooled thymus and spleen cells (6×10^8 cells/ml) from DBA/1 mice primed 3 days earlier with $10 \mu\text{g}$ GAT in Maalox. The extracts were diluted and added to spleen cells from normal DBA/1 mice at culture initiation. IgG PFC responses to SRBC and GAT were assayed after 5 days. (panel A) Activity of a single extract on the IgG PFC response to SRBC (○—○) and GAT-MBSA (●—●). PFC responses in cultures containing no extracts are given to the left of the titration curves. (panel B) Suppressive activity of four separate extracts expressed as the percent inhibition of the response to GAT-MBSA in cultures containing no extracts. Number of experiments performed in parentheses. (○—○) no. 28 (7); (△—△) no. 34 (3); (■—■) no. 35 (4); (●—●) no. 39 (10). (panel C) Expression of inhibitory activity as the inverse of the dilution of extract causing 50% inhibition of the PFC response to GAT-MBSA S_{50} units/ml.

$>0.1 \mu\text{g}$ GAT/ml of culture medium (7). Therefore, we conclude that an insufficient amount of GAT is present in extracts from GAT-primed nonresponder mice to account for the specific suppression observed.

Correlation Between the Presence of Suppressor Cells (T_s) and the Suppressive Activity in Lymphoid Cell Extracts. Spleen cells from DBA/1 mice injected with $10 \mu\text{g}$ GAT in Maalox specifically suppress the immune responses to GAT-MBSA by an equal number of spleen cells from normal DBA/1 mice in vitro (8). T_s activity can be detected from 3 to 21 days after the injection of GAT (Fig. 3). Similarly, soluble suppressive factor can be extracted from lymphoid cells of

TABLE I
 Suppressive Component in Extracts Is Not Native GAT

Group*	0.5 ml extract from mice primed with‡	Extract dilution	Soluble GAT added μg	GAT-specific IgG PFC/spleen§ <i>arith. mean \pm SE</i>	Suppression %	P
A	No extract	—	—	7,900 \pm 900	—	—
B	Maalox	1:5	—	7,400 \pm 1,500	7	—
C	Maalox	1:5	2.5	6,200 \pm 500	21	0.482
D	Maalox	1:10	1.25	5,900 \pm 800	26	0.391
E	Maalox	1:20	0.6	4,900 \pm 1,000	38	0.205
F	Maalox	1:120	0.1	5,000 \pm 500	37	0.321
G	GAT	1:5	—	2,200 \pm 1,200	72	0.022
H	GAT	1:10	—	1,900 \pm 1,100	76	0.014
I	GAT	1:20	—	4,800 \pm 1,200	39	0.219

* Six mice per group.

‡ Extracts injected i.v. within 1 h of injection of GAT-MBSA in Maalox-pertussis i.p.

§ PFC response day 7.

|| P value determined by Student's *t* test comparing all experimental groups with Group B.

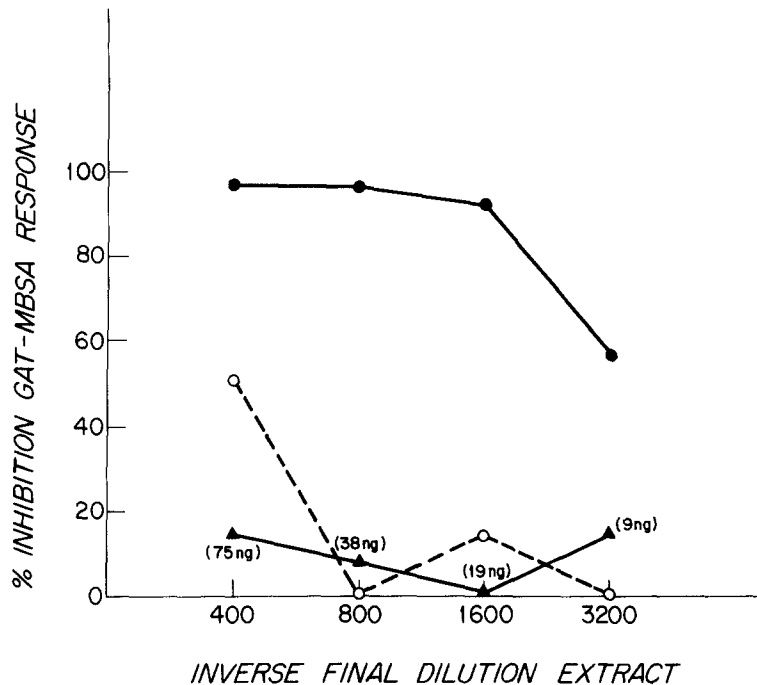


FIG. 2. Suppressive factor in extracts of GAT-primed DBA/1 mice is not native GAT. Normal DBA/1 spleen cells (8×10^6) were incubated with various dilutions of a control extract (○- -○), a control extract to which 25 μg GAT was added to 1 ml of undiluted extract, (▲—▲) or an extract from GAT-primed mice (●—●). Extracts were added to spleen cells at culture initiation and IgG PFC responses to SRBC (not shown) and GAT-MBSA were assayed after 5 days.

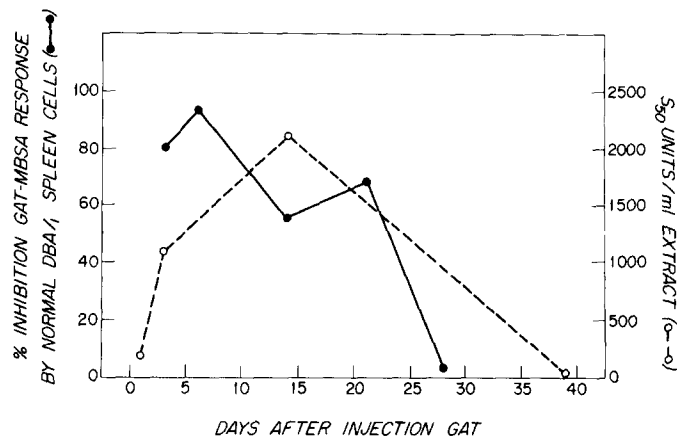


FIG. 3. Comparison of suppressive activity of spleen cells (●—●) vs. lymphoid cell extracts (○--○) at various times after injection of 10 μ g GAT in Maalox. Primed spleen cells (8×10^6) or dilutions of the extract were added to normal DBA/1 spleen cells (8×10^6) at culture initiation, and the IgG PFC responses to SRBC (not shown) and to GAT-MBSA were assayed after 5 days.

DBA/1 mice from days 3 to 14 after injection of GAT (Fig. 3). (Days between 14 and 38 were not tested for suppressive extract.)

Cellular Source of Suppressive Factors. The correlation between the presence of T_s and the suppressive activity of extracts prepared from spleen and thymus cells of GAT-primed nonresponder mice suggests that the suppressive factor is a T-cell product. To test this hypothesis more directly, two approaches were taken.

The contribution of contaminating lymph node cells to the activity in thymus extracts was excluded by removing peri- and intrathymic lymph nodes before preparation of the extracts. DBA/1 mice were injected with 10 μ g GAT in Maalox, 2 days later half of the mice were injected i.p. with 0.2 ml dialyzed India ink (13). All mice were sacrificed 1 day later, their thymuses removed, the black lymph nodes removed from the thymuses of mice that received India ink, and extracts prepared and titrated as usual. The suppressive activity of extracts prepared from thymuses without lymph node cells was equivalent to the activity in thymuses prepared in the usual manner (Table II).

To identify the cellular source of the suppressive factor directly, spleen and thymus cells from GAT-primed DBA/1 mice were purified in two steps. First, adherent cells were removed by passage over plastic dishes. Second, B and T cells were prepared by passage of the nonadherent cells over columns containing rabbit anti-mouse Ig bound to Sephadex G-200 (8). Extracts prepared from unseparated cells, nonadherent cells, B cells, and T cells were titrated for suppressive activity in vitro (Table III). Specific suppressive activity was detected in all extracts except that prepared from B cells. Dilutions of the extracts were adjusted to an equivalent number of cells and the recovered activity increased in this order: unseparated lymphoid cells < nonadherent cells < T cells. Therefore, we conclude that the suppressive component in lymphoid cell extracts is a T-cell product.

TABLE II
Thymic Lymph Node Cells Are Not the Source of Suppressive Factor

Extract from mice primed with GAT	S ₅₀ units/ml*	
	SRBC	GAT-MBSA
Spleen	<200	650
Thymus‡	<200	1,200
Thymus without lymph nodes§	<200	1,150

* S₅₀ units/ml determined at day 5 as described in Fig. 1.

‡ Extract prepared from thymuses of GAT-primed DBA/1 mice.

§ Extract prepared from thymuses of GAT-primed DBA/1 mice after removal of peri- and intra-thymic lymph nodes.

TABLE III
Suppressive Factor Is a T-Cell Product

Extract prepared from lymphoid cells of GAT-primed mice*	S ₅₀ units/ml‡	
	SRBC	GAT-MBSA
Spleen + thymus	<200	540
Nonadherent cells	<200	750
T cells	430	1,200
B cells	<200	<200

* Spleen and thymus cells from GAT-primed DBA/1 mice were pooled and sequentially fractionated into nonadherent cells, T cells, and B cells. Extracts were prepared from each of these populations, the dilutions were adjusted such that the undiluted extracts were equivalent to 6×10^8 cells/ml.

‡ S₅₀ units/ml determined at day 5 as described in Fig. 1.

Suppressive Activity of DBA/1 Extract on Immune Responses by Semi-Syngeneic and Allogeneic Spleen Cells. Dilutions of an extract from GAT-primed DBA/1 mice were added to cultures containing spleen cells from DBA/1 (*H-2^a*), C57BL/6 (*H-2^b*), A.SW (*H-2^s*), (C57BL/6 × DBA/1)F₁ (*H-2^{b/a}*), or (SJL × DBA/1)F₁ (*H-2^{s/a}*) mice. The results demonstrate that the DBA/1 extract can specifically suppress responses of A.SW mice to GAT-MBSA across histocompatibility barriers (Table IV). However, restrictions were demonstrated in that suppression of responses by allogeneic and semi-syngeneic spleen cells occurred only in spleen cells that were nonresponders to GAT, i.e. (A.SW and (SJL × DBA/1)F₁).

Discussion

The immunosuppressive activity of extracts from lymphoid cells of GAT-primed nonresponder mice can be assessed *in vivo* and *in vitro* (12). Titration of several different extracts in many separate experiments has shown that the *in vitro* assay provides a reproducible, semi-quantitative measure of specific suppressive activity (Fig. 1). This assay required only small amounts of extract which was essential for the molecular characterization of the suppressive moiety described in the accompanying paper (16).

Other soluble, immunospecific factors have been recently characterized in-

TABLE IV
*Effect of DBA/1 Extract on PFC Responses by Allogeneic and Semi-Syngeneic Mice
 In Vitro*

Strain*	<i>H-2</i>	Response to GAT	S_{50} units/ml‡	
			SRBC	GAT-MBSA
DBA/1	<i>q</i>	—	<400	>1,600
C57BL/6	<i>b</i>	+	<400	400
(C57BL/6 × DBA/1) F_1	<i>b/q</i>	+	<400	<400
A.SW	<i>s</i>	—	<400	1,500
(SJL × DBA/1) F_1	<i>s/q</i>	—	<400	1,600

* Dilutions of an extract from GAT-primed DBA/1 mice were added to 10^7 spleen cells from various strains of mice at culture initiation.

‡ S_{50} units/ml determined at day 5 as described in Fig. 1.

cluding helper T-cell-replacing factors (17, 18) and suppressor factors (19, 20). These molecules share certain properties: mol wt of approximately 50,000, antigen-binding sites, the lack of Ig determinants, and the presence of determinants recognized by alloantisera specific for I-region gene products. It seemed likely that the factor identified in lymphoid cell extracts from GAT-primed nonresponder mice belonged to this new group of mediators. However, since native GAT and the suppressive factor have similar, specific suppressive activities as well as mol wt of 30,000–50,000, it was necessary to determine whether the suppressive factor in these lymphoid cell extracts was native GAT.

If all the GAT injected into the donor mice were recovered in the spleen and thymus, an unlikely possibility, the maximum concentration of GAT would be 20–30 $\mu\text{g/ml}$ of extract. Therefore, this concentration of GAT was added to a control extract and its suppressive activity determined. No significant suppression was mediated by control extract or the control extract containing GAT at dilutions that extracts from GAT-primed mice were suppressive (Table I and Fig. 2). Inasmuch as the minimum amount of GAT required to suppress the response to GAT-MBSA was previously shown to be $\geq 1.0 \mu\text{g/mouse}$ and $> 0.1 \mu\text{g/ml}$ of culture medium (7), we conclude that the concentration of native GAT in extracts from GAT-primed mice is insufficient to account for the observed suppression. Furthermore, data presented in the following paper (16) confirm that this suppressive factor belongs to a new class of antigen-specific immunoregulatory factors.

After injection of 10 μg GAT in Maalox, the presence of the suppressive factor in thymus and spleen cell extracts is correlated with the appearance of T_s . Furthermore, soluble suppressive factor can be extracted from T cells, but not B cells, of GAT-primed nonresponder mice. Thus, the active moiety in these extracts is a T-cell product. Experiments are currently in progress to confirm the hypothesis that the factor is a suppressor T-cell product.

An extract prepared from GAT-primed DBA/1 mice specifically suppresses the immune response to GAT-MBSA by normal spleen cells from DBA/1, A.SW, and (SJL × DBA/1) F_1 mice (Table IV). These data demonstrate that a GAT-specific factor from DBA/1 mice can suppress antibody responses by *H-2* histoincompatible cells, but that the suppression is restricted to strains that are nonresponders

to GAT. This contrasts to the observations of Tada and Taniguchi (21) who found that a KLH-specific factor can effectively suppress antibody responses of *H-2* compatible strains, but not those of *H-2* incompatible strains. However, we have previously reported that a factor obtained from GAT-primed A.SW mice failed to suppress the response to GAT-MBSA by nonresponder DBA/1 mice (22). These differential effects of extracts from mice of different nonresponder haplotypes may be of considerable significance; however, more studies are necessary to rule out trivial explanations such as differences in relative concentrations of suppressive factors in each extract. Experiments are currently in progress to titrate the suppressive activity in extracts prepared from mice of all three nonresponder haplotypes (*H-2^{p,q,s}*) on immune responses by spleen cells from a panel of various inbred strains of mice *in vitro*. When trivial explanations for this observation are eliminated, it will be of interest to determine whether the ability to be suppressed by a given extract is genetically controlled.

Summary

The synthetic terpolymer of L-glutamic acid⁶⁰-L-alanine³⁰-L-tyrosine¹⁰ (GAT) fails to stimulate development of GAT-specific antibody responses in nonresponder strains of mice, but does stimulate the development of GAT-specific suppressor T cells that inhibit the development of normal anti-GAT antibody responses to GAT complexed to methylated bovine serum albumin (GAT-MBSA). Furthermore, extracts prepared from lymphoid cells of GAT-primed, but not control, nonresponder mice inhibit the development of antibody responses to GAT-MBSA by normal nonresponder mice. This suppression is specific, dose-dependent, and can be readily analyzed *in vitro*. The suppressive factor is a T-cell product. An extract from GAT-primed DBA/1 mice inhibits the response to GAT-MBSA by spleen cells from histoincompatible strains of mice that are nonresponders to GAT, but not strains that are responders to GAT.

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