ANTIGEN-SPECIFIC T-CELL-MEDIATED SUPPRESSION

I. Induction of L-Glutamic Acid^{60}-L-Alanine^{30}-L-Tyrosine^{10} Specific Suppressor T Cells In Vitro Requires Both Antigen-Specific T-Cell-Suppressor Factor and Antigen*

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T lymphocytes are responsible for regulatory control of both cellular and humoral immune responses (1, 2). Studies from many laboratories have demonstrated the importance of the murine *H-2* complex, particularly the *I* region, in these activities of T cells (3). Tada and co-workers (4, 5) and Murphy et al. (6) have defined the crucial role of the *I-J* subregion in various aspects of T-cell-mediated suppression. In addition, Debré et al. (7) have demonstrated the importance of two complementing genes mapping in the *I-A* or *I-B* and *I-C* subregions in determining suppressor T-cell (T$_s$)[1] responses.

In addition, it has become apparent that soluble materials either secreted by T cells in culture (8) or obtained from T cells after mechanical disruption can mediate suppressor function. Tada and associates (9, 10) and this laboratory (11) have described the presence of soluble materials with antigen-specific suppressive activity in sonicates of suppressor T lymphocytes specific for protein antigens and for synthetic polypeptide antigens, respectively.

The soluble T cell-derived suppressor factors (T$_s$F) obtained from genetic nonresponder mice challenged with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) or L-glutamic acid^{50}-L-tyrosine^{50} (GT) have been extensively characterized (12-15). The GAT-T$_s$F has been shown to be at least partially protein in nature, to possess a molecular weight of approximately 45,000, to display binding specificity for GAT, and to possess *I*-region, but not conventional immunoglobulin determinants. In addition, the crude T$_s$F is bound by a rabbit anti-GAT immunoadsorbent, indicating the presence of a fragment of GAT in the suppressive complex. GAT-T$_s$F binds to a GAT-Sepharose adsorbent (Pharmacia Fine Chemicals, Piscataway, N. J.), and can be eluted with 2 M KCl or with pH 2.5 acid glycine buffer. This purified GAT-T$_s$F no longer binds to rabbit anti-

[1] *Abbreviations used in this paper:* C, complement; GAT, random terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-MBSA, GAT complexed to methylated bovine serum albumin; GT, random copolymer L-glutamic acid^{50}-L-tyrosine^{50}; i.p., intraperitoneally; i.v., intravenously; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; KLH, keyhole limpet hemocyanin; MD$_H$, modified Mishell-Dutton medium containing Hepes; OVA, ovalbumin; PFC, plaque-forming cell(s); S$_{50}$, inverse dilution of T$_s$F needed to give 50% suppression of GAT-MBSA responses in vitro; SRBC, sheep erythrocytes; TNP$_5$-OVA, trinitrophenylated ovalbumin; T$_s$, suppressor T lymphocyte(s); T$_s$F, T-lymphocyte-derived suppressor factor (extract).

GAT-Sepharose, demonstrating that the serologically detectable GAT fragment(s) is (are) lost during such purification. The GT-T_sF prepared from H-2^k mice can be bound by an anti-I-J^k Sepharose adsorbent, confirming for the polypeptide-specific T_sF the presence of *I-J* determinants reported by Tada et al. to be on protein-specific T_sF. Studies by Waltenbaugh et al. (16) on the mechanism of action of crude T_sF preparations demonstrated that in vivo administration of GT-T_sF or GAT-T_sF to naive animals induces antigen-specific suppressor T cells in such recipients. This finding suggested that an important function of these T cell-derived antigen-specific factors is the stimulation of a second set of specific T cells to become the effector suppressor T cells actually responsible for inhibition of immune responses. In agreement with these findings, Tada (17) reported that the specific T_sF studied in his laboratory was produced by Ly 2,3+ cells and stimulated the generation of T_s.

The earlier finding that GAT-T_sF in the crude extract was complexed to GAT or a GAT fragment raised the possibility that both GAT-T_sF and antigen might be required for efficient induction of GAT-T_s in this model. To resolve this issue and better understand the precise nature and interactions of the cells and factors involved in T-cell-mediated antigen-specific suppression, an in vitro system was designed for inducing T_s under defined conditions. The current study focuses on the production of Ts by GAT-T_sF. The data demonstrate that GAT-T_sF does indeed induce normal spleen cells to develop antigen-specific T_s in vitro, and that both H-2 determinant-bearing T_sF and a small amount of the GAT antigen itself are necessary for this T_s induction.

Materials and Methods

Mice. DBA/1 (H-2^q) mice were purchased from The Jackson Laboratory (Bar Harbor, Me.). Mice were maintained on laboratory chow and acidified-chlorinated water ad lib., and used at 10–16 wk of age. Animals were always rested for 7–14 days after receipt before inclusion in experiments, and were sex- and age-matched in any single experiment.

Antigens. GAT, ~50,000 mol wt (lot VI), was purchased from Miles Laboratories Inc. (Elkhart, Ind.). Methylated bovine serum albumin (MBSA) was obtained from Worthington Biochemical Corp. (Freehold, N. J.) and GAT-MBSA complexes, sheep erythrocytes (SRBC; GIBCO, Grand Island, N. Y.), and GAT were prepared for use as previously described (18). Ovalbumin (OVA; Miles Laboratories) was trinitrophenylated by reacting the protein, at 10 mg/ml in 0.5% sodium carbonate, with picryl chloride (Nutritional Biochemicals Corporation, Cleveland, Ohio) equal to 5% of the total protein weight for 2 h at room temperature, followed by exhaustive dialysis against phosphate-buffered saline. The preparation used in these studies contained approximately 6 trinitrophenyl groups per OVA molecule (TNP$_6$OVA).

Preparation and Immunoabsorbent Purification of Cell-Free Extracts. Cell-free extracts were prepared as previously reported (13). Briefly, DBA/1 mice were inoculated intraperitoneally (i.p.) with Maalox (William H. Rorer Inc., Fort Washington, Pa.) alone or with 10 μg GAT in Maalox. 3–7 days later, cell suspensions were prepared from thymus and spleen. The cells were sonicated at 50–60 W for 5 min at 4°C, and the lysate centrifuged at 40,000 g at 4°C for 1 h. The supernates (Maalox [control] extract or crude GAT-T_sF, respectively) were collected and stored at −80°C until use. Purified GAT-T_sF was prepared by 2 M KCl elution of the suppressive material bound to a GAT-Sepharose column followed by gel filtration to remove excess salt, as reported previously (13).

The presence of H-2 determinants in the active extracts was assessed by passage of the suppressive material over Sepharose immunoabsorbents prepared with the immunoglobulin fraction of heat-inactivated specific alloantisera, as described (13). The antisera used in these studies were obtained from Dr. Martin E. Dorf (Department of Pathology, Harvard Medical School) and consisted of anti-H-2^b serum: (DBA/1 × DBA/2)F$_1$ anti-D1.LP, and anti-K^{q}I^q serum: (DBA/2 × B10.BR)F$_1$ anti-B10.T(6R), raised by repeated inoculation with lymphoid cells, followed by pooling of high-titered individual sera.

In Vitro Assay of GAT-T$_s$F.   The direct suppressive activity of Maalox extract or of the crude and purified GAT-T$_s$F was assayed by a modification of the previously reported Mishell-Dutton culture system (18, 19). In brief, replicate 1-ml cultures of 7.5×10^6 viable DBA/1 spleen cells in Mishell-Dutton medium plus 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes; MD$_H$) were established in the 16-mm wells of a 24-sample multiwell dish (Linbro FB-16-24TC; Linbro Chemical Co., New Haven, Conn.). Cultures contained either no extract or various dilutions (1/400-1/51,200) of Maalox extract or GAT-T$_s$F, and either 1 μg GAT as GAT-MBSA or 2×10^6 SRBC. The cultures were incubated at 37°C with rocking in 7% O_2, 10% CO_2, 83% N_2, and the GAT or SRBC plaque-forming cell (PFC) responses measured 5 days later, as previously described (18). Results are expressed as IgG GAT-PFC per culture of 7.5×10^6 cells.

In Vitro Induction and in Vivo Assay of GAT-T$_s$.   Single cell suspensions of washed DBA/1 spleen cells were prepared in MD$_H$ at 20×10^6/ml. 1.5 ml of this cell suspension was mixed in 60-mm tissue culture Petri dishes (Falcon Plastics, Oxnard, Calif.) with 1.5 ml of MD$_H$ containing either no additions, various amounts of GAT, or various dilutions of Maalox extract or crude or purified GAT-T$_s$F. These cultures were incubated at 37°C with rocking in 7% O_2, 10% CO_2, 83% N_2 for 2 days, then washed four times by centrifugation and resuspended in serum-free Hanks' balanced salt solution at 6×10^6 viable cells/ml. DBA/1 mice then received 0.5 ml of the washed cell suspensions containing 3×10^6 viable cells intravenously (i.v.), followed within 1 h by immunization i.p. with the appropriate antigen. PFC per spleen were determined 7 days later with unmodified SRBC, GAT-SRBC, or TNP-horse erythrocytes (20).

Treatment with Anti-Thy 1.2.   T lymphocytes were removed from cell suspensions after T$_s$ induction and before transfer in vivo by treatment with anti-Thy 1.2 plus complement (C) as previously described (21). Briefly, 50×10^7 cultured cells were mixed with 0.2 ml of anti-Thy 1.2 (AKR anti-C3H) or normal mouse serum diluted 1:5 with Eagle's Minimum Essential Medium containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes) and 10% heat-inactivated fetal calf serum, incubated 30 min at 4°C, washed once and resuspended in selected rabbit serum diluted 1:5 as a complement source. After 45 min at 37°C, the cells were washed three times with serum-free Hanks' balanced salt solution and resuspended to a volume calculated to give 6×10^6 cells/ml before treatment. 0.5 ml of treated cell suspension was then given i.v. to each mouse, followed by immunization with antigen.

Preparation and Use of Purified T Cells.   T cells were partially purified from normal DBA/1 spleen cells by passage over nylon wool, essentially as described by Julius et al. (22). Such "T-cell" preparations contained less than 5% surface immunoglobulin positive cells, as assessed by fluorescence microscopy. These T cells were then cultured as described above for induction of T$_s$ after addition to culture dishes containing Maalox extract or GAT-T$_s$F and X-irradiated (1,500 R) splenic adherent cells from 30×10^6 spleen cells to replace the macrophages lost by nylon wool passage.

Immunization.   Mice were immunized i.p. with 0.2 ml of 10% washed SRBC in Hanks' solution, or either 10 μg of GAT as GAT-MBSA or 200 μg of TNP$_6$OVA in Maalox plus 2×10^9 pertussis organisms (Eli Lilly & Company, Indianapolis, Ind.).

Results

Induction of Suppressive Activity in Spleen Cells Cultured in GAT-T$_s$F.   Previous studies with GT-T$_s$F demonstrated that the i.v. inoculation of even greatly diluted GT-T$_s$F preparations caused long-lasting suppression of GT-MBSA PFC responses, and that this suppression could be transferred with Thy 1.2 positive cells (16). These data indicated that GT-T$_s$F was able to induce GT-specific Ts in vivo. In vivo induction with GAT-T$_s$F of specific T$_s$ has also been demonstrated. A similar ability for GAT-T$_s$F to stimulate the development of GAT-T$_s$ can be demonstrated in vitro, as shown in Fig. 1. Transfer of 3×10^6 syngeneic spleen cells incubated with 1/200 or 1/400 dilutions of crude DBA/1 GAT-T$_s$F for 2 days completely suppressed the subsequent GAT-specific PFC response to GAT-MBSA in DBA/1 mice. Cultivation of spleen cells with a 1/600 dilution of GAT-T$_s$F yielded cells with marginal suppressive activity, whereas
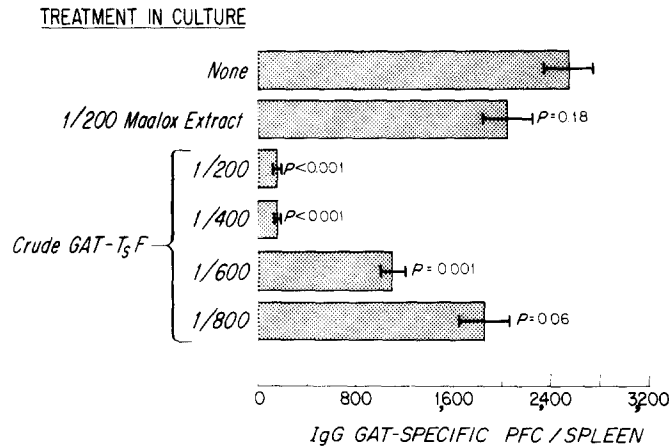
TREATMENT IN CULTURE

FIG. 1. Ability of various concentrations of DBA/1 GAT-T$_s$F to induce suppressive activity in DBA/1 spleen cells in vitro. DBA/1 spleen cells were incubated with the given final concentrations of GAT-T$_s$F or control extract (6 × 10^8 cell equivalents/ml = undiluted) as described in Materials and Methods, harvested 2 days later, and washed. 3 × 10^6 viable recovered cells were transferred i.v. to each recipient, each of which then received 10 μg GAT as GAT-MBSA in Maalox with pertussis vaccine. IgG GAT-specific PFC per spleen were determined 7 days later. P values calculated by Student's t test.

1/800 GAT-T$_s$F was ineffective at the 3 × 10^6 cell dose transferred in this experiment. The S$_{50}$ (inverse dilution of T$_s$F needed to give 50% suppression of GAT-MBSA responses in vitro) of the GAT-T$_s$F used for these experiments was found to be 1,000–1,200, and thus, only slightly greater (<twofold) than the suppressor induction activity assayed here under quite different conditions. Control (Maalox) extracts did not induce suppressor cells under similar conditions; in fact, in several experiments mice receiving cells incubated with control extracts gave slightly higher responses than mice receiving no cells (data not shown). In other experiments, the number of cells needed to obtain 50% suppression after induction with 1/200 GAT-T$_s$F ranged from 3 × 10^5 to 1 × 10^6, with 2–3 × 10^6 cells always giving >85% suppression. Hence, 3 × 10^6 cells and 1/200 GAT-T$_s$F were chosen for all subsequent experiments.

Evidence That the Active Suppressor Cell Induced by GAT-T$_s$F is a T Cell. To establish that the suppression of the GAT-MBSA response seen after transfer of spleen cells incubated with GAT-T$_s$F was indeed due to T lymphocytes, as predicted by analogy with the in vivo GT-T$_s$F studies, two approaches were used. In the first, spleen cells cultured in 1/200 GAT-T$_s$F for 2 days were treated with anti-Thy 1.2 plus C before transfer, and their residual suppressive activity measured. Fig. 2 presents data from a representative experiment of this type. Spleen cells incubated in control extract were not suppressive, whereas cells incubated in 1/200 GAT-T$_s$F caused complete suppression of the GAT-MBSA response. Transfer of cells after anti-Thy 1.2 plus C treatment did not cause a detectable suppression. In several other similar experiments, the same elimination of suppressor cell activity was seen with a different preparation of anti-Thy 1.2 serum, and no reduction in suppression seen after treatment with C alone or C plus normal mouse serum (data not shown). Table I gives
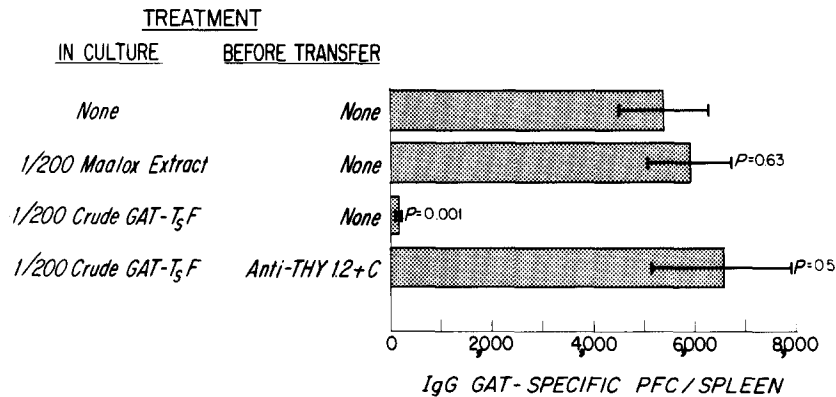
TREATMENT

IN CULTURE   BEFORE TRANSFER

FIG. 2. Sensitivity of in vitro GAT-T$_s$F-induced suppressor cells to treatment with anti-Thy 1.2 plus C. Same protocol as for Fig. 1, except that some cells were treated as described in Materials and Methods with anti-Thy 1.2 plus C before transfer.

TABLE I

Induction of GAT-Specific T$_s$ in Vitro using Purified T Cells and Crude GAT-T$_s$F

Culture*	Cells transferred‡	IgG GAT-specific PFC/spleen§	P Value‖
None	None	2,456 ± 503	—
1/200 Maalox extract and whole spleen	3 × 10⁶	2,006 ± 659	0.607
1/200 Crude GAT-T$_s$F and whole spleen	3 × 10⁶	413 ± 155	0.008
1/200 Maalox extract and purified T cells¶	2 × 10⁶	1,893 ± 192	0.336
1/200 Crude GAT-T$_s$F and purified T cells	2 × 10⁶	450 ± 137	0.008

* See Materials and Methods.
‡ Number of viable cells after culture transferred i.v. per recipient.
§ Mean IgG GAT-specific PFC per spleen ± SEM detected 7 days after cell transfer and immunization with 10 µg GAT as GAT-MBSA in Maalox with pertussis vaccine.
‖ Calculated by Student's t test. Comparison to group receiving no transferred cells.
¶ Nylon wool-passed cells with <5% B cells and irradiated splenic adherent cells.

data from a second type of experiment, establishing the T-cell dependence of the observed suppression of the GAT-MBSA response. Nylon wool column-passed cells containing <5% B cells were incubated with 1/200 GAT-T$_s$F or control extract for 2 days, then 2 × 10⁶ cells were transferred and the recipients challenged with GAT-MBSA. The purified T cells cultured with GAT-T$_s$F gave the same complete suppression of the GAT-MBSA response as seen when 3 × 10⁶ GAT-T$_s$F cultured whole spleen cells were transferred. Taken together, the anti-Thy 1.2 and nylon wool purification experiments are strong evidence that the suppression of the GAT-MBSA response seen after transfer of spleen cells incubated with GAT-T$_s$F is due to T lymphocytes.

Antigen Specificity of Suppression Mediated by GAT-T$_s$F-Induced Suppressor T Cells. Although T$_s$ cells and T$_s$F in the GAT-GT systems have always proved to be antigen specific, reports of nonspecific T suppressor cells (2, 23) make necessary documentation of the specificity of the T$_s$ induced by GAT-T$_s$F. Fig. 3 demonstrates that transfer of 3 × 10⁶ T$_s$ induced by 1/200 GAT-T$_s$F is
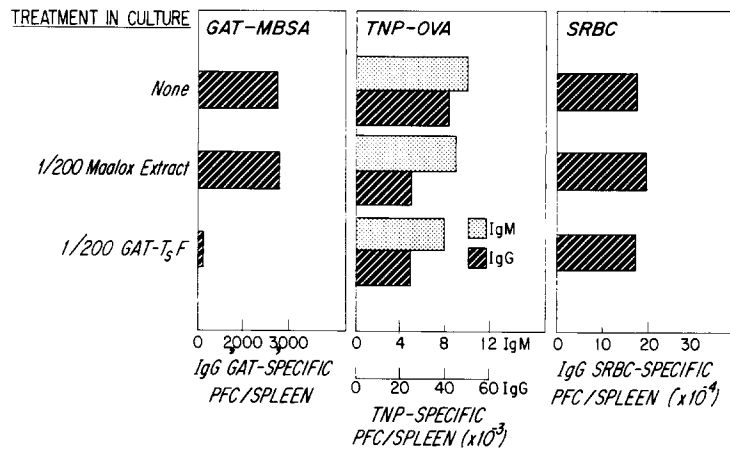
FIG. 3. Antigen specificity of in vitro GAT-T$_s$F induced suppressor T cells. Same protocol as Fig. 1, except some groups challenged with 0.2 ml 10% SRBC in Hanks' balanced salt solution or 200 μg TNP$_6$OVA in Maalox with pertussis vaccine, and assayed on SRBC or TNP-horse erythrocytes, respectively.

able to selectively suppress the IgG GAT-specific PFC response to GAT-MBSA and not the IgG SRBC or IgM TNP$_6$OVA response. Some decrease in the IgG TNP$_6$OVA response is observed upon transfer of DBA/1 cells cultured with control extract or GAT-T$_s$F, but no differences in the effects of cells incubated with these two materials were found, in contrast to their quite distinct effects on the GAT-MBSA response. Although the cause of this mild nonspecific suppression is not known at present, the data clearly reveal the GAT-specific activity of the GAT-T$_s$F-induced T$_s$ in this system.

The GAT-T$_s$F Which Stimulates the Development of T$_s$ Bears H-2 (K and/or I) Determinants. Characterization of antigen-specific T$_s$F revealed the presence of H-2, and more specifically, I-J determinants on the active material (13, 15). To ascertain whether such H-2 determinants were present on the moiety responsible for T$_s$ induction by T$_s$F, GAT-T$_s$F from DBA/1 (H-2^q) mice was passed over an anti-H-2^b or anti-K^{q}I^q Sepharose immunoadsorbent. The material that passed through the column was tested for induction of T$_s$ in vitro. The data from one such experiment are presented in Fig. 4. As can be seen, 1/200 GAT-T$_s$F induces active T$_s$, as does 1/200 GAT-T$_s$F passed over a control anti-H-2^b immunoadsorbent. However, the ability to induce T$_s$ is removed from GAT-T$_s$F by passage over an anti-K^{q}I^q column, indicating that at least one component necessary for stimulation of T$_s$ bears K^q and/or I^q determinants, and thus is identical to previously studied T$_s$F in this regard.

Role of Antigen in the Induction of T$_s$ in Vitro by GAT-T$_s$F. It was previously demonstrated (13) that the material in crude GAT-T$_s$F able to suppress GAT-MBSA responses could be removed by a rabbit anti-GAT Sepharose column, implying that GAT was complexed to the suppressive moiety. It was also shown that crude GAT-T$_s$F bound to and could be eluted from a GAT-Sepharose adsorbent. Furthermore, column-purified GAT-T$_s$F retained full suppressive activity upon direct in vitro testing in the presence of GAT-MBSA. Further studies revealed that the purified GAT-T$_s$F recovered from such a
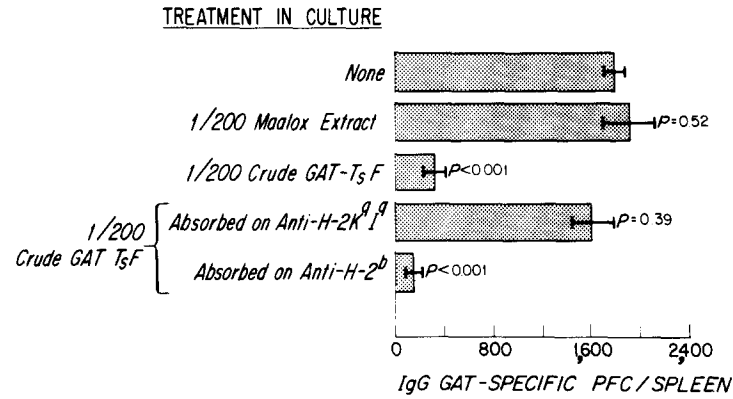
TREATMENT IN CULTURE

FIG. 4.   Presence of $H\text{-}2\text{-}K^q I^q$ determinants on active material in $GAT\text{-}T_sF$ able to induce T_s in vitro. Same protocol as Fig. 1 except portions of $GAT\text{-}T_sF$ were passed through anti-$H\text{-}2^b$ or anti-$H\text{-}2K^q I^q$ Sepharose immunoadsorbents before use in T_s induction.
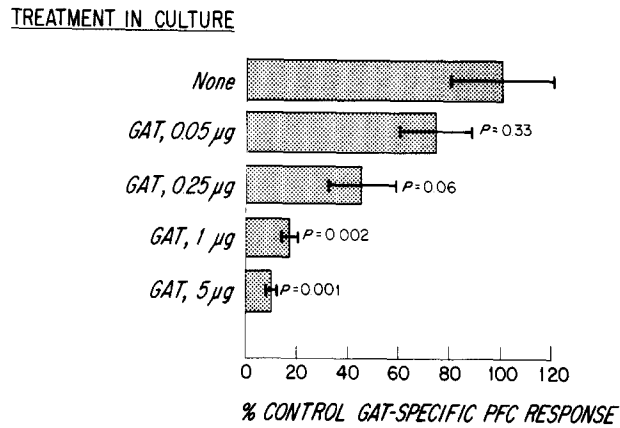
TREATMENT IN CULTURE

FIG. 5.   Induction of T_s in vitro by GAT. The given total amounts of GAT were added to 3-ml cultures of DBA/1 spleen cells containing 30×10^6 cells. 2 days later, the cells were recovered and washed, and 3×10^6 transferred per recipient. These mice were then challenged with 10 μg GAT as GAT-MBSA in Maalox with pertussis vaccine, and IgG GAT-specific PFC per spleen measured 7 days later.

GAT-Sepharose column lacked the serologically detectable piece of GAT. These findings raised the issue of whether $GAT\text{-}T_sF$ needs to be bound to GAT for the efficient stimulation of GAT-specific suppressor T cells. As a first step in answering this question, free native GAT, known to induce $GAT\text{-}T_s$ in vivo, was tested for T_s induction in vitro. Fig. 5 reveals that spleen cells incubated with 1-5 μg of GAT per culture (0.33-1.67 μg/ml) developed $GAT\text{-}T_s$ that were capable of complete suppression of the GAT-MBSA response when 3×10^6 cells were transferred per recipient. Spleen cells cultured with 50 ng GAT were not significantly suppressive under these conditions. These data are consistent with earlier work on direct suppression of DBA/1 responses in culture by free GAT, in which >0.1 μg/ml was found necessary for elimination of GAT-MBSA PFC responses (24). They also indicate that the amount of GAT in the crude extract at the dilution used was insufficient to be responsible by itself for T_s
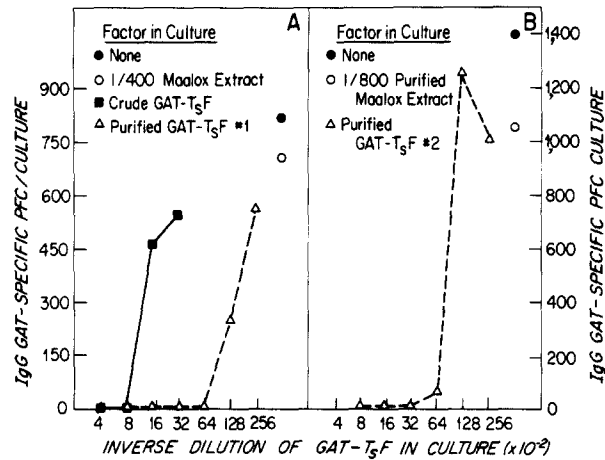
FIG. 6.   Direct in vitro suppression of the GAT-MBSA responses mediated by crude or purified GAT-T$_s$F. Cultures containing the indicated final concentrations of crude or purified GAT-T$_s$F or control extracts were established and assayed on day 5 as described in Materials and Methods. No significant effects on SRBC responses were seen, and these data are omitted for clarity. Panels A and B represent separate experiments.

induction in vitro, because 1/200 dilutions of the crude extracts would contain a maximum of 100 ng of GAT if all the antigen injected in the mice from which the GAT-T$_s$F was prepared were recovered in the extract. This is consistent as well with the requirement for an *H-2* coded product for the induction of suppressor cells.

Fig. 6 shows the results of titrations of the crude GAT-T$_s$F used in these studies and of two different preparations of purified GAT-T$_s$F directly on the in vitro PFC response of DBA/1 spleen cells to GAT-MBSA. None of the extracts suppressed the control SRBC responses at any concentration tested (data not shown). Control (Maalox) extracts had no effect or slightly suppressed GAT-MBSA responses at the highest concentration tested, and had either no effect or enhanced GAT-MBSA responses at greater dilutions. The crude GAT-T$_s$F had an S$_{50}$ of between 1,000–1,600 on repeated testing, whereas the two purified GAT-T$_s$F had S$_{50}$ of between 10,000–16,000, 10-fold higher than the crude material from which they were derived. This increase was due in part to concentration of the material during column purification (fivefold) and a repeatedly observed increase in suppressive activity of purified material of about twofold, probably due to the removal of nonspecific culture-enhancing materials which partially mask the suppressive activity of crude extracts (13).

The two purified GAT-T$_s$F preparations were then tested for induction of T$_s$ in vitro in the absence of added antigen. Fig. 7A presents data for one preparation of purified GAT-T$_s$F, and 7B for the second, with the data taken from one of three or four replicate experiments. The crude GAT-T$_s$F at 1/200 final dilution clearly induced T$_s$ capable of suppressing the GAT-MBSA response in each experiment. The purified materials were used as 1/200 dilution of the concentrated, 10-fold more active material and yet both failed to induce any significant T$_s$ activity. 10 or 50 ng of GAT alone yielded no significant
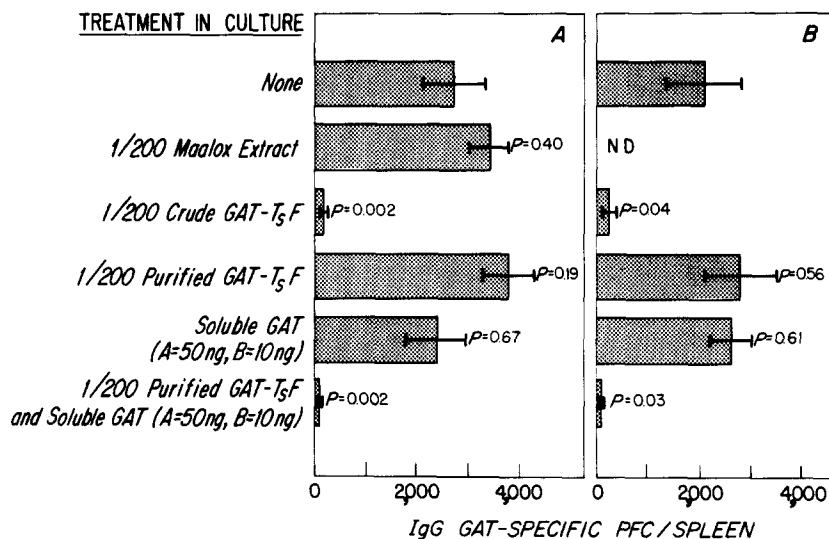
FIG. 7. Requirement for both purified GAT-T$_s$F and GAT for in vitro GAT-specific T$_s$ induction. Protocol is essentially identical to Figs. 1 and 5. Panels A and B represent separate experiments with different preparations of purified GAT-T$_s$F.

suppression, as expected. However, cells incubated with both low doses of GAT and purified GAT-T$_s$F demonstrated almost complete suppression of GAT-MBSA responses upon transfer. Thus, these data indicate that for induction of GAT-specific T$_s$ in vitro by GAT-T$_s$F, both an H-2 coded antigen-specific material and a small amount of GAT are required.

Discussion

The activity of both crude and purified soluble GAT-suppressive extracts was investigated by a combination of in vitro and in vivo techniques. In agreement with earlier in vivo studies on the mechanism of action of GT-T$_s$F and GAT-T$_s$F by Waltenbaugh et al. (16), we have shown that addition of low concentrations of GAT-T$_s$F to cultures of syngeneic spleen cells stimulates the development of GAT-T$_s$ which after transfer are able to suppress the GAT-MBSA response of recipient mice. The cells responsible for this suppression were shown to be T lymphocytes, both by using purified T cells for suppressor-cell induction in vitro and by elimination of suppressive activity of cells cultured with GAT-T$_s$F by treatment with anti-Thy 1.2 plus C before cell transfer. The suppression was demonstrated to be antigen specific with both particulate (SRBC) and soluble (TNP-OVA) control antigens. For the induction of GAT-T$_s$, a moiety bearing H-2 (K^q and/or I^a) determinants and also GAT itself, either bound to the crude GAT-T$_s$F or added in nanogram amounts to antigen-free purified T$_s$F, were both required. Purified GAT-T$_s$F, which lacks detectable GAT, was shown to be suppressive when tested directly on spleen cell cultures containing GAT-MBSA (Fig. 6), but not to be able to induce T$_s$ in the absence of added GAT (Fig. 7). This suggests that the GAT-MBSA provides the requisite GAT antigen in the assay cultures for induction by purified GAT-T$_s$F of GAT-specific T$_s$ which in turn suppress the GAT-MBSA response.

These results extend earlier observations on the ability of extracts of T-cell preparations containing suppressor T lymphocytes to interfere with immune responses. Several laboratories have reported on such T_sF. Tada et al. originally demonstrated that mice receiving two injections of soluble protein antigen, such as keyhole limpet hemocyanin (KLH) had T_s and could yield T_sF with properties of antigen specificity, size, and the presence of Ia determinants similar to those recently described for the GAT- and GT-T_sF (9, 10, 25). They did not, however, describe any antigen to be present in their active T_sF materials, in contrast with crude GAT-T_sF. The KLH T_sF described by Tada also appears to induce certain antigen-primed T cells to become mature T_s in the presence of added KLH (17). This resembles the results reported here, and careful analysis of the material necessary for this effect may reveal whether both antigen and I-coded factor are always necessary for the induction of suppressor T cells by T_sF. In this regard, it is important to determine the reason for the discrepancy between the strict I-J matching required for KLH-T_sF activity (4) and the lack of such restrictions reported for GAT- and GT-T_sF (12, 14). The possibility that the presence of antigen in the latter T_sF preparations may mask inherent I-J restrictions must be considered. Also, because KLH-T_sF is assayed with secondary responses whereas GAT-T_sF is assayed on primary responses, it is possible that the I-J restriction is a result of the primed state of the target T cells in the KLH model.

The present experiments do not establish definitively that the active material in the GAT-T_sF responsible for T_s induction has both Ia (more particularly I-J) determinants and antigen-binding activity in the same molecular complex, as was shown for GT-T_sF responsible for direct in vitro suppression (15). Purified GT-T_sF eluted sequentially from GT-Sepharose and anti-I-J Sepharose is now being tested for T_s induction to answer this precise question and also to test for the requirement for additional GT antigen, to confirm the findings for purified GAT-T_sF reported here. The ability of GAT-T_sF and GT-T_sF to act across H-2 barriers in T_s induction is being examined with preliminary data suggesting no absolute requirements for either immune response gene or I-J subregion matching (R. N. Germain, unpublished observations).

Several other soluble T-cell-derived suppressor substances have been described (26–29). One of these is a suppressive supernate from T_s-containing spleen cells in the allotype suppression model studied by Herzenberg et al. (30). This factor is not antigen-specific, but seems to directly inactivate allotype-specific helper T cells without the induction of allotype-specific T_s. It is thus clearly distinct in effect from the KLH- or GAT-T_sF. Whether a similar material active directly on helper T cells is present in extracts from antigen-, as opposed to allotype-suppressed mice is not yet known, but might be postulated to be the product of the T_s induced by the GAT-T_sF studied here.

The ability of GAT-T_sF to induce GAT-specific T_s raises several other questions concerning the pathways of cellular and soluble factor activity responsible for T lymphocyte-dependent suppression. The studies of Debré et al. (31) have shown that mice which normally produce T_s in response to GT fail to do so after cyclophosphamide pretreatment. However, such mice and A/J mice (which have a genetic inability to be suppressed by GT) can be suppressed by GT-T_sF (14, 31). Taken together with the data in this paper, it would seem

that at least two distinct T cells are involved in immune suppression, one of which produces T_sF in response to antigen, and the second of which is activated by this T_sF plus antigen to become the effector T_s. The experiments of Feldman and co-workers on T1-T2 cell interaction in suppression (32), and Ly and Ia phenotyping of T_s (33), support this concept. In addition, this two-cell model of producer and acceptor cells superficially resembles that described by Munro and Taussig (34) for factor production by T helper cells and factor acceptance by B cells. The existence of distinct *Ir* gene defects at these two sites has been documented in the branched copolymer models and suggests that similar types of defects may exist in mice that fail to produce suppression after GAT or GT immunization. A possible example of a GT factor-deficient mouse able to yield T_s when given active $GT-T_sF$ is the A/J mouse, and studies are currently underway to detect the reciprocal defect, in the hope that such mice will help map the cellular loci of action of the complementing *Is* genes in the *I-A* or *I-B* and *I-C* subregions responsible for GT suppression.

It should be noted, however, that acceptors for the *I* region portion only of these various factors is not a complete explanation for producer-acceptor cell interactions because the current work reveals a need for antigen as well in T_s induction. The role of this antigen may be to aid in focusing the T_sF on the appropriate antigen-specific T_s precursor in high concentrations. The *I* region acceptor could then recognize the T_sF and trigger the cell. This would be analogous to the model for specific B-cell activation by low concentrations of haptenated polyclonal B-cell activators proposed by Coutinho and Möller (35). Conversely, a two-signal model can be proposed in which both antigen receptor binding and factor acceptor binding of their respective ligands is necessary for T_s triggering. The potential roles of T_sF acceptors and antigen receptors in genetic restriction phenomena and T_s triggering can now be explored by sequential pulsing of lymphocytes with antigen and T_sF, using the in vitro model detailed here.

Summary

A combination of in vitro and in vivo techniques were used to explore the mode of action of both crude and purified suppressive extracts specific for the random copolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} ($GAT-T_sF$) obtained from nonresponder DBA/1 (H-2^q) mice. Normal DBA/1 spleen cells were incubated under modified Mishell-Dutton culture conditions for 2 days together with crude or purified $GAT-T_sF$, and in the presence or absence of free GAT. These cells were then washed extensively and 3×10^6 viable cells transferred to syngeneic recipients, which were challenged at the same time with the immunogenic form of GAT complexed to methylated bovine serum albumin (GAT-MBSA). GAT-specific IgG plaque-forming cells (PFC) in the spleen were assayed 7 days later. In agreement with earlier in vitro studies on the action of $GAT-T_sF$, it was demonstrated that under these conditions, low concentrations of $GAT-T_sF$ stimulated the development of cells which, after transfer, are able to suppress the GAT PFC response to GAT-MBSA. The cells responsible for this suppression were shown to be T lymphocytes by using nylon wool-purified T cells for suppressor cell induction and by eliminating suppressive activity in cells cultured with crude $GAT-T_sF$ by treatment with anti-Thy 1.2 plus C before

transfer. The suppressor T cells act in a specific manner failing to suppress significantly either anti-sheep erythrocyte or trinitrophenyl-ovalbumin primary PFC responses. For the induction of GAT-specific suppressor T cells in culture, a moiety bearing H-2(K^q or I^q) determinants and also GAT, either bound to the crude GAT-T_sF or added in nanogram amounts to antigen (GAT)-free purified GAT-T_sF, were both required.

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