Brief Definitive Report

SUPPRESSION OF ANTIBODY AND T CELL PROLIFERATIVE RESPONSES TO L-GLUTAMIC ACID^{60}-L-ALANINE^{30}-L-TYROSINE^{10} BY A SPECIFIC MONOCLONAL T CELL FACTOR*

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The immune response to the synthetic terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) is controlled by an H-2-linked immune response gene. In mice bearing the H-2$^{a, b, d, f, k}$ haplotypes, GAT stimulates antibody formation and primes for subsequent T cell proliferative responses to GAT in vitro (1, 2). GAT does not stimulate antibody production or prime for T cell-proliferative responses in mice bearing nonresponder, H-2^{p,q,s}, haplotypes. Nevertheless, nonresponder mice can develop GAT-specific antibody and T cell proliferative responses if immunized with GAT complexed to methylated bovine serum albumin (MBSA) (GAT-MBSA) (1, 2). To date, our studies suggest that the lack of responses to GAT in nonresponder mice is a result of the preferential development of GAT-specific suppressor T cells (3).

The mechanism(s) by which GAT-specific suppressor T cells regulate immunity have been investigated by analysis of T cell extracts from GAT-primed nonresponder mice (4–7). These extracts contain a GAT-specific soluble T cell suppressor factor(s) (GAT-TsF) that mimics the effects of suppressor T cells by inhibiting development of GAT-specific plaque-forming cell (PFC) responses stimulated by GAT-MBSA in nonresponder mice in vivo and in vitro. Recent experiments demonstrated that extracts that contain GAT-TsF also inhibit GAT-specific proliferative responses by T cells from GAT-MBSA-primed, nonresponder mice. The materials in the extracts that inhibit PFC and proliferative responses appear to copurify (8). These data suggest that a single mediator, or closely related set of mediators, can inhibit both GAT-specific responses.

Experiments described in this communication characterize a monoclonal, GAT-specific suppressor factor produced by a hybrid T cell line derived by fusing the HAT-sensitive AKR thymoma, BW5147, with splenic T cells from GAT-primed nonresponder DBA/1 (H-2^{q}) mice. This hybrid T cell line produces a factor that is representative of the specific suppressive material extracted from nonresponder T

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cells. More importantly, both GAT-specific PFC and proliferative responses are inhibited by this monoclonal T cell product.

Materials and Methods

Mice. DBA/1 (H-2^q) mice were bred in the animal facilities at The Jewish Hospital (St. Louis, Mo.) and were used when they were 3–6 mo old. DBA/1 mice are nonresponders to GAT.

Antigens and Immunization. GAT was purchased from Vega-Fox Biochemicals Div., Newbery Energy Corp., Tuscon, Ariz.; MBSA was purchased from Sigma Chemical Co., St. Louis, Mo. The insoluble complex, GAT-MBSA, was prepared as previously described (1). DBA/1 mice were injected in the hind footpads with 5–20 μg of GAT as GAT-MBSA emulsified in complete Freund's adjuvant that contained *Miliary tuberculosis* H37Ra.

Production of T Cell Hybrid Cell Lines. DBA/1 mice were injected i.p. with 10 μg of GAT mixed with Maalox (Wm. H. Rorer, Inc., Fort Washington, Pa.). 4 d later the mice received an injection of 2.5 mg of cortisone acetate i.p. (Merck Sharp & Dohme, Div. of Merck & Co., Inc., West Point, Pa.), and 3 d later they were sacrificed. Cortisone was injected 4 d after injection of GAT because we have found that the concentration of GAT-specific suppressor T cells is three- to fourfold greater in spleens from these mice compared with spleens from mice injected with GAT alone (J. A. Kapp. Unpublished observation.). Splenic suppressor T cells were further enriched by filtration through nylon-wool columns (9). The nonadherent cells were fused with the hypoxanthine-guanine phosphoribosyl transferase-deficient AKR thymoma, BW5147, with polyethylene glycol (Carbowax 1,500; Union Carbide Corp., Chemicals & Plastics, New York) according to the technique of Galfre et al. (10). Cells were cultured in HAT-selection media that contained 10% gamma globulin-free horse serum and 5% calf serum. After 3–6 wk, hybrid cell growth was detectable, and supernatant fluids from confluent cultures were assayed for suppressor activity (see below). Cells from primary wells that contained GAT-specific suppressive activity were cloned by limiting dilution in soft agar over 3T3 feeder layers. Isolated colonies were picked, replated in liquid medium, and supernatant fluids were again tested for suppressive activity. Selected clones have been maintained in tissue culture, and samples of each cell line have been frozen in liquid nitrogen. The GAT-specific suppressor factor to be described in this communication was produced by clone C4≠4, a clone that has grown continuously for 6 mo without loss of suppressor activity. This clone also has been reestablished from frozen stocks without difficulty or loss of suppressor activity, and, upon recloning, all of the sublines produced suppressor factor.

Antisera. Alloantisera specific for the I^q subregion was prepared by injecting (AQR × A)F_1 mice with lymphocytes from B10.T (6R) mice. Anti-I-J^s was prepared by immunizing [A.TL × B10.S(9R)]F_1 mice with B10.HTT lymphocytes. These sera were prepared by Dr. Donald Shreffler, Washington University School of Medicine (St. Louis, Mo.). Preparation and characterization of rabbit anti-mouse Ig serum has been described previously (6).

Preparation and Use of Immunoadsorbents. GAT was coupled to aminohexyl Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) and bovine serum albumin (BSA), and antibodies were coupled to CNBr-Sepharose 4B as previously described (6). Undiluted culture supernates were applied to antigen-coupled Sepharose 4B columns and incubated at 4°C for 1 h. The unbound material was collected, the columns were washed extensively, and bound material was eluted with a discontinuous gradient of 0.5–2.0 M KCl (6). Material eluted from GAT-Sepharose will be referred to as purified factor. The purified factor was diluted 1:100 in phosphate-buffered saline that contained 1 mg/ml BSA and applied to columns that contained antibody-coupled Sepharose 4B, incubated 1 h at 4°C, and the unbound material was collected.

Estimate Molecular Weight. Supernate from C4≠4 was affinity purified and concentrated by filtration with a YM-10 filter (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). The concentrated factor was filtered through a Sephacryl G150 column (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) that had been calibrated with BSA, ovalbumin, and cytochrome *c*.

Cell Culture and Assay of Hybrid T Cell Supernates. Development of primary splenic PFC responses was measured in vitro under modified Mishell-Dutton conditions previously described (1). PFC responses in cultures stimulated with GAT-MBSA or sheep erythrocytes (SRBC) were

assayed on day 5 with GAT-SRBC or SRBC as indicator cells (1). T cell proliferative responses to GAT-MBSA by lymph node cells from DBA/1 mice immunized with 5–20 μg of GAT, as GAT-MBSA were measured as previously described (2). No differences have been detected in proliferative responses or suppressive effects of hybrid T cell supernates between cultures that contained unfractionated lymph node cells and nylon wool-enriched lymph node T cells (8); thus, these cells have been used interchangeably in this report. Incorporation of [^{3}H]thymidine ([^{3}H]TdR) was measured on day 5.

Supernatant fluids from hybrid T cell cultures were diluted and added to PFC or proliferation cultures at initiation. In each experiment, the relative suppressive activity of C4#4 was determined by titration, and the specificity was verified by addition to cultures stimulated with an irrelevant antigen. Some of the data have been expressed as the inverse of the final dilution of supernatant fluid that causes 50% suppression of the response (S_{50} U/ml), Each of the experiments reported in this manuscript has been performed several times, and the results were highly reproducible. Therefore, the data presented are from representative experiments.

Results and Discussion

Suppression of GAT-specific PFC and Proliferative Responses. Fusion of T cells from GAT-primed DBA/1 mice and the AKR thymoma resulted in hybrid cells that grew in HAT-selection medium from which three clones that produced specific suppressive factor were derived. Supernates from other clones and the thymoma did not inhibit in vitro responses when assayed at dilutions ≥1:500, although dilutions ≤1:50 frequently augmented cell growth in culture. Soluble suppressor activity of clone C4#4 has been characterized and is reported here. Supernatant fluids from clone C4#4 inhibited GAT-specific PFC responses to GAT-MBSA (Fig. 1 A) and GAT-specific proliferative responses to GAT (Fig. 1 B) in cultures of DBA/1 lymphoid cells. 50% inhibition of PFC and proliferative responses was obtained with supernatant fluids diluted 1:10,000 to 1:20,000. This supernate was a rich source of suppressor factor compared with extracts of lymphoid cells from a single mouse that usually caused 50% inhibition at a dilution ≃1:500 (4). The suppressive activity was specifically removed from this supernate by filtration through GAT-Sepharose columns (Fig. 1); none of the activity was absorbed by BSA-Sepharose (not shown). The factor absorbed to GAT-Sepharose was eluted with 2.0 M KCl, and the recovered material was enriched in suppressive activity (Fig. 1). Enrichment of suppressive activity has been previously observed during affinity purification of extracts (6). The most likely explanation for this observation is that both extracts and culture supernates contain undefined, nonspecific enhancing materials that compete with GAT-specific suppressor factors, thereby causing an underestimate of the original suppressive activity.

Characterization of Monoclonal GAT-TsF. To further characterize this hybrid T cell product, affinity-purified material was subsequently filtered through insolubilized rabbit anti-mouse Ig or murine alloantisera specific for the I^q or I-J^s subregion. The PFC responses to GAT-MBSA and proliferative responses to GAT were specifically inhibited by the purified factor, and the suppressive moiety contained determinants encoded by the I^q subregion but no determinants encoded by Ig-constant region genes (Table I). This factor was not bound by antibody directed against the I-J^s haplotype; this antibody previously has been shown to bind GAT-TsF extracted from GAT-primed B10.S lymphoid cells (8). Although the I-J subregion of the H-2^q haplotype has not been defined by recombination, we assume that the determinants bound by the polyvalent anti-I^q sera are encoded by the I-J subregion because GAT-TsF from

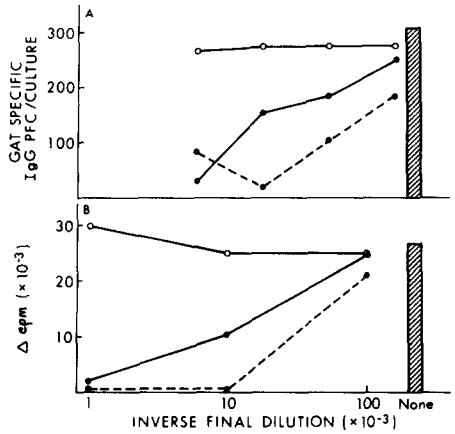

FIG. 1. Inhibition of GAT-specific responses by culture supernate from clone C4#4. Suppression of the PFC response was determined by addition of supernate to cultures that contained normal DBA/1 spleen cells that were stimulated with GAT-MBSA (A). Suppression of the proliferative response was determined by addition of supernate to cultures that contained GAT-MBSA-primed DBA/1 lymph node cells stimulated with GAT (B). The responses of culture without supernates are shown by the vertical bars; responses in the presence of untreated supernate (●——●), supernate filtered through GAT-Sepharose (○——○), and affinity-purified supernate (●- - -●).

TABLE I

Inhibition by Monoclonal GAT-TsF

C4#4 1:5,000	Responses			
	IgG PFC/culture*		[^{3}H]TdR Δcpm‡	
	GAT-MBSA	SRBC	GAT	MBSA
None	505	600	26,431	89,931
Purified§	151	540	1,585	78,174
Purified → RAMIG ‖	49	495	3,856	96,443
Purified → α-I^q	585	800	21,748	81,195
Purified → α-I-J^s	125	650	1,097	75,640

* PFC responses by spleen cells from normal DBA/1 mice.
‡ Proliferative responses by lymph node cells from GAT-MBSA-primed DBA/1 mice.
§ Affinity-purified culture supernate.
‖ Rabbit anti-mouse Ig.

H-2^s mice and GT-TsF from H-2^k mice (11) bear determinants encoded by the I-J subregion.

GAT-TsF from sonicated cells has an estimated 45,000–60,000 mol wt determined by Sephadex chromatography (6). Chromatography of affinity-purified supernate from C4#4 demonstrated that all of the suppressive activity was contained in a pooled fraction containing 20,000- to 60,000-mol wt proteins (Table II). Although this was a crude fractionation, the results demonstrated that GAT-TsF extracted from lymphoid cells and GAT-TsF isolated from supernatant fluids from hybrid T cells are approximately the same size. This contrasts with the report from Taussig and Holliman (12) that demonstrated that a T cell hybrid suppressor factor specific for SRBC had a ≥100,000 mol wt.

From these experiments, we conclude that this cloned T cell hybrid line produces

TABLE II

Molecular Size of Monoclonal GAT-TsF

C4#4	S_{50} U/ml*
Purified‡	1,000,000
Purified → G150	
Pooled fractions	
80,000–200,000	<1,000
60,000–80,000	<1,000
20,000–60,000	1,300,000
10,000–20,000	<1,000

* Specific suppressive activity determined by inhibition of PFC responses to GAT-MBSA in vitro.

‡ Affinity-purified, concentrated supernate, 1.0 ml of which was applied to a Sephacryl G150 column.

a constitutive, GAT-specific suppressor factor that functionally and serologically resembles GAT-TsF isolated from extracts of GAT-primed nonresponder T cells. Both GAT-specific PFC responses and GAT-specific T cell proliferative responses are inhibited by this factor. Because this factor was produced in vitro by a cloned hybrid T cell line, these data provide evidence that a single mediator, GAT-TsF, is capable of inhibiting at least two different GAT-specific responses. We consider the possibility that individual hybrid cells produce two functionally distinct, GAT-specific suppressor factors unlikely, but proof of a single mediator must await biochemical analysis. Whether the target cell of GAT-TsF in these two assays is the same cell or whether GAT-TsF can inhibit multiple T cell subsets is currently under investigation.

Several investigators have produced hybrid T cell lines that synthesize antigen-specific regulatory factors continuously (12–14), and we anticipate that such cell lines will produce enough material for characterization in the near future. We are particularly interested in analyzing T cell products with the same antigenic specificity derived from mice that bear different H-2 haplotypes and, consequently, bear serologically distinct I-J antigens. Comparison of hybrid suppressor T cell products specific for GAT and the related copolymer L-glutamic acid^{50}-L-tyrosine^{50} derived from several haplotypes is in progress.

Summary

The synthetic terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) stimulates GAT-specific suppressor T cells in nonresponder mice. Extracts from these T cells contain a GAT-specific soluble T cell suppressor factor (GAT-TsF) that inhibits development of GAT-specific plaque-forming cell (PFC) responses by spleen cells from nonresponder mice stimulated with GAT complexed to methylated bovine serum albumin (GAT-MBSA). These extracts also contain a factor that inhibits development of GAT-specific proliferative responses by GAT-MBSA-primed, nonresponder lymph node T cells. Experiments reported in this manuscript show that a hybrid T cell line, produced by fusion of the AKR thymoma, BW5147, with spleen cells that contain GAT-specific suppressor T cells, produces a constitutive GAT-specific suppressor factor that functionally and serologically resembles GAT-TsF extracted from T cells. More importantly, both GAT-specific PFC and T cell proliferative responses are inhibited by this factor.

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References

1. Kapp, J. A., C. W. Pierce, and B. Benacerraf. 1973. Genetic control of immune responses in vitro. I. Development of primary and secondary plaque-forming cell responses to the random terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) by mouse spleen cells in vitro. *J. Exp. Med.* **138:**1107.
2. Araneo, B. A., and J. A. Kapp. 1980. H-2-linked Ir gene control of T cell proliferative responses to the synthetic terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). I. Requirements for T cell activation in responder and nonresponder mice. *J. Immunol.* **124:** 1492.
3. Kapp, J. A., C. W. Pierce, S. Schlossman, and B. Benacerraf. 1974. Genetic control of immune responses in vitro. V. Stimulation of suppressor T cells in nonresponder mice by the terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). *J. Exp. Med.* **140:**648.
4. Kapp, J. A., C. W. Pierce, F. DeLa Croix, and B. Benacerraf. 1976. Immunosuppressive factor(s) extracted from lymphoid cell of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). I. Activity and antigenic specificity. *J. Immunol.* **116:**305.
5. Kapp, J. A., C. W. Pierce, and B. Benacerraf. 1977. Immunosuppressive factor(s) extracted from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). II. Cellular source and effect on responder and nonresponder mice. *J. Exp. Med.* **145:**828.
6. Theze, J., J. A. Kapp, and B. Benacerraf. 1977. 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. *J. Exp. Med.* **145:**839.
7. Kapp, J. A. 1978. Immunosuppressive factors from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}. IV. Lack of strain restrictions among allogeneic, nonresponder donors and recipients. *J. Exp. Med.* **147:**997.
8. Araneo, B. A., and J. A. Kapp. Immunosuppressive factor(s) from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). V. Inhibition of T cell proliferative responses. *J. Immunol.* In press.
9. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus derived murine lymphocytes. *Eur. J. Immunol.* **3:**645.
10. Galfre, G., S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard. 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature (Lond.).* **266:**550.
11. Germain, R. N., J. Theze, C. Waltenbaugh, M. E. Dorf, and B. Benacerraf. 1978. Antigen-specific T cell mediated suppression. II. *In vitro* induction by I-J coded L-glutamic acid^{50}-L-tyrosine^{50} (GT)-specific T cell suppressor factor (GT-TsF) of suppressor T cells (Ts_2) bearing distinct I-J determinants. *J. Immunol.* **121:**602.
12. Taussig, M. J., and A. Holliman. 1979. Production of an H-2-related suppressor factor by a hybrid T cell line. *Nature (Lond.).* **277:**305.
13. Taniguchi, M., and J. F. A. P. Miller. 1978. Specific suppressive factors produced by hybridomas derived from the fusion of enriched suppressor T cells and a T lymphoma cell line. *J. Exp. Med.* **148:**373.
14. Kontiainen, S., E. Simpson, E. Bohrer, P. C. L. Beverley, L. A. Herzenberg, W. C. Fitzpatrick, P. Vogt, A. Torano, I. F. C. McKenzie, and M. Feldman. 1978. T cell lines producing antigen-specific suppressor factor. *Nature (Lond.).* **274:**477.