IDENTIFICATION OF Igh-C-LINKED DETERMINANTS ON SUPPRESSOR T CELL HYBRIDS AND FACTORS SPECIFIC FOR L-GLUTAMIC $ACID_{60}$-L-$ALANINE_{30}$-L-$TYROSINE_{10}$ (GAT)

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B lymphocytes and certain subpopulations of T lymphocytes respond to stimulation with antigen by synthesis and secretion of unique glycoproteins capable of binding the stimulating antigen. In the case of the B cell, these glycoproteins are the classic antibody molecules, which have been well characterized in terms of structure, function, and organization, both at the protein and nucleic acid levels. The antibody nature of the B cell receptor for antigen is also well documented (1, 2). The equivalent molecule(s) that serve as T cell receptors for antigen or are secreted by T cells in response to antigen are not as well characterized. Recently, however, several laboratories have reported (3–5) initial characterization of putative T cell receptors for antigen on helper and cytotoxic T cells. Also partially characterized are those biologically active molecules synthesized and secreted by suppressor T (Ts)[1] cells, the suppressor T cell factors (TsF). Structural characterization of these secreted molecules and their relationship to receptors for antigen on Ts cells is still incomplete, and largely descriptive in nature (6–11), however, it is known (12) that Ts cells do not use the β chain of the T cell receptor used by helper and cytotoxic T cells. In spite of the inherent difficulties in studying TsF molecules, which have very high biological activity in very small quantities of material ($\sim 10^{14}$ units of suppressor activity per nanogram of protein), certain facts concerning their structural organization have emerged from a variety of systems where TsF have been derived from T cell hybridomas. The T cell–derived molecules specific for the synthetic antigen, L-glutamic $acid_{60}$-L-$alanine_{30}$-L-$tyrosine_{10}$ (GAT) are composed of either one or two polypeptide chains, depending on their cellular source (9), and are relatively hydrophobic molecules (8, 10, 11) that serologically crossreact with serum antibody against the same antigen when analyzed with antiidiotypic antibody

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[1] *Abbreviations used in this paper:* ABC, antigen-binding chain; BSA, bovine serum albumin; Con A, concanavalin A; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; GAT, random terpolymer of L-glutamic $acid_{60}$-L-$alanine_{30}$-L-$tyrosine_{10}$; Igh, Ig heavy chain locus; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PFC, plaque-forming cells; PNA, peanut agglutinin; SDS, sodium dodecyl sulfate; SRBC, sheep red blood cell; Ts, suppressor T; TsF, Ts factor.

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(13, 14). The basis of this latter relationship to B cell antibody is unclear, but does not involve the transcription of shared genes (15). What is implied by these data is that the T cell molecules, or GAT-TsF, also bear a variable region.

Recently, Owen (16) and Taniguchi et al. (17) have reported a series of serological reagents that recognize determinants linked to the immunoglobulin heavy chain locus (Igh) on chromosome 12 in the mouse on subpopulations of T cells. These T cell determinants behave in a manner suggesting a constant region, or a relatively conserved structure, and have been called IgT-C determinants.

We generated a similar set of monoclonal antibodies (mAb) for use in analysis and dissection of the structural relationships among the antigen-binding TsF of the various Ts cell subsets. Herein, we describe the initial characterization of these reagents, their reactivity with antigen-binding TsF, and we discuss some of the implications of these findings in light of what is known about Ts cell subsets and their products. We also describe results using these reagents to analyze Ts cells for the presence of antigen-binding structures in the cell membrane with potential receptor function.

Materials and Methods

Mice. BALB/c, C.B20, and C57BL/10 mice were bred in the Animal Resources Facility of the Jewish Hospital of St. Louis, maintained on water and laboratory chow ad libitum, and used at 10–20 wk of age. Mice were vaccinated at 5 wk of age with the IHD-T strain of vaccinia virus.

Antigens. GAT (45,000 mol wt) (lot 111F; Sigma Chemical Co., St. Louis, MO) was prepared for use as antigen in culture (18), and coupled to sheep red blood cells (SRBC) for use as indicator cells in the hemolytic plaque assay (19), as described. SRBC (Colorado Serum Co., Denver, CO) were used as antigen in culture and as indicator cells in the plaque assay as described (20).

Culture System and Hemolytic Plaque Assay. Single-cell suspensions of spleen in completely supplemented Eagle's minimal essential medium with 10% fetal calf serum (lot 100361; HyClone Laboratories, Logan, UT) were incubated at 6×10^6 cells in 0.5-ml volumes, in 16-mm wells of a 24-well tissue culture plate (76-033-05; Linbro Chemical Co., Hamden, CT) for 5 d at 37°C in an atmosphere of 10% CO_2, 7% O_2, 83% N_2, under modified Mishell-Dutton conditions (18, 20). Soluble GAT (2 μg/culture) or 10^7 SRBC and diluted samples of GAT-TsF were added to the spleen cells at the start of culture. GAT-specific IgG and SRBC-specific IgM antibody responses were assayed using the slide modification of the Jerne hemolytic plaque assay (18–20). Data are presented as plaque forming cells (PFC)/culture, or as percent suppression of control responses.

Generation of Hybridomas. Generation and characterization of the suppressor T cell hybridomas specific for GAT have been described in detail (7–11, 21, 22). T cell hybridomas from: (*a*) C57BL/10 mice, 372B3.5 single polypeptide chain GAT-TsF_1, and 372D6.5 two polypeptide chain GAT-TsF_2; (*b*) DBA/1, 258C4.4 single polypeptide chain GAT-TsF; (*c*) B10.S, 342B1.11 single polypeptide chain GAT-pTsF_1; (*d*) B10.Q 762B3.7 two polypeptide chain GAT-TsF_2; and (*e*) BALB/c, 371A1.5 two polypeptide chain GAT-TsF_2 were used in this study. C57BL/10 (H-2^b, Igh-1^b) are responders to GAT, whereas DBA/1 (H-2^q, Igh-1^c), B10.S (H-2^s, Igh-1^b), and B10.Q (H-2^q, Igh-1^b) are nonresponders to GAT.

Concanavalin A (Con A)-stimulated blasts were prepared by incubating single-cell suspensions of C.B20 spleen at a concentration of 1.2×10^7 cells/ml in RPMI 1640 with 10% newborn calf serum and 2 μg/ml Con A (Sigma Chemical Co.) for 24 h at 37°C in an atmosphere of 5% CO_2 in air. Cells were harvested, washed extensively with phosphate buffered saline (PBS) at pH 7.2, and adjusted to 10^7 cells/ml with PBS. 10^7 cells were injected intraperitoneally into BALB/c mice every 2 wk for 3–4 mo. Individual mice were tested for serum antibody by complement-dependent cytotoxicity (16, 17). B cell hybrid-

omas were constructed by fusing spleen cells from BALB/c mice (H-2^d, Igh-1^a), which had been hyperimmunized with C.B20 (H-2^d, Igh-1^b) Con A blasts, with the BALB/c-derived nonsecreting myeloma Sp 2/0-Ag 14. Individual wells were screened for the presence of antibody by enzyme-linked immunosorbent assay (ELISA), and positive wells were tested for the ability to be absorbed by thymocytes and splenic T cells, but not B cells from C.B20 mice. Cells from wells that were positive by these criteria were cloned in soft agar, screened for desired reactivity, and expanded and further characterized. Several clones reactive with B cells, but not T cells, in C.B20 spleen were identified and frozen for future analysis.

ELISA. Isotypes of mAb were determined using an indirect ELISA. Briefly, 96-well ELISA plates (Fisher Scientific Co., Pittsburgh, PA) were coated with rabbit anti–mouse IgM, rabbit anti–mouse IgG1, rabbit anti–mouse IgG2a, rabbit anti–mouse IgG2b, or rabbit anti–mouse IgG3 (Litton Bionetics Inc., Kensington, MD) at a concentration of 2 μg/ml, followed by blocking of remaining unreacted sites with 1% bovine serum albumin (BSA). Hybridoma supernatant fluids were then added to the plates and serially diluted in PBS containing 1% BSA and 0.01 percent NaN_3. Alkaline phosphatase–coupled goat anti–mouse Ig was added, followed by the substrate (*p*-nitrophenyl)phosphate disodium (Sigma Chemical Co.). Absorption at 405 nm was determined using a Dynatech ELISA Reader (Dynatech Laboratories, Inc., Alexandria, VA). Concentration of antibody in culture supernatant fluids was determined by comparison with an appropriately diluted samples of mouse Ig of known concentration.

Preparation of Cell Populations. B cells and T cells were obtained as cells adherent or nonadherent, respectively, to goat anti–mouse Ig plates, as described by Wysocki and Sato (23). Thymocytes and hepatocytes were obtained following dissociation of thymus and liver with ice-cold Hank's balanced salt solution. Cortisone-resistant thymocytes were those cells remaining in the thymus 2–8 d after intraperitoneal treatment with 2.5 mg hydrocortisone 21-acetate per mouse. Lipopolysaccharide (LPS)-stimulated blasts were prepared by incubating suspensions of spleen cells at a concentration of 1.2×10^7 cells/ml in RPMI 1640 with 10% newborn calf serum (lot 29121077; M. A. Bioproducts, Walkersville, MD) and 50 μg/ml LPS (Sigma Chemical Co.) for 3 d at 37°C in an atmosphere of 5% CO_2 in air.

Cell Absorptions. mAb were absorbed by incubating 100 μl of hybridoma supernatant fluids with 10^7 cells from the appropriate source in V-bottom, 96-well microtiter plates (76-223-05; Linbro Chemical Co.) for 60 min at 4°C. Following centrifugation at 1,000 rpm for 10 min, the supernatant fluids were removed and tested for the presence of antibody by ELISA.

Preparation of Cell Membranes. Cell pellets from hybridomas 372B3.5 and 372D6.5 were washed three times with PBS, resuspended in 5 ml PBS, to which phenylmethylsulfonyl fluoride and aprotinin (Sigma Chemical Co.) were added to a final concentration of 100 μg/ml each. Cells were disrupted by 15 strokes in a Dounce homogenizer, or until visual examination showed that >90% of the cells were disrupted. Nuclei and remaining intact cells were removed in the pellet after centrifugation at 400 *g* for 10 min; the remaining sample, containing membranes, was applied to a sucrose gradient, as described by Kinzel and Mueler (24). The fraction containing membranes was collected, washed, and the proteins were extracted on ice for 90 min in PBS containing 0.5% Nonidet P-40 (NP-40). Debris was removed by centrifugation at 100,000 *g*, and the supernatant fluid, which contained the extracted proteins, was used in subsequent analyses as the soluble membrane preparation.

Immunoabsorbents. BALB/c anti C.B20 T cell mAb were coupled to CNBr-Sepharose 4 B (Sigma Chemical Co.) according to the manufacturer's instructions. Peanut agglutinin (PNA)-agarose was purchased from E-Y Laboratories, San Mateo, CA. Samples (GAT-TsF or soluble membrane preparations) were applied to the immunoabsorbents, and eluted with 500 mM D-galactose (Sigma Chemical Co.) in the case of PNA-agarose, or 2 M KCl for antibody coupled to Sepharose, and either tested for biological activity, or analyzed by gel electrophoresis. GAT-TsF_2 were separated into isolated chains, as previously described (9), by reaction with 5 mM dithiothreitol (DTT) (Sigma Chemical Co.)

for 30 min. at 4°C, and then applying to the relevent immunoabsorbent that had been pre-equilibrated with 5 mM DTT; sample was eluted as before. DTT was removed by dialysis against PBS before subsequent immunoabsorption or biological analysis.

Gel Electrophoresis. Samples were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or nonreducing conditions, in 15% polyacrylamide gels (25). Isoelectric focusing was performed as described by Sidman (26), using a pH gradient of 5–8. Gels were fixed in a solution of methanol/acetic acid/water 50:10:40 (vol/vol/vol), and stained with silver by the method of Morrisey (27).

Results

Generation and Characterization of BALB/c Anti-C.B20 mAb. Hyperimmunization of BALB/c mice with Con A blasts from allotype congenic C.B20 mice resulted in low but significant titers of antibodies reactive in a complement-dependent lysis assay with thymocytes and splenic T cells from C.B20, but not BALB/c mice. Lysis of 20–30% of T cells was generally observed against backgrounds of 8–10% lysis. Spleen cells from mice with the highest titers were fused with the nonsecreting BALB/c myeloma SP 2/0-Ag14; the resulting clones were again screened for reactivity on splenic T cells and thymocytes. Those seven clones most strongly positive were expanded, and are listed in Table I. In addition to the hybridomas reactive only with T cells, several hybridomas reactive only with B cells (not shown) were also identified. Antibodies produced by these hybridomas were characterized by isotype using an indirect ELISA. The results of these assays are summarized in Table I. All antibodies were restricted to one of two subclasses; IgM (1322 A2.1, 1322 B3.2, 1322 B3.3, 1322 B1.3, 1322 B3.5) or IgG1 (1322 C5.3, 1322 D4.7). Concentrations of antibodies ranged from 16 to 125 μg/ml (data not shown).

Tissue Distribution of 1322 mAb. The reactivity of BALB/c anti-C.B20 mAb with a variety of cell types was examined. Antibody-containing hybridoma supernatant fluids were absorbed with the appropriate cells, and were then assayed for the presence of antibody by ELISA. The results of such an absorption

TABLE I
Tissue Distribution of BALB/c Anti-C.B20 mAb

mAb	Cells used for absorption							
	Spleen	T cells	B cells	Con A blasts	LPS blasts	Cortisone-resistant thymocytes	Liver	Control (unabsorbed)
1322 A2.1 (μ, k)	0.4	0.2	3.0	0.2	2.5	0.5	2.9	3.0
1322 B3.2 (μ, k)	0.75	0.8	1.6	0.5	1.5	0.6	1.6	1.6
1322 B3.5 (μ, k)	1.8	1.0	5.5	0.8	6.0	0.9	5.7	6.0
1322 B3.3 (μ, k)	2.0	0.5	2.5	0.3	2.8	1.0	3.0	3.0
1322 C5.3 (μ, k)	2.0	0.8	12.0	0.5	11.9	1.0	12.0	12.5
1322 D4.7 (μ, k)	1.0	1.2	2.5	1.5	3.0	1.5	2.9	3.0
1322 B1.3 (μ, k)	0.2	1.5	1.9	1.0	1.8	2.9	3.0	3.0

10^7 of the indicated cell type were incubated with 100 μl of the individual BALB/c anti-C.B20 mAb for 90 min at 4°C in V-bottom 96-well Linbro plates. Absorbed supernatant fluids were collected and tested for presence of antibody by ELISA. Data are presented as grams of mAb remaining after absorption by the indicated cell type. Isotypes of mAb were determined by indirect ELISA analysis, as described in Materials and Methods.

are summarized in Table I. B cells, LPS blasts, and liver failed to absorb antibody, whereas spleen, T cells, Con A blasts from spleen, and cortisone-resistant thymocytes (except 1322 B1.3) absorbed significant amounts of antibody. Taken together, we interpret these results to indicate the 1322 mAb are T cell–specific, and fail to have significant reactivity with other cell types.

Reactivity of 1322 mAb with Monoclonal GAT-TsF. Immunoabsorbents prepared from BALB/c anti–C.B20 T cell mAb were tested for their ability to absorb GAT-TsF activity using a panel of monoclonal GAT-TsF derived from T cell hybridomas from mice that differ at major histocompatibility complex (MHC) and Igh loci. Three basic conclusions can be made from the data presented in Table II: (*a*) six of the seven mAb reagents were capable of absorbing the monoclonal GAT-TsF, and all but one, mAb 1322 B3.5, absorbed at least two of the GAT-TsF tested; (*b*) the ability to be absorbed by the 1322 mAb required expression of the Igh-1^b haplotype in the GAT-TsF-producing hybridoma, but was independent of the MHC haplotype, as seen in the ability to absorb GAT-TsF from 372B3.5 (H-2^b, Igh-1^b), 372D6.5 (H-2^b, Igh-1^b), 342B1.11 (H-2^s, Igh-1^b) and 762B3.7 (H-2^q, Igh-1^b), but not 258C4.4 (H-2^q, Igh-1^c) or 371A1.5 (H-2^d, Igh-1^a); and, (3) the mAb could be divided into those capable of absorbing only GAT-TsF_1 single-chain suppressor factors (1322 A2.1, and B3.2 absorbed 372B3.5 and 342B1.11), and those capable of absorbing only GAT-TsF_2, two-chain suppressor factors (1322 B3.3 and C5.3 absorb 372D6.5 and 762B3.7). This point is further illustrated in Table III, in which the GAT-TsF_1, 372B3.5 and GAT-TsF_2, 372D6.5 were sequentially absorbed by 1322 B3.2 and 1322 C5.3. As can be seen in the data presented, GAT-TsF_1 is 1322 C5.3^-, 1322 B3.2^+, while GAT-TsF_2 is 1322 C5.3^+, 1322 B3.2^-. These data support the interpretation that the reagents recognize independent, nonoverlap-

TABLE II

Absorption of Hybridoma-derived GAT-specific Suppressor Factors by BALB/c Anti-C.B20 mAb

Immunoabsorbent*	GAT-specific PFC/culture in presence of					
	372 B.35 single-chain (H-2^b, Igh-1^b)	372 D6.5 two-chain (H-2^b, Igh-1^b)	258 C4.4 single-chain (H-2_q, Igh-1^c)	342 B1.11 single-chain (H-2^s, Igh-1^b)	371 A1.5 two-chain (H-2^d, Igh-1^a)	762 B3.7 two-chain (H-2^q, Igh-1^b)
None	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)
1322 A2.1	278 (117)	43 (18)	<10 (0)	320 (134)	<10 (0)	24 (10)
1322 B3.2	253 (106)	13 (5)	18 (8)	345 (145)	18 (8)	<10 (0)
1322 B3.5	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	243 (102)
1322 B3.3	45 (19)	155 (65)	<10 (0)	<10 (0)	<10 (0)	219 (92)
1322 C5.3	20 (8)	215 (90)	<10 (0)	<10 (0)	<10 (0)	219 (92)
1322 D4.7	25 (11)	60 (25)	18 (8)	<10 (0)	<10 (0)	ND
1322 B1.3	205 (86)	245 (103)	<10 (0)	ND	ND	ND

Hybridoma-derived GAT-TsF were absorbed with the indicated immunoabsorbent before addition to culture at a final dilution of 1:10,000. B10 spleen cells (6×10^6) were cultured with 2 μg GAT and GAT-TsF absorbed with the indicated immunoabsorbent; data are presented as IgG GAT-specific PFC/culture. Control response was 238 GAT-specific IgG PFC/culture; percent of control response to GAT is in parentheses. ND, not done.

* Culture supernatant fluids from the indicated hybridomas coupled to Sepharose 4B.

TABLE III
Sequential Absorption of Hybridoma-derived GAT-TsF BALB/c Anti-C.B20 mAb

TsF absorbed	First absorption*	Second absorption‡	GAT-specific PFC/culture
None	—	—	390
GAT-TsF_1	1322 C5.3 EFF	1322 B3.2 EFF	420
	1322 C5.3 EFF	1322 B3.2 EL	<10
	1322 C5.3 EL	1322 B3.2 EFF	310
	1322 C5.3 EL	1322 B3.2 EL	330
	1322 C5.3 EL	1322 C5.3 EFF	340
	1322 B3.2 EFF	1322 C5.3 EL	410
	1322 B3.2 EL	1322 C5.3 EFF	<10
	1322 B3.2 EL	1322 C5.3 EL	418
GAT-TsF_2	1322 C5.3 EFF	1322 B3.2 EFF	405
	1322 C5.3 EFF	1322 B3.2 EL	365
	1322 C5.3 EL	1322 B3.2 EFF	<10
	1322 C5.3 EL	1322 B3.2 EL	565
	1322 B3.2 EFF	1322 C5.3 EFF	325
	1322 B3.2 EFF	1322 C5.3 EL	<10
	1322 B3.2 EL	1322 C5.3 EFF	285
	1322 B3.2 EL	1322 C5.3 EL	413

5×10^6 B10 spleen cells were cultured with 2 μg GAT and GAT-TsF (1:10,000 final dilution) absorbed with the indicated immunoabsorbents; data are presented as IgG GAT-specific PFC/culture.

* The indicated mAb coupled to CNBr-Sepharose 4 B was used to absorb GAT-TsF (372B3.5) or GAT-TsF2 (372D6.5). Column effluents (EFF) and 2.0 M KCl eluates (EL) were collected.

‡ Effluent or eluate fractions from the first immunoabsorbent were applied to the indicated second immunoabsorbent and column effluents (EFF), or 2.0 M KCl eluates (EL) were collected and added to culture at a final dilution of 1:10,000.

ping determinants restricted to either TsF_1 or TsF_2 molecules. Finally, one mAb, 1322 B1.3, absorbed both single-chain GAT-TsF_1 (372B3.5) and two-chain GAT-TsF_2 (372D6.5 and 762B3.7).

Mapping of Determinant(s) Recognized by 1322 mAb. The GAT-TsF_2 from hybridoma 372D6.5 was separated into its component antigen-binding chain (ABC^+) and I-J-bearing chain ($I\text{-}J^+$), as shown previously (9), and as described in Materials and Methods. Isolated chains were then applied to Sepharose columns coupled with the 1322 C5.3 mAb; column effluents and 2.0 M KCl eluates were assayed for their ability to complement the reciprocal unabsorbed chain and restore suppressive activity (general flow chart illustrated in Fig. 1). The results of this experiment are summarized in Table IV. As seen previously (9), the isolated antigen-binding chain (ABC^+, eluate B) and $ABC^-/I\text{-}J^+$ chain (effluent A, previously shown [9] to be the $I\text{-}J^+$ chain) are unable to suppress individually (groups 3 and 4), but are highly suppressive when combined (group 5). Isolated antigen-binding chains and $ABC^-/I\text{-}J^+$ chains were applied to a 1322 C5.3–

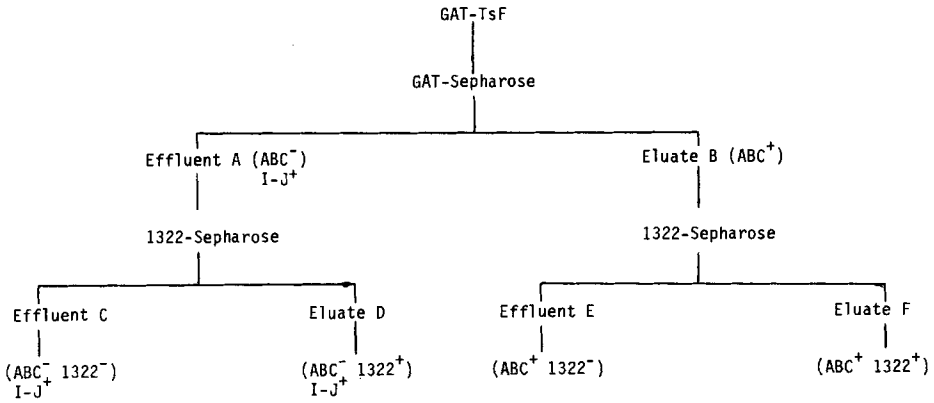

FIGURE 1. GAT-TsF was applied to GAT-Sepharose in the presence or absence of 5 mM DTT. The effluent and 2.0 M KCl eluate were collected and dialyzed. Effluent and eluate material was then applied to a second immunoabsorbent composed of 1322 mAb linked to Sepharose, in the absence of DTT. Effluents and 2.0 M KCl eluates were collected (C–F) and analyzed for biological activity alone or in various combinations.

TABLE IV

Reactivity of 1322 C5.3 with the Antigen-binding Chain of the GAT-TsF_2, 372 D6.5

Group	Immunoabsorbent fraction added*	GAT-specific PFC/culture‡
1	None	265
2	Unfractionated 372 D6.5	<10 (1)
3	$ABC^-/I\text{-}J^+$ (A)	243 (92)
4	ABC^+ (B)	288 (109)
5	$ABC^-/I\text{-}J^+$ (A) + ABC^+ (B)	<10 (1)
6	$ABC^-/I\text{-}J^+$, 1322^- (C) + ABC^-, 1322^+ (D)	253 (95)
7	ABC^+, 1322^- (E) + ABC^+, 1322^+ (F)	243 (92)
8	$ABC^-/I\text{-}J^+$, 1322^- (C) + ABC^+, 1322^- (E)	223 (84)
9	$ABC^-/I\text{-}J^+$, 1322^+ (D) + ABC^+, 1322^+ (F)	333 (126)
10	ABC^+, 1322^- (E) + ABC^+, 1322^+ (D)	228 (86)
11	$ABC^-/I\text{-}J^-$, 1322^- (C) + ABC^+, 1322^+ (F)	<10 (2)

* GAT-TsF_2 was reduced with 5 mM DTT, and filtered through GAT-Sepharose; adsorbed material was eluted with 2.0 M KCl. Effluent and eluate fractions were then filtered through 1322 C5.3-Sepharose and eluted. Samples were dialyzed to remove DTT before addition to culture. Letters in parentheses refer to the effluent and eluate fractions diagrammed in Fig. 1.

‡ Unfractionated GAT-TsF_2 and isolated chains were added to cultures of spleen cells from C57BL/10 mice at a final dilution of 1:10,000. Cultures were stimulated with 2 μg GAT; data are presented as GAT-specific PFC/culture. Percent of control response is in parentheses.

Sepharose immunoabsorbent; effluent and eluates were collected and tested in various combinations. Suppression was seen only when $ABC^-/I\text{-}J^+$ chain effluents from 1322 C5.3 were combined with eluates of antigen-binding chain from 1322 C5.3–Sepharose (group 11). These data support the conclusion that the Igh linked determinant(s) recognized by the 1322 C5.3 mAb is borne by the antigen-binding chain of GAT-TsF_2.

In a similar fashion, localization of the determinant recognized by 1322 A.21 on GAT-TsF_2 was examined. The data presented in Table V demonstrate two points: GAT-TsF_1 behaves as a single polypeptide, as previously reported (9) and demonstrated here by recovery of suppressive activity in the eluate from the GAT-Sepharose column (group 4) in the presence of DTT. Identical results are seen after absorption and elution from a anti-I-J^b mAb–Sepharose column under the same conditions (data not shown) (9). Second, absorption of the GAT-TsF_1 eluted from GAT-Sepharose (or anti-I-J–Sepharose) on 1322 A2.1–Sepharose (in the presence of DTT) quantitatively removes suppressor activity that is recovered in the 2.0 M KCl eluate, again supporting the conclusion that GAT TsF_1 is composed of a single polypeptide chain.

Cell Membrane Analysis. Cell membranes were prepared from 372B3.5 and 372D6.5 by sucrose density gradient fractionation, and membrane proteins were extracted with PBS, pH 7.2, containing 0.5% NP-40, followed by centrifugation at 100,000 *g* for 30 min to remove cell debris. The extracted membrane proteins were applied to 1322 A2.1–Sepharose in the case of the GAT-TsF_1-producing hybridoma 372B3.5, or to 1322 C5.3–Sepharose for the GAT-TsF_2-producing hybridoma 372D6.5. After incubation for 1 h at 4°C, samples were eluted with 15% glycerol containing 6% SDS, and were applied to a 12.5% acrylamide gel. When indicated, samples were first reduced with 2-mercaptoethanol before applying to the gel. Individual lanes were divided into 3-mm slices that were eluted with PBS; eluted material was tested for direct suppression of GAT-specific PFC responses when material isolated from 372B3.5 was analyzed, or for the ability to complement isolated I-J^+ chains and reconstitute suppression when membrane isolates from 372D6.5 were analyzed. Results presented in Fig.

TABLE V

Subunit Structure of GAT-TsF_1 Defined by 1322 A2.1

Group	Immunoabsorbent fraction added*	GAT-specific PFC/culture‡
1	None	265
2	Unfractionated 372 B3.5	<10 (1)
3	ABC^- (A)	268 (101)
4	ABC^+ (B)	8 (3)
5	ABC^- (A) + ABC^+ (B)	<10 (1)
6	ABC^-, 1322^- (C)	245 (92)
7	ABC^-, 1322^+ (D)	318 (120)
8	ABC^+, 1322^- (E)	333 (126)
9	ABC^+, 1322^+ (F)	20 (8)

* GAT-TsF_1 was equilibrated with 5 mM DTT and applied to a GAT-Sepharose column; adsorbed material was eluted with 2.0 M KCl. Effluent and eluate fractions were then applied to a 1322 A2.1-Sepharose column; effluent and eluate fractions were collected. Samples were dialyzed to remove DTT before addition to culture. Letters in parentheses refer to individual effluent and eluate fractions according to the flow chart diagrammed in Fig. 1.

‡ Unfractionated or fractionated GAT-TsF_1 was added to cultures of C57BL/10 spleen at a final dilution of 1:10,000, and cultures were stimulated with 2 μg GAT. Data are presented as GAT-specific PFC/culture; percent of control response is in parentheses.

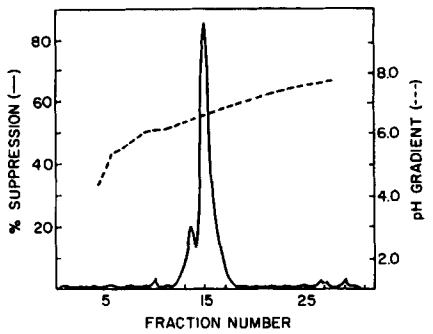

FIGURE 2. Solubilized membrane proteins from 372D6.5 (*A* and *C*) and 372B3.5 (*B* and *D*) were applied to 1322 C5.3–Sepharose or 1322 A2.1–Sepharose, respectively, eluted with sample buffer, applied to a 12.5% SDS–polyacrylamide gel, and electrophoresed under nonreducing (*A* and *B*) or reducing (*C* and *D*) conditions. Lanes containing sample were divided into 3-mm slices, eluted with PBS, and individual fractions were tested for biological activity. Material isolated from 372B3.5 was tested for direct suppression of a GAT-specific PFC response, whereas material isolated from 372D6.5 was assayed by the ability to complement independently isolated I-J$^+$ chains, and restore antigen-specific suppressive activity. Molecular masses (in kD) of known proteins are indicated by arrows. Data are expressed as percent suppression of control.

2, *A* and *C* show that 1322 C5.3–Sepharose absorbs a biologically active molecule from the membrane of 372D6.5 that reconstitutes suppression in the presence of an I-J$^+$ chain, and has an apparent molecular mass of 31.5–34 kilodaltons (kD) under reducing and nonreducing conditions. The discrepancy in molecular mass between reduced individual chains and nonreduced samples is most probably due to a combination of differences in folding of the reduced molecules and aberrant migration of glycoproteins in SDS–polyacrylamide gels. The molecular mass for the antigen binding chain (1322 C5.3$^+$ chain) isolated from the membrane of 372D6.5 appears to be ~10 kD larger than the equivalent chain obtained from the secreted protein (data not shown). This discrepancy could be due to differences in glycosylation, presence of lipid, or extraneous membrane material covalently attached to the membrane material, or could represent two unique molecules; one secreted, the other membrane bound. The available data do not allow us to address this point. Results with 372B3.5 membrane proteins absorbed by 1322 A2.1–Sepharose are presented in Fig. 2, *B* and *D*. Under nonreducing conditions, a biologically active molecule with an apparent molecular mass of 48–50 kD is obtained. Under reducing conditions, the same sample migrates at a lower molecular mass of ~24–25 kD.

The possibility that the suppressor molecule isolated from the membrane of 372B3.5 exists as a disulfide-linked dimer was partially addressed by eluting material from 1322 A2.1–Sepharose with 2.0 M KCl, dialyzing to remove excess salt and applying the sample, after reduction with 2-mercaptoethanol, to an isoelectric focusing gel with a pH gradient of 3–10. The sample lane was divided into 3-mm slices, eluted, and the material from individual slices was tested for direct suppression of GAT-specific PFC responses. The results illustrated in Fig. 3 show that all suppressive activity focused with a single pI of 6.6.

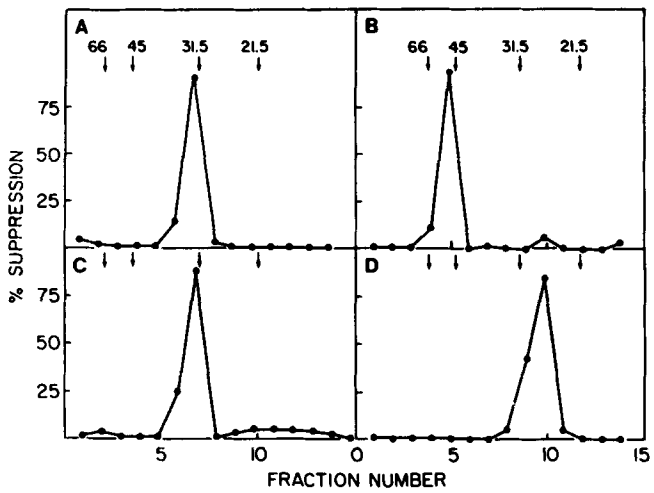

FIGURE 3. Solubilized membrane proteins from 372B3.5 were applied to 1322 A2.1–Sepharose, eluted with 2.0 M KCl, dialysed, and reduced with 2-mercaptoethanol before applying to an isoelectric focusing gel with a pH gradient of 3–10. The lane containing sample was divided into 3-mm slices, eluted with PBS, and the material was tested for antigen-specific suppression of a GAT-PFC response. Data are presented as percent of control.

Discussion

Fusion of spleen cells from BALB/c mice hyperimmunized with Con A blasts from allotype congenic C.B20 mice yielded a series of hybridomas secreting mAb reactive in an allotype-restricted manner with T cells and not B cells. Further analysis showed the determinants to be expressed on cortisone-resistant thymocytes, and on Con A blasts, but not on LPS blasts or hepatocytes. In other experiments (C. W. Pierce, C. M. Sorensen, and J. A. Kapp, manuscript in preparation) these reagents were shown, individually, to react with functionally defined subpopulations of either Lyt-1$^+$ or Lyt-2$^+$ regulatory T cells, but not with both. Using a panel of Igh-V- and Igh-C-region recombinant mice, we have begun mapping the positions of the genes on chromosome 12 that are involved in expression of these determinants. Initial results with our reagents suggest that the genetics are more complicated than those originally reported (28, 29), and will be the subject of another report. Immunoabsorbents made from these hybridomas absorbed monoclonal suppressor factors in an allotype-restricted manner, independent of MHC haplotype. These same immunoabsorbents were used to isolate proteins from the membranes of suppressor T cell hybridomas that were capable of suppressing in vitro antibody responses in an antigen-specific manner.

Previous reports (17, 27, 28) have described antibodies raised in a similar fashion that are reactive exclusively with T cells in an allotype-restricted manner. In these studies (27, 28), several reagents raised against the Igh-1^c allotype were identified and shown to react with determinants on certain mature functional subsets of T cells, or were reactive with T cells and thymocytes in a manner reminiscent of differentiation antigens (reviewed in 16). These determinants have been termed IgT-C determinants, and have been suggested as possible

constant region determinants on the T cell receptor for antigen, based on these data and on proximity of these apparent structural genes encoding a T cell–restricted antigenic determinant to the Ig constant region gene complex. Hedrick et al (5) have reported the cloning of a gene that appears to encode the β chain of the T cell receptor for antigen on cytotoxic T cells and helper T cells. The gene shows rearrangement in the appropriate functional T cell clones, as would be predicted for a functional T cell receptor. Chromosomal mapping has placed the gene on chromosome 6, in linkage with the Ig light chain locus (29). These findings do not preclude our results, which suggest the involvement of genes linked to chromosome 12 in coding for or controlling the expression of the antigen receptor on suppressor T cells. In fact, a cDNA probe generated from the receptor gene isolated by Hedrick and coworkers (12) fails to hybridize and show rearrangement with DNA from any functional suppressor hybridomas examined to date, including several Ts cell hybridomas used in this report. This result, along with results of experiments showing the failure of anti–suppressor factor mAb to react with T helper cells (C. W. Pierce, C. M. Sorensen, J. A. Kapp, manuscript in preparation), suggests that the receptor structure on suppressor T cells may be distinct from that found on helper cells and cytotoxic cells, and in fact, may use entirely different genes. This is also suggested by the failure of cloned, functional influenza-specific cytotoxic T cells to absorb significant amounts of antibody, implying the absence of the relevent determinants of this T cell subpopulation (our unpublished observation).

In this report, we have described a set of mAb, similar to those described previously (17, 27, 28), raised across an Igh-1^b allotype barrier, that have been used to further characterize soluble factors derived from suppressor T cell hybridomas. The antibodies identified segregated into two nonoverlapping functional classes; those reactive with the single–polypeptide chain GAT-TsF_1 molecules, and those reactive with the two–polypeptide chain TsF_2 molecules. The one exception to this observation is the ability of 1322 B1.3 to absorb both TsF_1 and TsF_2 molecules derived from Igh-1^b mice. This observation is consistent with the idea of constant region determinants shared between the two classes of suppressor factors, perhaps analogous to the concept of a suppressor determinant. Alternatively, this would indicate that 1322 B1.3 is not a true clone, but in fact is composed of two separate cell types with independent activities. This latter possibility may be addressed most directly by recloning and repeating the absorption experiment. An interesting observation to emerge from these data is the isotype distribution among the mAb that was limited to the IgM and IgG1 subclasses. Furthermore, those antibodies reactive with GAT-TsF_1 molecules were restricted to the IgM subclass, while those reactive with GAT-TsF_2 molecules were of the IgG1 subclass. Experiments are currently in progress to characterize the products of separate fusions of spleen cells from BALB/c mice hyperimmunized with C.B20 blasts to determine whether the isotype restriction is a general phenomena, or reflects the limited size of the sample in the results presented here. That this may represent a general phenomena representative of this class of serological reagents is suggested by identical observations by Owen (16), where only antibodies of the IgM and IgG isotypes were isolated.

Serologically, some of the anti-IgT-C reagents that we have described react

with a determinant that segregates with the antigen-binding chain of GAT-TsF_2 molecules, and not with the I-J^+ chain. From a serological standpoint, the structure of the antigen-binding chain is reminiscent of the chains of the antibody molecule, where a polymorphic or variable region, capable of binding specifically to antigen, is detected by antiidiotypic reagents, and a less polymorphic or constant region is detected by the 1322 mAb. The serologic reactivity of Ts cells, and more specifically the antigen-binding chain of GAT-TsF_2, with antibodies raised against idiotypic determinants on anti-GAT antibodies is presumably crossreactive at the level of the three-dimensional structure of the idiotype, since analysis at the nucleic acid level fails to show any homology by Northern or Southern analysis, suggesting that T cells and B cells do not use the variable region genes (15). The GAT-TsF_1 molecule is composed of a single polypeptide chain, as shown by the 1322 mAb: the IgT-C determinant, antigen-binding portion, and I-J determinant all cosegregate, under reducing or nonreducing conditions, on appropriate immunoabsorbents. This confirms our previous data (9) on the chain structures of the GAT-TsF_1 and GAT-TsF_2 molecules.

The 1322 mAb were also used to analyze two suppressor T cell hybridomas, 372B3.5 and 372D6.5, for the presence of suppressor factor–like activity in the cell membranes. Using an NP-40 extract from sucrose gradient–purified plasma membranes, suppressor activity was recovered from T cell hybridomas, which was reactive with 1322 mAb. The biologically active protein isolated from 372D6.5 membranes had a molecular mass of 31.5–34 kD, and alone was not suppressive. Combination of this protein with independently isolated I-J^+ chain from supernatant fluid derived from 372D6.5 GAT-TsF_2, alone also not suppressive, resulted in reconstitution of GAT-specific suppressor activity. This result is consistent with data showing the IgT-C determinant recognized by mAb 1322 C5.3 to be on the antigen-binding chain of the secreted GAT-TsF_2 molecule. Two implications follow from these results: based on these data and other studies which show that unfractionated membrane extracts are specifically suppressive, it appears that a GAT-TsF_2-related structure, composed of two polypeptide chains associated in a noncovalent fashion, exists in the cell membrane (C. Sorensen and C. Pierce, manuscript in preparation). This result is in contrast to previous data (9, 21) showing the covalent association of the two chains in hybridoma supernatant fluid GAT-TsF_2, but has precedent in results of Taniguchi and colleagues (6), in which a keyhole limpet hemocyanin–specific suppressor factor extracted from cells was also shown to exist as two polypeptide chains associated in a noncovalent fashion. The second point concerns the apparent molecular mass of the membrane-derived antigen-binding chain, which is ~6 kD larger than the secreted antigen-binding chain (26–28 kD) (C. Sorensen and C. Pierce, manuscript in preparation). This difference is consistent with the interpretation that the antigen-binding chain extracted from membranes is a transmembrane protein responsible for the anchoring the suppressor molecule in the membrane. No differences in molecular mass were detected between membrane- and supernatant-derived I-J^+ chain, both of which have molecular masses of 42–44 kD (data not shown). The existence of two molecular species of antigen-binding chains is also suggested by results of mRNA translation studies, in which two independent mRNA species, of 11 S and 17.5 S, encoding the

antigen-binding chain are demonstrated (C. Sorensen and C. Pierce, manuscript in preparation).

The protein isolated from hybridoma 372B3.5 membranes using 1322 A2.1–Sepharose had a molecular mass of 48–50 kD under nonreducing conditions, and 25 kD under reducing conditions. Similar to the results with the GAT-TsF_2-producing hybridoma, the molecule in the membrane of hybridoma 372B3.5, which reacted with mAb 1322 A2.1 had a greater molecular mass than its supernatant-derived equivalent (19 kD) (8), suggesting that it may be an integral membrane protein. Unlike the GAT-TsF_2, the molecule derived from the membrane of 372B3.5 suppressed in an antigen-specific manner, and in the absence of additional polypeptide products. The presence of a disulfide bond in the membrane-derived protein from 372B3.5 is suggested by the reduction in molecular mass in the presence of reducing agents. This observation may indicate that the membrane-derived suppressor activity is unique from the hybridoma supernatant fluid–derived GAT-TsF_1, and may be due to different content of cysteine residues. However, analysis at the mRNA level shows only a single mRNA species for the suppressive activity isolated from 372B3.5 (31). Experiments are currently in progress to investigate the relationship between the membrane-bound and soluble forms of the two suppressor proteins, but the final answer requires isolation of the two molecules independently, and comparison of their primary sequences.

The data support the concept of constant region determinants on at least two subsets of biologically active proteins derived from T cells, which may represent some form of antigen receptors. The prediction or demonstration of constant region determinants on or associated with the T cell receptor for antigen has been suggested by data from several laboratories. Using xenoantisera, Kontiainen and Feldmann (32) have shown suppressor factor–specific determinants shared among factors with different antigen specificities. Using reagents similar to those reported here, others have also demonstrated allotype-linked determinants present on suppressor cells or suppressor factors with different antigen specificities. Biochemical analysis (3) of receptor structures isolated from a variety of human cytotoxic T lymphocyte clones has shown the presence of conserved peptides common to all clones, in addition to clonally unique peptides. In studies by Owen and coworkers, a single class of suppressor T cell–restricted determinants or allotypes, Tsu, with linkage to the Ig allotype locus was identified. Two additional determinants linked to Ig allotype were also described, Tind and Tpre, neither of which appear to be expressed on the T suppressor cells. One interpretation of the results reported here is that the suppressor allotype locus is in fact more complex, with possibly two independent determinants mapping to this region; those restricted to the single-chain GAT-TsF_1 molecules, and those restricted to the antigen-binding chain of the GAT-TsF_2 molecules. Such an interpretation, however, depends upon the results of genetic mapping studies currently in progress using several chromosome 12–recombinant mice. Alternative explanations for our results, such as recognition of alternative glycosylation of a common peptide, do of course exist, and have not been ruled out. If the determinants recognized by the 1322 mAb are constant region determinants on suppressor T cell receptors for antigen, one prediction is that they should have general

usefulness as reagents for defining specific suppressor T cell subsets independent of antigen specificity or MHC haplotype. One drawback to this potential application(s) has been our inability to use these reagents in immunofluorescent studies on normal spleen cell or lymph node populations. These studies were done using culture supernatant fluids, and may require further purification of the specific antibody for fluorescence to be detectable. Alternatively, these results may reflect a normal low-level expression of the relevant determinants on what is probably a small subpopulation of the T cell pool. This is not to say that these reagents have no practical use (based on preliminary data [33 and C. Pierce, manuscript in preparation] suggesting that individual, functionally distinct subpopulations of T cells may be selected using the mAb reported here) as panning reagents.

Summary

Hyperimmunization of BALB/c mice with concanavalin A–stimulated blasts from the Ig allotype-congenic strain, C.B20, results in the production of antibodies reactive with T cells in an allotype-restricted manner. Spleen cells from these hyperimmune BALB/c mice were used to generate a panel of hybridomas that secrete monoclonal antibodies, reactive, in an allotype-restricted manner, exclusively with T cells subpopulations, and in particular, reactive with suppressor T cell hybridomas and their secreted soluble factors. Two functional classes of antibodies were identified: those that react with single polypeptide–chain suppressor T cell factors (TsF_1) and the suppressor T cell hybridomas that produce such factors, and those that react with two polypeptide–chain suppressor T cell factors (TsF_2) and their corresponding suppressor T cell hybridomas. These two classes of antibody were used to isolate molecules from the membranes of the respective suppressor T cell hybrids that are functionally and structurally related to the secreted suppressor T cell factors, suggesting a receptor function for these molecules.

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