REGULATION OF IMMUNE RESPONSES
BY *I-J* GENE PRODUCTS

I. Production and Characterization of
Anti-I-J Monoclonal Antibodies*

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Genes located in the *I* region of the murine major histocompatibility complex (*H-2*) regulate specific immune responses to a number of antigens (1, 2). Immune response-associated (Ia) molecules found on most B cells, and to varying degrees on macrophages and T cells, are coded by genes in the *I-A* and *I-E* subregions and might well be the products of immune response (Ir)[1] and immune suppressor (Is) genes (2–5). Gene products of the *I-J* subregion, on the other hand, are found on suppressor T (Ts) cells and their soluble factors (TsF) as well as on some helper T cells (Th) and macrophages, all of which are intimately involved in the regulation of the immune response (6). Due in part to a relatively higher degree or frequency of expression, a great deal of information has been amassed as to the function, serology, and biochemistry of *I-A* and *I-E* molecules (3, 4). In contrast, the low frequency (~5%) with which I-J-bearing cells are found in normal cells populations and the difficulty in producing anti-I-J alloantisera (7) have severely limited similar studies of *I-J* gene products.

I-J gene products affect the immune response profoundly. Tada et al. (8) showed that a TsF specific for keyhole limpet hemocyanin (KLH) bears determinants encoded by the *I-J* subregion. These findings were subsequently confirmed by Theze et al. (9) for a poly(Glu^{50}Tyr^{50})(GT)-specific TsF and by Greene et al. (10) for an azobenzene arsonate-specific TsF. Pierres et al. (11, 12) demonstrated that the administration of microliter quantities of anti-I-J serum to GT-suppressor strain mice allowed them to make a GT-specific antibody response apparently due to the elimination or inactivation of I-J-bearing Ts. Likewise, Greene et al. (13) showed that alloanti-I-J, when administered to S1509a fibrosarcoma-bearing mice, caused regression of the tumor due to the inactivation of tumor-induced Ts. In addition, Tada et al. (14) demonstrated that KLH-specific Ts bear antigenic determinants distinct from those found

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on I-J$^+$ Th, suggesting that at least two distinct genes are located within the *I-J* subregion. It may well be that the *I-J* subregion is composed of a cluster of regulatory genes whose products are selectively expressed on Ts, Th, and macrophages. Although cell absorbtion studies have been useful in the identification of differing *I-J* gene products, critical evaluation of the heterogeneity of the *I-J* subregion genes and their products is limited when alloantisera are used. The introduction of somatic B cell hybridization technology has allowed the production of large quantities of monoclonal antibodies (mAb). These monospecific antibodies should allow the further elucidation of the function and biochemistry of Ia molecules.

In this paper I report the preparation and properties of a series of mAb directed against gene products of the *I-J*^{b} and *I-J*^{k} subregions. These mAb were selected for their functional effect(s) upon normal spleen cell populations: first, for their ability to allow GT-suppressor mice to respond to GT immunization both in vitro and in vivo, and subsequently for their ability to bind GT-TsF of the appropriate haplotype. Some mAb bind GT-TsF, indicating a heterogeneity of *I-J* gene products. In addition, an I-J^k monoclonal GT-TsF (mGT-TsF) has been produced that is specifically bound by anti-I-J^k mAb. The implications of these findings and reagents to the further characterization of the *I-J* subregion and its products are discussed.

Materials and Methods

Mice. BALB/c (*H-2*^{d}) and AKR/Cum (*H-2*^{k}) mice were purchased from Cumberland View Farms, Clinton, Tenn. C57BL/6 (B6, *H-2*^{b}), C57BL/10 (B10, *H-2*^{b}), and B10.BR (*H-2*^{k}) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Breeding pairs of B10.A(3R) (3R, *H-2*^{i3}) and B10.A(5R) (5R, *H-2*^{i5}) were the gift of Dr. N. Ponzio, Department of Microbiology-Immunology, Northwestern University. (DBA/2 × 3R)F$_1$ and (DBA/2 × 5R)F$_1$ mice were bred in our animal facilities. Mice used were 8–12 wk of age at the initiation of these experiments and were maintained on standard laboratory chow and water ad lib.

Antigens. Poly(Glu^{50}Tyr^{50})(GT), lot 9, originally purchased from Miles Laboratories, Inc., Elkhardt, Ind., was the generous gift of Dr. Baruj Benacerraf, Harvard Medical School. Poly(Glu^{60}Ala^{30}Tyr^{10})(GAT), lot 1, and methylated bovine serum albumin (MBSA) were purchased from Vega-Fox Biochemicals Div., Newberg Energy Corp., Tucson, Ariz. GAT, GT, and their MBSA complexes were prepared as previously described (15).

Alloantisera. Alloantisera to the *I-J*^{k} subregion were prepared by injecting (DBA/2 × 3R)F$_1$ mice biweekly with 10^7 spleen and thymus cells from 5R female mice. Anti-I-J^b were similarly prepared by injecting (DBA/2 × 5R)F$_1$ mice biweekly with 10^7 lymphoid cells from female 3R mice. In the preparation of both types of alloantisera, recipient mice were bled following the seventh biweekly immunization and were immunized and bled on alternate weeks thereafter.

Production of Hybridomas. B cell hybridomas secreting anti-I-J antibodies were derived from the somatic cell fusion of hyperimmune spleen cells with the hypoxanthine phosphoribosyl transferase-negative myeloma cell line, P3X63Ag8. Briefly, a single cell suspension of (DBA/2 × 3R)F$_1$ spleen cells was prepared 13 d after the 12th biweekly immunization of these F$_1$ mice with 10^7 5R lymphocytes. 39 × 10^6 (DBA/2 × 3R)F$_1$ hyperimmune spleen cells were admixed with 3.9 × 10^6 P3X63Ag8 myeloma cells in serum-free Dulbecco's minimum essential medium (DME) and centrifuged into a loosely packed pellet. 1 ml of polyethylene glycol (PEG, diluted to 50% [vol/vol] with serum-free DME) was added dropwise to fuse the cell membranes (16). The fusion mixture was diluted to 50 ml with serum-free DME over 15 min. The cells were washed to remove the PEG, resuspended to 30 ml in DME plus 10% fetal calf serum (FCS), and dispensed into 96-well microculture plates (Linbro Chemical Co., Hamden, Conn.; 76-003-05) to a total of 384 wells. Each well received 5 × 10^4 normal spleen cells from (DBA/2 × 3R)F$_1$ mice as "feeder" cells. The cultures were incubated for 48 h at 37°C in 10% CO$_2$. Hybridoma cells were positively selected by elimination of the parent myeloma using hypoxanthine-aminopterin-thymidine (HAT)-containing selective medium (17). Cultures were fed every

other day by one-half medium replacement with HAT-DME plus 10% FCS for 7 d, followed by HT-DME plus 10% FCS for 7 d, and, finally, the cells were maintained in DME plus 10% FCS. Approximately 95% of the seeded wells showed cellular growth after the HAT-selection procedure. Anti-I-J^b-secreting hybridomas were similarly prepared from the fusion of 3R-immunized (12th immunization) (DBA/2 × 5R)F$_1$ spleen cells with P3X63Ag8. Anti-I-J-secreting hybrids were selected as detailed in Results. Selected cell lines were established as monoclonal by the isolation of individual colonies in soft agar, as previously described (18). At various stages of the selection procedure, hybrid cell lines were cryopreserved in 2-ml aliquots containing no less than 10^6 viable cells/ml in DME plus 20% FCS plus 10% dimethylsulfoxide and stored at −85°C.

T cell hybridomas producing GT-TsF were prepared in a manner similar to that described above for the B cell hybridomas. In brief, B10.BR (H-2^k,I-J^k) mice were primed i.p. 4 d before fusion with 100 μg GT in aluminum-magnesium hydroxide gel (Maalox, Wm. F. Rorer, Ft. Washington, Pa.). 170 × 10^6 GT-primed B10.BR spleen cells were fused with 8.4 × 10^6 BW5147.G.1.4 (HAT-sensitive AKR thymoma cells obtained from the Cell Distribution Center, Salk Institute, San Diego, Calif.) cells. The HAT selection procedure was similar to that used for the selection of B cell hybrids, as outlined above, and will be described in further detail in a forthcoming report.

Preparation of GT-TsF. GT-TsF was prepared as previously described (19–21). In brief, BALB/c, B10.BR, or B6 mice were primed with 100 μg GT in Maalox i.p. 3–7 d before use. A single-cell suspension was prepared of the spleens and thymuses of these animals, washed once in Hanks' balanced salt solution (HBSS), resuspended to 6 × 10^8 cells/ml and sonicated (50 W for 5 min) by a Sonifier Cell Disrupter equipped with a microtip (Ultrasonic Industries, Westbury, N. Y.). Sonicated material was centrifuged at 40,000 g for 45 min at 4°C. GT-TsF-containing supernates were stored at −85°C until used.

Preparation and Use of Immunoadsorbent Columns. Immunoadsorbent columns were prepared as previously described (20). Hybridoma culture supernates or ascites fluids from hybridoma-bearing mice were heat inactivated for 30 min at 56°C. Immunoglobulin-rich fractions were prepared by precipitation with 50% ammonium sulfate and extensively dialyzed against 0.5 M NaCl-0.1 M NaCO$_3$. The globulin-rich fractions were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) at a final protein concentration of 2 mg/ml packed gel. In all cases, coupling efficiency, as judged by UV absorbtion, was >90%. Immunoadsorbents were stored in phosphate-buffered saline (PBS) containing 0.2% NaN$_3$. Before use, gels were packed in 3-ml plastic syringes to serve as columns and washed with 10–25 vol of PBS in the absence of azide. GT-TsF, diluted 1:10, was applied to the columns always in a volume equal to or less than one-half the void volume of the gel. The GT-TsF were incubated in the gel bed for 15 min at 24°C, fresh PBS was applied to the column, and the effluent was collected. Columns were then washed with at least 10 vol of PBS, 0.1 M glycine-HCl buffer, pH 2.5, applied to the column, and the acid eluate was collected and immediately neutralized to pH 7.1–7.3 with the appropriate quantity of 2 M Tris. Effluent and eluate materials were immediately filter sterilized (0.45-μm filter) and added to Mishell-Dutton cultures at the final concentrations indicated in Tables I and III. In one series of experiments, an anti-idiotype immunoadsorbent column was used. This column was the generous gift of Dr. S.-T. Ju and Dr. M. E. Dorf, Department of Pathology, Harvard Medical School. Anti-idiotype antibodies (anti-CGAT) (22) for the common anti-GAT idiotype were bound to CNBr-activated Sepharose 4B at 1 mg protein/ml packed gel. Adsorptions and elutions from the column were performed exactly as described above.

Immunizations. AKR/Cum and B10.BR mice were immunized with 10 μg GT in Maalox and *Bordetella pertussis* i.p. as adjuvant immediately after intravenous injection of monoclonal anti-I-J antibodies. 7 d later the number of GT-specific plaque-forming cells (PFC) per spleen were determined.

In Vitro Cultures. Spleen cells of BALB/c, AKR/Cum, B6, B10, or B10.BR mice were placed in a modified Mishell-Dutton culture system. In brief, 8 × 10^6 spleen cells were cultured for 5 d in 0.5-ml cultures in a 24-well tissue culture plate (Linbro 76-033-05) in standard Mishell-Dutton culture medium (23) containing 5 × 10^{-5} M 2-mercaptoethanol and 10% FCS (lot 2851, Pel-Freez Biologicals Inc., Rogers, Ark.). 5 μg GAT, 10 μg GT, or 10 μg GT as GT-MBSA

were added at culture initiation. Alloanti-I-J antisera, hybridoma culture supernates, ascites fluids from hybridoma-bearing mice, and/or GT-TsF was/were added as indicated in the figures and the table legends. Cultures were fed daily with 120 µl of a mixture of 50% nutritional cocktail and 50% FCS. Cultures were incubated at 37°C with rocking in a moist atmosphere containing 83% N_2, 10% CO_2, and 7% O_2. 5 d after the establishment of culture, the number of GT-specific PFC per culture was enumerated.

Hemolytic Plaque Assay. The anti-GAT and anti-GT PFC response was assayed by a modification of the Jerne hemolytic plaque technique (20). Indicator cells were prepared by coupling GAT to sheep erythrocytes by the $CrCl_3$ method. Data obtained are expressed as number of PFC per spleen or PFC per culture.

Results

Effect of Anti-I-J Alloantisera In Vitro. In our laboratory we are studying the regulatory role(s) of *I-J* gene products expressed selectively on different macrophage and lymphoid cell populations. To this end, we have produced a panel of monoclonal antibodies (mAb) to gene products of the *I-J* subregion. The major obstacle to the identification of mAb directed against *I-J* gene products by the microcytotoxicity assay is the low frequency with which I-J-bearing cells are found in normal cell populations (7). We decided not to use selected Ts or I-J-bearing tumor cell populations because this might not have expressed a full repertoire of I-J specificities and, consequently, might have skewed our results by allowing us to detect only a limited subset of mAb directed against I-J specificities. Instead, we chose to use a functional assay to screen for anti-I-J mAb. Although the binding of TsF to an anti-I-J immunoadsorbent column was one of the criteria originally used to identify *I-J* gene

TABLE I

Effect of Anti-I-J Alloantisera on the Anti-GAT and Anti-GT PFC Responses of C57BL/6 and B10.BR Spleen Cells In Vitro

Strain	Antigen*	Alloantiserum‡	Antigen-specific PFC per culture§
C57BL/6 ($H-2^b$)	GAT	None	285
	GAT	Anti-I-J^b 1:200	750
	GAT	Anti-I-J^b 1:400	412
	GAT	Anti-I-J^b 1:800	337
	GAT	Anti-I-J^k 1:200	158
	GAT	Anti-I-J^k 1:400	195
B10.BR ($H-2^k$)	GT	None	<20
	GT	Anti-I-J^b 1:400	<20
	GT	Anti-I-J^b 1:800	390
	GT	Anti-I-J^b 1:1,600	160
	GT	Anti-I-J^k 1:400	1,535
	GT	Anti-I-J^k 1:800	1,305
	GT	Anti-I-J^k 1:1,600	1,265
	GT	Anti-I-J^k 1:3,200	325

* 5 µg of GAT or 10 µg of GT was added at culture initiation. Spleen cells were cultured for 5 d at 8 × 10⁶ cells per 0.5 ml in Mishell-Dutton culture medium containing 5 × 10⁻⁵ M β-mercaptoethanol.

‡ Alloantisera were prepared as described in Materials and Methods and added to cultures at the indicated final dilutions at culture initiation.

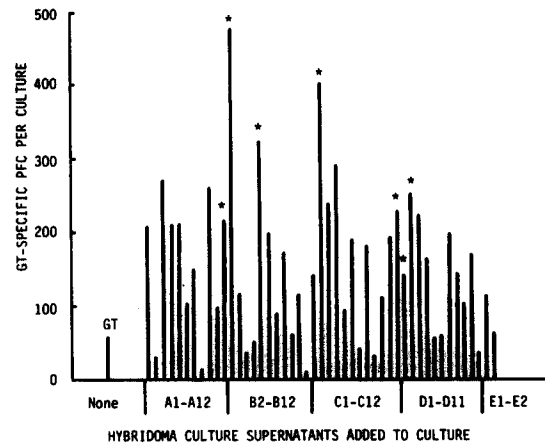§ Total PFC response on day 5 after culture initiation.

Fig. 1.   In vitro augmentation of GT-specific PFC by hybridoma culture supernates. AKR/Cum spleen cells were placed in Mishell-Dutton-type culture conditions for 5 d in the presence of 10 μg GT/culture well and 25 μl of WF8 series culture supernates were added to cultures at a 1:400 final dilution. The letter-numbers designate the hybridoma. The asterisk above the verticle bar denotes the cell lines whose supernates were tested for their ability to bind GT-TsF, Table II, experiment I.

products (8), the large number of hybrids obtained from a somatic B cell fusion makes this type of initial screening approach both costly and difficult to perform. Therefore, we asked whether anti-I-J antibody would augment PFC response in vitro. B6 ($I\text{-}J^{b}$) spleen cells were placed in Mishell-Dutton-type cultures with suboptimal concentration (5 μg/ml) of GAT (Table I). The addition of anti-I-J^{b} alloantiserum at a final concentration of 1:200 to 1:400 caused a marked augmentation of the anti-GAT PFC response. Anti-I-J^{k} alloantiserum, on the other hand, caused no enhancement of the B6 anti-GT PFC response. B10.BR ($I\text{-}J^{k}$) spleen cells normally are unresponsive to GT in vitro (9). The addition of anti-I-J^{k} antibodies to cultures of B10.BR spleen cells causes a marked increase in the number of anti-GT PFC (Table I), whereas the addition of anti-I-J^{b} antibody caused only minimal enhancement. This provided us with a rapid and simple method for the detection of anti-I-J activity.

Effect of Hybridoma Culture Supernates In Vitro.   Anti-I-J^{k}-secreting hybridoma cell lines were derived from the fusion of hyperimmune (DBA/2 × 3R)F$_1$ anti-5R spleen cells with the P3X63Ag8 myeloma cell line, as described in Materials and Methods. Initial screening for anti-I-J-secreting hybridomas utilized an X-Y coordinate system. In brief, two 96-well culture plates containing hybrid B cells were arranged to form a grid of 12 vertical columns and 16 horizontal rows. 12 X-axis pools were made, each pool containing 20 μl of culture supernate from each well in an individual column. Similarly, 16 Y-axis pools were made, each containing 15 μl of culture supernate from each well of an individual row. 20 μl from each X-axis pool or 15 μl from each Y-axis pool was added to individual 0.5-ml Mishell-Dutton-type cultures of AKR/Cum (I-J^{k}) spleen cells; in addition, each culture contained 10 μg of GT. Each individual hybridoma culture supernate was thus tested at a 1:400 final dilution. Positive (i.e., GT-response) cultures were identified by matching positive X-Y coordinates, and 47 putative anti-I-J-secreting hybridomas were isolated. These 47 hybridomas were expanded in 16-mm tissue culture wells and assigned letter-number designations. Supernates from these wells were used for the second screening phase. 2-d-old culture

TABLE II

Adsorption of GT-TsF by Monoclonal Anti-I-J Antibodies Bound to Sepharose

	Immuno-adsorbent‡	GT-specific PFC per culture*		
		Untreated	Effluent§	Eluate‖
Experiment I: test for anti-I-J^k activity in WF8 series				
Control GT-MBSA response	—	1,510	—	—
B10.BR (*H-2^k*) GT-TsF	—	<20	—	—
B10.BR (*H-2^k*) GT-TsF	WF8.A12		852	<20
B10.BR (*H-2^k*) GT-TsF	WF8.B2		<20	777
B10.BR (*H-2^k*) GT-TsF	WF8.B6		1,225	<20
B10.BR (*H-2^k*) GT-TsF	WF8.C2		1,900	<20
B10.BR (*H-2^k*) GT-TsF	WF8.C12		763	<20
B10.BR (*H-2^k*) GT-TsF	WF8.D1		<20	513
B10.BR (*H-2^k*) GT-TsF	WF8.D2		863	<20
Experiment II: test for anti-I-J^b activity in WF9 series				
Control GT-MBSA response	—	251	—	—
C57BL/6 (*H-2^b*) GT-TsF	—	<15	—	—
C57BL/6 (*H-2^b*) GT-TsF	WF9.1.4		735	<15
C57BL/6 (*H-2^b*) GT-TsF	WF9.5.2		870	<15
C57BL/6 (*H-2^b*) GT-TsF	WF9.11.6		585	<15
C57BL/6 (*H-2^b*) GT-TsF	WF9.20.8		488	<15
C57BL/6 (*H-2^b*) GT-TsF	WF9.24.8		270	105
C57BL/6 (*H-2^b*) GT-TsF	WF9.40.5		998	<15

* GT-specific PFC response of BALB/c spleen cells 5 d after culture initiation, each culture containing 2.5 μg of GT as GT-MBSA, GT-TsF (1:400 final), or no factor. GT-TsF was incubated on immunoadsorbent columns, and effluents were collected. Columns were washed with PBS, and glycine-HCl, pH 2.5, was used to elute bound material.

‡ Immunoadsorbents prepared from hybridoma culture supernatants (see Materials and Methods).

§ Effluents from supernatant-Sepharose columns were added at 1:400 final dilution.

‖ Bound material was eluted with glycine-HCl buffer, pH 2.5. Eluate was neutralized with 2 M Tris to pH 7.2 before addition to culture.
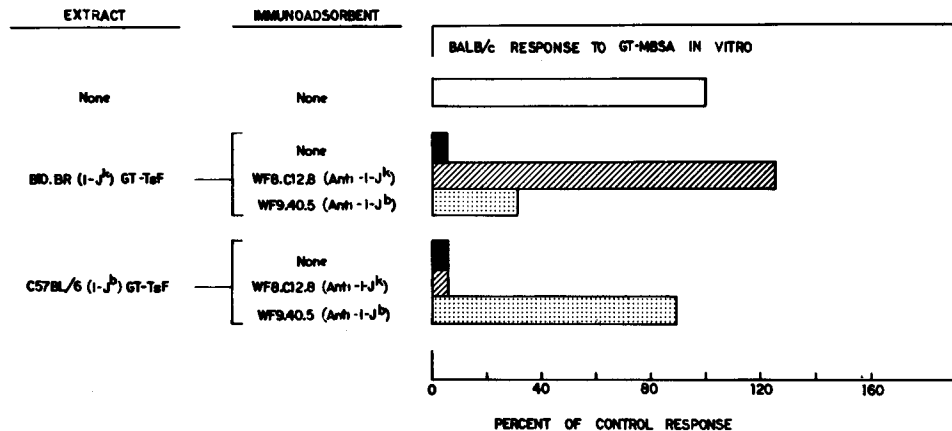

FIG. 2. Specificity of two anti-I-J mAb. I-J^k (B10.BR) GT-TsF totally suppresses the GT-MBSA response of BALB/c mice. This suppressive activity can be totally removed by WF8.C12.8 (anti-I-J^k mAb) but not by WF9.40.5 (anti-I-J^b mAb) immunoadsorbent columns. Conversely, an I-J^b (B6) GT-TsF is adsorbed by WF9.40.5 but not by WF8.C12.8 immunoadsorbent columns.

ANTI-I-J MONOCLONAL ANTIBODIES

TABLE III

Absorption of Monoclonal Anti-I-J Antibody Activity by B10.A(3R) and B10.A(5R) Lymphocytes

Strain	Antigen§	Additions to culture‡		GT-specific PFC per culture*			
		Antibody	Absorption	Con-trol	Final anti-I-J dilution		
					1:4,000	1:40,000	1:400,000
B10.BR (*H-2^k*)	GT-MBSA	—	—	600	—	—	—
	GT	—	—	217	—	—	—
	GT	WF8.C12.8 (α*I-J^k*)	None		367	1,365	345
	GT	WF8.C12.8	B10.A(3R)		698	NT‖	660
	GT	WF8.C12.8	B10.A(5R)		<15	<15	<15
C57BL/10	GT-MBSA	—	—	232	—	—	—
(*H-2^b*)	GT	—	—	52	—	—	—
	GT	WF9.40.5 (α*I-J^b*)	None		352	495	190
	GT	WF9.40.5	B10.A(3R)		<15	<15	90
	GT	WF9.40.5	B10.A(5R)		825	375	712

* GT-specific PFC responses on day 5 of culture.

‡ Sterile ascites fluid containing monoclonal anti-I-J antibodies was added at culture initiation at the indicated final dilutions. Where indicated, 0.5 ml of ascites was absorbed for 1 h at 4°C with 300 × 10^6 spleen and thymus cells, before addition to culture.

§ 2.5 μg of GT as GT-MBSA or 10 μg of GT was added at culture initiation. Spleen cells, either B10.BR or C57BL/10, were cultured for 5 d at 8 × 10^6 cells per 0.5 ml in Mishell-Dutton medium containing 5 × 10^{-5} M 2-mercaptoethanol.

‖ Not tested.

supernates were screened for their effect on the GT responsiveness of AKR/Cum spleen cells in vitro (Fig. 1). Cultures containing only GT produced <60 PFC per culture. A number of those wells receiving hybridoma culture supernates showed a marked enhancement of GT-specific PFC. The great frequency with which these supernates allow a GT-suppressor strain to make a GT response, shown in Fig. 1, is most likely due both to the use of hyperimmune animals in the fusion and to the fact that this is a second-phase screening from preselected wells. Subsequent screenings performed in this manner revealed that some hybridomas showed a more consistent pattern of augmentation than others. Seven of these, each indicated by an asterisk, were selected for subsequent testing.

Adsorption of GT-TsF by Hybridoma Culture Supernates. Although indicative of anti-I-J activity, the screening procedure described above is by no means conclusive proof. Because determinants encoded by the *I-J* subregion have been shown to be associated with soluble TsF (8–10), we wished to establish whether those supernates that augment a PFC response in vitro can also bind GT-TsF. Accordingly, the heat-inactivated immunoglobulin fractions of the hybridoma cultures indicated by an asterisk in Fig. 1 were coupled to CNBr-Sepharose and used as immunoadsorbent columns for the third screening phase. B10.BR (*I-J^k*) GT-TsF was incubated on the hybridoma immunoglobulin-Sepharose columns. B10.BR GT-TsF normally causes near total suppression of the GT-MBSA PFC response of BALB/c spleen cells in vitro at a 1:400 final dilution (Table II, experiment I). Supernates from five of the anti-I-J^k hybridoma cultures (WF8 series; A12, B6, C2, C12, and D2) bound GT-TsF and two (B2 and D1) did not. Adsorbed GT-TsF can be eluted from the columns with glycine-HCl buffer, pH 2.5, with no loss of suppressive activity (Table II and unpublished

observations), indicating that the loss of suppressive activity of GT-TsF when incubated on the Sepharose columns was not due to the inactivation of GT-TsF by the column adsorbtion procedure. Monoclonal cell lines were then established by soft agar cloning of the hybridoma cells, and the monoclonally derived cell lines were rescreened according to the procedures described above.

Screening procedures for the identification of hybridoma anti-I-J^b antibodies were similar to those used for the detection of anti-I-J^k antibodies, except that hybridoma culture supernates were tested on either B6 (I-J^{b}) or B10 (I-J^{b}) spleen cell cultures receiving a suboptimal, 5 μg/culture, concentration of GAT, similar to that shown in Table I. Six hybridoma-containing wells were further tested for their ability to bind I-J^b GT-TsF (Table II, experiment II). Similar to our findings with the WF8 series of

TABLE IV

In Vivo Activity of Monoclonal Anti-I-J^k Antibodies in AKR and B10.BR Mice

Strain*	Number of mice per group	Treatment‡	GT-specific PFC per spleen§	P‖
Experiment I				
AKR/Cum (H-2^k)	3	—	3,067 ± 260	—
AKR/Cum	7	WF9.11.6 (αI-J^{b})	4,128 ± 688	0.65
AKR/Cum	7	WF8.A12.4 (αI-J^{k})	11,842 ± 989	<0.01
AKR/Cum	7	WF8.D2.3 (αI-J^{k})	8,585 ± 782	<0.01
Experiment II				
B10.BR (H-2^k)	4	—	3,450 ± 1,021	—
B10.BR	4	WF8.C12.8 (αI-J^{k})	6,888 ± 1,169	0.04

* Mice were immunized with 100 μg of GT in Maalox and *Bordetella pertussis* immediately after i.v. injection of monoclonal anti-I-J antibodies.
‡ 20 μl of hybridoma culture supernatant (experiment I) or 20 μg of DEAE-Sephacel-purified antibody from ascites fluid was injected i.v. in 0.5 ml HBSS.
§ 7 d after injection, GT-specific PFC were enumerated.
‖ All comparisons made with Student's t test.

TABLE V

Adsorption of Monoclonal GT-TsF by Monoclonal Anti-I-J^k Antibody

		GT-specific PFC per culture*		
	Immunoadsorbent‡	Un-treated	Effluent	Eluate
Control GT-MBSA response	—	310	—	—
WF11.3.A1 (GT-TsF)§	—	<10	—	—
WF11.3.A1	WF8.C12.8 (anti-I-J^k)		250	<10‖
Eluate from WF8.C12.8‖	Anti-CGAT (anti-idiotype)		570	<10

* GT-specific PFC were counted on day 5 of Mishell-Dutton culture. WF11.3.A1 was incubated with the appropriate immunoadsorbent, and the effluent was collected. The column was washed with PBS, and bound material was eluted with glycine-HCl, pH 2.5. The eluate was neutralized with 2 M Tris to pH 7.2 before addition to culture.
‡ Immunoadsorbent columns were prepared as described in Materials and Methods. The anti-CGAT (anti-idiotype) columns were generously provided by Dr. S.-T. Ju and Dr. M. Dorf, Harvard Medical School
§ Monoclonal GT-TsF (WF11.3.A1) was derived from the fusion of GT-primed B10.BR (H-2^k) spleen cells with BW5147.G.1.4 and added to cultures at a 1:2,000 final concentration.
‖ Material eluted from WF8.C12.8 was neutralized and sequentially adsorbed on an anti-CGAT (anti-idiotype) immunoadsorbent column.

hybridoma mAb, six of the seven anti-I-J^b mAb anti-I-J^b tested (WF9 series; 1.4, 5.2, 11.6, 20.8, and 40.5) bound B6 GT-TsF.

Specificity of Monoclonal Anti-I-J Antibodies. The specificity of two anti-I-J mAb is shown in Fig. 2. An I-J^k GT-TsF is bound by anti-I-J^k mAb (WF8.C12.8) but not by anti-I-J^b mAb (WF9.40.5). Conversely, an I-J^b GT-TsF is bound by WF9.40.5 but not by WF8.C12.8. Table III shows that the in vitro PFC-enhancing ability of WF8.C12.8 is completely absorbed by 5R (I-J^k) spleen and thymus cells but not by 3R (I-J^b) lymphoid cells. Similarly WF9.40.5 activity is completely removed by absorption by 3R but not by 5R spleen and thymus cells. Together, these data show that these two anti-I-J mAb are noncross-reactive, indicating that they do not recognize common "framework" portions of I-J^b and I-J^k gene products. Furthermore, neither the WF8 nor the WF9 series of hybridoma anti-I-J antibodies cross-reacts with BALB/c (H-$2^d,I$-J^d)-derived GT-TsF (unpublished observation). Although cross-reactivity between I-J^b and I-J^d specificities has been reported for alloantiserum (24) and, most recently, for another anti-I-J^b mAb (25), we have observed no such cross-reactivity. This is not surprising in light of the fact that the WF8 and WF9 series of anti-I-J mAb were derived from the fusion of hyperimmune spleen cells from F$_1$ mice that carried the H-2^d as part of their genome. The use of DBA/2 (H-$2^d,I$-J^d) as one of the parents of the F$_1$ was purposely included to help eliminate this cross-reactivity. Further studies are currently in progress to determine whether these or any other anti-I-J mAb are cross-reactive with I-J gene products of "inappropriate" haplotypes.

In Vivo Activity of Anti-I-J^k Monoclonal Antibodies. It remained to be determined whether these mAb have in vivo as well as in vitro activity. Table IV, experiment I, illustrates that the intravenous injection of 20 μl of culture supernate from 2-d-old cultures of WF8.A12.4 or WF8.D2.3 (both anti-I-J^k mAb) into AKR (H-2^k) allows these mice to make a significant anti-GT PFC response. Injection of anti-I-J^b mAb (WF9.11.6) into AKR mice caused no significant augmentation of the anti-GT PFC response. Hybridoma cells were grown in ascites form in irradiated mice. The resultant ascites fluid was fractionated with 50% ammonium sulfate, dialyzed, and purified on a DEAE-Sephacel column equilibrated with 0.04 M PO$_4$ buffer, pH 8.2. Experiment II shows that the intravenous administration of 20 μl of this purified mAb caused B10.BR (H-2^k) mice to respond to GT challenge. Therefore, the anti-I-J^k mAb act in vivo in much the same manner as alloantisera (12).

Monoclonal GT-TsF Bears Both I-J and Idiotypic Determinants. A number of antigen-specific TsF have been shown to bear antigenic determinants of the I-J subregion (8–10). Several of these extracts from Ts have been shown to have idiotypic determinants in common with antibody (26, 27). Direct evidence that both I-J and idiotypic determinants are present on the same TsF molecule or molecular complex is lacking. The establishment of monoclonal cell lines producing TsF allows us to resolve this question. Monoclonal GT-TsF was produced by a hybridoma T cell line resulting from the fusion of BW5147.G.1.4 (HAT-sensitive) AKR thymoma cells with GT-primed B10.BR (I-J^k) spleen cells. Culture supernates containing GT-TsF derived from the resultant hybridoma T cell line WF11.3.A1 are able to totally suppress the GT-MBSA PFC response of BALB/c spleen cells in vitro at a 1:2,000 final dilution (Table V). This suppressive material can be adsorbed by and eluted from a monoclonal anti-I-J^k (WF8.C12.8) immunoadsorbent column. Recently, Ju et al. (22) described an anti-idiotype antibody (termed anti-CGAT) that recognizes the predom-

inant idiotypic determinants on anti-GAT and anti-GT antibodies. Because initial studies showed that our monoclonal GT-TsF specifically binds to GT-Sepharose columns, we asked whether WF11.3.A1-derived GT-TsF bears idiotypic determinants recognized by anti-CGAT anti-idiotype antibodies. The suppressive material eluted from the WF8.C12.8 immunoadsorbent column (Table V) was incubated on an anti-CGAT column. Table V shows that our monoclonal GT-TsF, which is bound by anti-I-J^k mAb, is also bound by and can be eluted from the anti-idiotype column. Therefore, the same monoclonal GT-TsF bears both *I-J* and idiotypic determinants. Studies are currently in progress to determine the biochemical association of these two determinants.

Discussion

I-J subregion genes or their products appear to be intimately involved in several aspects of immune regulation. The marker locus for the *I-J* subregion, *Ia-4*, codes for antigenic determinants on Ts and TsF (7–10, 24), whereas other *Ia* loci contained within the *I-J* subregion apparently code for determinants found on Th and macrophages (7). The selective expression of different *I-J* gene products on functionally distinct populations of lymphocytes and macrophages provides a potentially powerful model for the investigation of genetic regulation of the immune response. Major drawbacks to such studies have been both the limited availability of adequate anti-I-J alloantisera (28) and the relative heterogeneity of such sera with respect to *I-J* gene products (14). Lack of intra-I-J recombinant mouse strains also hinders definition of additional *Ia* loci and/or gene products within the subregion. A promising approach to the identification of *I*-region gene products is to use monoclonal antibodies (mAb) directed against individual antigenic determinants. I report the establishment of a panel of mAb directed against gene products of the *I-J*^{b} and *I-J*^{k} subregions. These mAb are secreted by hybridoma B cell lines derived from the fusion of the P3X63Ag8 myeloma cell line with either (DBA/2 × 3R)F$_1$ anti-5R or (DBA/2 × 5R) anti-3R hyperimmune spleen cells. The resultant hybridoma antibodies were assayed in a two-stage functional assay procedure. Putative anti-I-J antibodies were first assayed for their ability to augment a humoral immune (PFC) response both in vitro and in vivo. The anti-I-J mAb are active in causing nonresponder mice, or mice receiving suboptimal concentrations of antigen, to respond to antigenic challenge both in vivo and in vitro. A select number of these mAb were tested in a second-stage functional assay for their ability to bind I-J-bearing GT-TsF. Some of the anti-I-J mAb bind TsF, indicating that they may recognize different *I-J* gene products. Our anti-I-J mAb are all of the IgG$_1$ class of antibody and are haplotype specific. No cross-reaction is seen between antibodies directed against the *I-J*^{b} and *I-J*^{k} subregions. Although cross-reactivity by anti-I-J^b antibodies to *I-J*^{b} and *I-J*^{d} subregion specificities has been reported (24, 25), I have observed no such cross reactivity with either the WF8 or WF9 series of mAb (unpublished observations). The lack of cross-reactivity with I-J^b products by our anti-I-J^b mAb is most likely due to the use of (*H-2*^{d} × *H-2*^{i3})F$_1$ mice as one of the fusion partners. Further studies are currently in progress to determine whether either the anti-I-J^k or anti-I-J^b mAb cross-react with other I-J specificities.

The two-stage functional screening assay for the identification of anti-I-J mAb is both simple and rapid. Furthermore, in those lacking the appropriate recombinational event that allows the production of monospecific anti-I-J alloantibody, monoclonal

anti-I-J hybridoma antibodies can be selected using this technique. We are currently producing a panel of mAb directed against the I-J^d subregion. Our approach has been to hyperimmunize (D2.GD × 5R)F$_1$ mice with B10.D2 spleen and thymus cells. Alloantisera produced from this immunization combination should recognize I-B^d, I-J^d, and I-E^d subregions. Using the two-stage functional screening assay, we have been able to isolate a series of hybridoma anti-I-J^d antibodies. We are presently establishing these as monoclonal hybridoma cell lines by soft-agar cloning techniques. The characterization and functional properties of this series of anti-I-J^d mAb will be the subject of a forthcoming report.[2]

I-J gene products have been shown to be present on antispecific TsF (8–10). In addition, intravenous administration of microliter quantities of alloanti-I-J antibodies to mice has been shown to augment humoral (11, 12) and cellular (13) immunity, apparently through the inactivation of Ts. There is no evidence that antibodies directed against I-J determinants on antigen-specific TsF are the same as those that augment an immune response. Our experiments show that anti-I-J mAb that binds TsF also causes augmentation of a PFC response (Table II and Fig. 1). This is not to say, however, that all hybridoma anti-I-J antibodies both augment a humoral response and bind TsF. Table II, experiment I, clearly shows that of seven hybridoma anti-I-J antibodies, all of which cause augmentation of the GT PFC response in vitro, only five bind I-J^k GT-TsF. This suggests that alloantisera contain antibodies directed against several different I-J gene products that are individually recognized by anti-I-J mAb. That some mAb bind TsF and augment the PFC response might indicate that these antibodies are directed against gene products expressed at only one stage in the suppressor pathway (i.e., the anti-I-J mAb might be directed against gene products expressed on the TsF-producing [Ts1] cell). Those mAb that augment a PFC response but do not bind TsF may be directed against I-J determinants selectively expressed by a different subset of cells, representing another stage in the suppressive pathway. The use of anti-I-J mAb should allow the further elucidation of the suppressor pathway.

Little is known about the biochemistry of I-J gene products. Monoclonal anti-I-J antibodies should provide a valuable tool in the biochemical characterization of I-J gene products. We have been successful in producing monoclonal GT-TsF by fusing spleen cells from GT-primed B10.BR (I-J^k) mice with the BW5147 thymoma. Table V shows that the culture supernate from one such hybridoma T cell line, WF11.3.A1, contains a suppressive material that can be sequentially bound by anti-I-J^k mAb (WF8.C12.8) and by anti-idiotype (anti-CGAT). This clearly demonstrates that monoclonal GT-TsF bears both I-J^k and idiotypic determinants on the same molecule or molecular complex. The use of both monoclonal GT-TsF and anti-I-J mAb should provide powerful tools in the further biochemical and functional characterization of a functional I-J gene product.

Finally, it should be noted that evidence accumulating from other laboratories that have been assaying our monoclonal anti-I-J antibodies has demonstrated that they are cytotoxic for azobenzene arsonate Ts1 cells and that they bind Ars-specific TsF (J. Bromberg and M. I. Greene, personal communication). These mAb also react strongly with TsF-producing T cell hybridoma cell surface antigens as detected by immunofluorescent staining (J. Trial and J. A. Kapp, personal communication).

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[2] Lei, H.-Y. et al. Manuscript in preparation.

Therefore, it appears that anti-I-J mAb are useful in the identification of *I-J* gene products in antigenic systems other than GT and that these antibodies might prove to be a powerful tool for the further delineation of *I-J* subregion gene(s) and their products.

Summary

Using a novel, two-step functional screening procedure, we have isolated hybridoma B cell lines secreting monoclonal antibodies directed against gene products of the $I\text{-}J^b$ and $I\text{-}J^k$ subregions of the mouse *H-2* complex. These monoclonal antibodies act in vitro by allowing nonresponder spleen cells to respond to normally suppressive quantities of $poly(Glu^{50}Tyr^{50})$ (GT) (WF8 series of anti-I-J^k monoclonal antibodies) or to suboptimal concentration of $poly(Glu^{60}Ala^{30}Tyr^{10})$ (WF9 series of anti-I-J^b monoclonal antibodies). Some of the culture supernates that show augmenting activity bind GT-specific T cell-derived suppressor factor (GT-TsF), indicating that some monoclonal antiantibodies display a nonspecific enhancing effect, or, more likely, that anti-I-J monoclonal antibodies have been produced against I-J determinants not found on TsF. It is this last possibility that is most intriguing and that might serve as a means for exploring the heterogeneity of the *I-J* subregion. It is also possible that some of our monoclonal anti-I-J antibodies might detect antigenic determinants selectively expressed on suppressor T cells, helper T cells, and/or macrophages. In addition, we have demonstrated that monoclonal anti-I-J antibodies should be useful in the biochemical characterization and purification of a monoclonal GT-TsF. These haplotype-specific anti-I-J monoclonal antibodies should prove to be powerful tools for future studies exploring the role of *I-J* gene products in the regulation of specific immune responses.

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