IMMUNOREGULATORY CIRCUITS THAT MODULATE RESPONSIVENESS TO SUPPRESSOR CELL SIGNALS

Failure of B10 Mice to Respond to Suppressor Factors Can Be Overcome by Quenching the Contrasuppressor Circuit*

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Taniguchi et al. (1) have shown that the in vitro antibody response of B10 and B10-H-2-congenic mouse strains (B10 strains) cannot be suppressed by a suppressor factor from T cells (TsF)[1] that has potent inhibitory effects when tested on cells from other mouse strains. This factor, which is keyhole limpet hemocyanin (KLH) specific, is extracted by sonication from primed Ly-1^-,2^+ (Ly-2) T cells. It induces primed Ly-1^+,2^+ (Ly-1,2) T cells to help Ly-2 express immunosuppressive activity, and is H-2 restricted in its action. This TsF as well as its producer and acceptor T cell subsets bear a determinant controlled by the I-J subregion of the H-2 gene complex (2). The ability of Ly-1.2 T cells to be activated by this TsF is regulated by a locus that is not linked to the H-2 gene complex. Cells from B10 strains are capable of producing this TsF, but are not suppressed by it. Failure of the TsF to suppress antibody responses of B10 strain spleen cells has been correlated with an inability of acceptor T cells to absorb the factor.

Recently, we have described another suppressor factor from Ly-2 T cells (Ly-2 TsF) which also fails to suppress in vitro antibody responses of spleen cells from B10 strains.[2] This factor is similar to one described above in that it is produced by primed Ly-2 T cells, and is H-2 restricted in its action. However, unlike the factor described by Tada et al. (1), the Ly-2 TsF we have studied has the following characteristics: (*a*) it is specific for a different antigen (sheep erythrocytes ([SRBC] vs. KLH), (*b*) it is

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[1] *Abbreviations used in this paper:* B6, C57BL/6; $B6AF_1$, $(C57BL/6 \times B10.A)F_1$; BRBC, burro erythrocytes; BSS, balanced salt solution; C′, complement; KLH, keyhole limpet hemocyanin; FCS, fetal calf serum; HRBC, horse erythrocytes; Ly-1, Ly-1^+,Ly-2^- T cell set; Ly-1,2, Ly-1^+,Ly-2^+ T cell set; Ly-2, Ly-1^-,Ly-2^+ T cell set; Ly-2 TsF, suppressor factor from Ly-2 T cells; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TsF, suppressor factor from T cells.

[2] Yamauchi, K., D. B. Murphy, F.-W. Shen, H. Cantor, and R. K. Gershon. Analysis of "I-J^-" MHC-restricted cell-free products from "I-J^-";Ly-2 T cell that suppress Ly-2 depleted spleen cells. Manuscript submitted for publication.

released by immune cells into culture supernates (i.e., it is not physically extracted by sonication), (*c*) it acts on a different acceptor cell (unprimed Ly-1^+,Ly-2^- T cell set [Ly-1] T cells vs. primed Ly-1,2 T cells), (*d*) it does not require Ly-1,2 T cells to express suppressive activity, and (*e*) is produced by I-J^-;Ly-2^3 (vs. I-J^+;Ly-2) T cells.

Mattingly et al. (3) have also described a TsF (SRBC specific) that differs by several criteria from both of the factors described above and that also fails to suppress in vitro antibody responses of B10 strain spleen cells.

Thus, the inability of various factors to suppress in vitro antibody responses of B10 strain spleen cells does not correlate with a unique immunogen or a unique interaction site in the suppressor circuit. We therefore felt that if we could determine the nature of the suppressor defect in B10 strains, we might gain further insight into the mechanism(s) by which TsF work. In particular, we wanted to know if the activity of cells in the newly described contrasuppressor circuit (4)[4] could be responsible for the failure of B10 strains to respond to TsF.

The results of our investigation, reported herein, indicate that factor preparations from Ly-2 T cells contain, in addition to TsF, a second factor which is more cross-reactive than is Ly-2 TsF (4) and induces contrasuppressor activity (TcsiF). Thus, the failure of Ly-2 TsF to suppress B10 spleen cells responses under ordinary circumstances is a result of the overriding activity of the contrasuppression system, which acts to oppose suppression. Experimental maneuvers that remove contrasuppressor activity render B10 strains indistinguishable from other mouse strains in their sensitivity to Ly-2 TsF-mediated suppression.

Materials and Methods

Mice. C57BL/6 (C57BL/6 × B10.A)F_1 ($B6AF_1$), and B10.A/Sn mice, 6–10 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, Maine. All other strains are maintained in our colony at Yale University School of Medicine, New Haven, Conn.

Antigens. SRBC and horse erythrocytes (HRBC) were obtained from Colorado Serum Co., Denver Colo.

Antisera. Anti-Ly-1.2 (C3H/An anti-C3H.CE-Lyt-1.2) and anti-Ly-2.2 (C3H/An × B6-Lyt-2.1 anti-ERLD) were prepared and tested for specificity as previously described (5). These sera were generously supplied by F.-W. Shen, Memorial Sloan-Kettering Cancer Center, New York. Monoclonal anti-Thy-1 reagents were generously provided by Dr. Phillip Lake, University College, London and Dr. Jonathan Sprent, University of Pennsylvania, Philadelphia, Pa. Anti-I-J^b serum was prepared by hyperimmunizing B10.A(5R) recipients with a mixture of B10.A(3R) spleen and lymph node cells (antiserum No. ASM-5). Anti-I-J^k sera were prepared by hyperimmunizing B10.A(3R) and [B10.A(3R) × A.BY]F_1 (antiserum No. ASM-46) recipients with B10.A(5R) cells (6).

Method of Cell Preparation. Spleen cells were washed in balanced salt solution and suspended in RPMI-1640 supplemented with 10% fetal calf (FCS) serum, 100 mM glutamine, 25 mM Hepes, and 5×10^{-5} M 2-mercaptoethanol for tissue culture. T cells were prepared by adding unprimed spleen cells to plastic petri dishes coated with goat anti-mouse immunoglobulin and harvesting the nonadherent fraction (7).

Cytotoxic Depletion of Cells. Depletion of cells bearing a given marker was achieved by

[3] The designation "I-J^-" is used to indicate that the cell type in question is not killed by complement-dependent treatment with our anti-I-J reagents. We cannot exclude the possibility that these I-J^- functional cell populations (*a*) express an I-J determinant not detected by our reagents or (*b*) express insufficient amounts of I-J antigen to be killed or bound.

[4] Green, D. R., D. D. Eardley, A. Kimura, D. B. Murphy, K. Yamauchi, and R. K. Gershon. Immunoregulatory circuits which modulate responsiveness to suppressor cell signals: characterization of an effector cell in the contrasuppressor circuit. Manuscript submitted for publication.

incubating cells with antiserum dilution in balanced salt solution (BSS) for 30 min at 37°C (1×10^7 cells/1 ml of 1:20-diluted anti-Ly sera, 1×10^7 cells/1 ml of 1:1,000-diluted and anti-Thy-1 serum, or 1×10^7 cells/1 ml of 1:5-diluted anti-I-J serum), washing, and incubating with rabbit complement for 45 min at 37°C (1×10^7 cells/1 ml of 1:10-diluted complement). The cells were then washed twice in BSS and resuspended in tissue culture medium (8).

Purification by Ly-2 TsF. The detailed method for production of this factor has been described elsewhere.[2] Briefly, mice were immunized with 0.2 ml the second immunization. Their spleen cells were treated with an anti-Ly-1 serum and rabbit complement (C′) and then cultivated in vitro for 48 h at 1×10^7 cells/1 ml in RPMI-1640 supplemented with 10% FCS. After incubation, the supernate was harvested, centrifuged at 2,500 rpm for 20 min, and passed through a Millipore filter (Millipore Corp., Bedford, Mass.). (The role of antigen in helping [or suppressing] the production of these factors has not yet been fully determined.)

Absorption of Soluble Factors. Absorption with erythrocytes was done by mixing 1 ml of culture supernates with 0.1 ml of a 50% erythrocyte suspension for 1 h on ice. The erythrocytes were removed after centrifugation. For absorption with anti-I-J sera, supernatant fluids were passed over anti-I-J immunoabsorbents prepared by conjugation of antisera to cyanogen bromide-activated Sepharose.

Assay Cultures. Suppressor activity by the supernates of primed, cultured Ly-2 T cells was determined by adding these materials to cultures containing unprimed spleen cells that had been treated with various test reagents. All cells were suspended in RPMI-1640 tissue culture medium (see above), at a concentration of 5×10^6/1 ml, and 1 ml of the cell suspensions was cultured with 0.05 ml of a 1% SRBC suspension in Falcon 3008 plates (Falcon Labware, Div. of Becton, Dickenson & Co., Oxnard, Calif.) in a 5% CO_2-95% air incubator at 37°C. At day 5, the anti-SRBC response was determined by enumerating the number of plaque-forming cells (PFC) per culture by the technique of Cunningham and Szenberg (9).

Results

Supernates from Cultured Immune Ly-2 T Cells Fail to Suppress the Primary Mishell-Dutton Response of Spleen Cells from B10 Mouse Strains (Table I). Results presented in Table I demonstrate the phenomenon we are investigating. Supernates from cultured immune Ly-2 T cells (Ly-2 factor preparation), which have potent suppressive activity at the concentrations used, when assayed on B6 (experiments A–C) or $B6AF_1$ (experiment D) spleen cells, fail to suppress responses by B10 (experiments A–C) or B10.A (experiment D) spleen cells. Similar results were obtained with Ly-2 factor preparations from strains B6 (experiments A and B), B10 (experiment C), and $B6AF_1$ (experiment D). Thus, although responses by B10 and B10.A spleen cells are not suppressed, both strains are capable of producing TsF (see Table II for results with B10.A Ly-2 factor preparation).

Ly-2 Factor Preparations Can Suppress the Response of Spleen Cells from B10 Strains If Ly-2^+ (Ly-1,2 and Ly-2) Cells are Removed from the Assay Cultures (Table II). Results presented in Table II show that cultures of B10 or B10.A spleen cells depleted of Ly-2^+ (Ly-1,2 and Ly-2) cells by treatment with anti-Ly-2 antiserum plus C′ (Ly-2^- spleen cells) are readily suppressed by Ly-2 factor preparations. Experiment D of Table II shows that B10.A Ly-2^- spleen cells are suppressed as easily as are $B6AF_1$ cells (note the contrast with experiment D in Table I; the data come from the same experiment). Experiments E, F, and G demonstrate that whereas unfractionated B10 and B10.A spleen cells are not suppressed by the Ly-2 factor preparation, Ly-2^- spleen cell cultures are significantly suppressed. Thus, resting, nonimmune Ly-2^+ (Ly-1,2 and/or Ly-2) cells block Ly-2 factor-mediated suppression in B10 strains.

B10 Strain Ly-2^- Spleen Cells Are as Easily Suppressed as Are Ly-2^- Spleen Cells of Other Mouse Strains (Table III). The results in Table III show that this exceptional blocking

TABLE I

Supernates from Cultured Immune Ly-2 T Cells Fail to Suppress the Primary Mishell-Dutton Response of Spleen Cells from B10 Mouse Strains

Exp.	Spleen cells in culture*	Ly-2 factor preparation‡	PFC/culture§	Suppression
				%
A	B6	−	1,500	
	B6	+ (B6)	600	60
	B10	−	2,400	
	B10	+	2,000	17
B	B6	−	1,300	
	B6	+ (B6)	400	69
	B10	−	1,000	
	B10	+	900	10
C	B6	−	1,900	
	B6	+ (B10)	900	53
	B10	−	850	
	B10	+	800	6
D	$B6AF_1$	−	1,300	
	$B6AF_1$	+ ($B6AF_1$)	300	77
	B10.A	−	1,500	
	B10.A	+	1,400	7

* 5×10^6 unprimed spleen cells.

‡ (−) without or (+) with culture supernate from in vivo primed anti-Ly-1 plus C′-treated spleen cells. Strain source of factor is in parentheses.

§ Mean PFC of triplicate cultures.

by B10 mice of suppression by Ly-2^+ cells is not indirectly caused by an insensitivity of their helper cells to TsF. In the absence of Ly-2^+ cells, they are at least as sensitive to suppressor factor as are B6 Ly-1 helper cells. The maximum level of suppression that can be achieved with Ly-2 TsF seems to be in the 75–80% range,[2] and this level is the one we have found in most situations where B10 Ly-2^- spleen cells have been tested.

B10 T Cells Are More Efficient in Contrasuppression Than Are B6 T Cells Even When Assayed on Ly-2-depleted B6 Spleen Cells (Table IV). The results in Table IV confirm the general conclusion drawn from the results presented in Table III. In addition they show that similar numbers of B10 T cells exert significantly more contrasuppression than do B6 T cells even when added to B6 Ly-2^- spleen cells in assay cultures (compare groups 3 and 5 with groups 4 and 6 in Table IV).

Cells Responsible for Blocking the Suppressive Activity of Ly-2 Factor Preparations Exhibit the Ly-1^+,2^+;I-J^+;Qa-1^+ Phenotype (Table V). Experiments designed to further characterize the cell surface phenotype of the Ly-2^+ cells responsible for neutralizing suppressive activity (Table V) show that cells responsible for overcoming suppressor activity express, in addition to the Ly-2 alloantigen, the Ly-1 alloantigen (groups 2), the Qa-1 alloantigen (group 2a), and an alloantigen controlled by the I-J subregion (groups 3). Results listed in groups 4 verify the specificity of the anti-I-J serum.

In sum, data in the preceding sections show that the failure of Ly-2 factor preparations to suppress in vitro SRBC responses by B10 strains is not a result of a

TABLE II

Ly-2 Factor Preparations Can Suppress the Response of Spleen Cells from B10 Strains If Ly-2$^+$ (Ly-1,2 and Ly-2) Cells Are Removed from the Cultures

Exp.	Spleen cells in culture*	Ly-2 factor preparation‡	PFC/culture§	Suppression
D	$B6AF_1$	−	1,300	
	$B6AF_1$	+ ($B6AF_1$)	300	77
	B10.A Ly-2$^-$ spleen‖	−	1,700	
	B10.A Ly-2$^-$ spleen‖	+	300	82
E	B10	−	1,700	
	B10	+ (B6)	1,700	0
	B10 Ly-2$^-$ spleen‖	−	1,700	
	B10 Ly-2$^-$ spleen‖	+	250	85
F	B10.A	−	1,150	
	B10.A	+ (B10.A)	1,300	0
	B10.A Ly-2$^-$ spleen‖	−	850	
	B10.A Ly-2$^-$ spleen‖	+	200	76
G	B10	−	2,100	
	B10	+ (B6)	2,400	0
	B10 Ly-2$^-$ spleen‖	−	2,200	
	B10 Ly-2$^-$ spleen‖	+	500	77

* 5×10^6 unprimed spleen cells.
‡ (−) without or (+) with culture supernate from in vivo primed anti-Ly-1 plus C′-treated spleen cells. Strain source of factor is in parentheses.
§ Mean PFC of triplicate cultures.
‖ Spleen cells treated with anti-Ly-2 serum + C′.

defect in the suppressor system. Rather, Ly-1$^+$,2$^+$;I-J$^+$;Qa-1$^+$ T cells present in normal spleen cell preparations interfere with suppressor activity. Removal of these cells results in efficient suppressor activity.

Coupled with our earlier work, which also showed that Ly-1$^+$,2$^+$;I-J$^+$ T cells could act to block suppressor cell activity (4), these results suggest that the defect in B10 strains may be a result of contrasuppressive activity. To further test this possibility, we utilized the knowledge that culture supernates from primed Ly-2 T cells contain two activities; one produced I-J;Ly-2 T cells (Ly-2 TsF) which suppresses cultures of Ly-1 T cells plus B cells and another (Ly-2 TcsiF) produced by I-J$^+$;Ly-2 T cells which induces Ly-2$^+$ T cells to express contrasuppressive activity. We reasoned that if B10 strains fail to respond to suppressor factor because of competing contrasuppressor activity, removal of Ly-2 TcsiF-inducing activity from the Ly-2 factor preparation would restore suppression. Data in the next three sections show that this is the case and provide further information on the nature of Ly-2 TcsiF.

Spleen Cells from B10 Strains Can Be Suppressed by Factor Preparations from Cultured I-J$^-$;Ly-2 T Cells (Table VI). Data in Table VI show that anti-I-J plus C′ pretreatment of the cellular source of TsF and TcsiF eliminates the I-J$^+$;Ly-2 T cells responsible for the production of Ly-2 TcsiF but does not hamper the production of Ly-2 TsF. Thus, Ly-2 TsF is inhibitory even when assayed on unfractionated B10 strain spleen cells (experiments D–G of Table VI) when Ly-2 TcsiF is not present.

TABLE III

B10 Strain Ly-2⁻ Spleen Cells Are as Easily Suppressed as Are Ly-2⁻ Spleen Cells of Other Mouse Strains

Spleen cells in culture*	Ly-2 factor preparation‡	PFC/culture§	Suppression
			%
B10	−	2,400	B10 standard
B6	−	1,500	B6 standard
B10	+	2,000	18
B6	+	600	60
B10 Ly-2⁻	−	4,300	B10 standard
B6 Ly-2⁻	−	1,400	B6 standard
B10 Ly-2⁻	+	1,200	82
B6 Ly-2⁻	+	800	43

* 5×10^6 unprimed spleen cells.

‡ (−) without and (+) with culture supernate from in vivo primed anti-Ly-1 plus C′-treated B6 spleen cells.

§ Mean PFC of triplicate cultures.

TABLE IV

B10 T Cells Are More Efficient in Contrasuppression Than Are B6 T Cells Even When Assayed on Ly-2-depleted B6 Spleen Cells

	T cells added to Ly-2⁻ splenic B6 cells in culture*	Ly-2 factor preparation‡	PFC/culture§		Percentage of suppressed response recovered‖	
			Exp. I	Exp. II	Exp. I	Exp. II
1	—	−	1,900	1,200	Control	
2	—	+	250	350	Standard	
3	2×10^5 B6	+	300	ND¶	3	ND
4	2×10^5 B10	+	700	ND	28	ND
5	5×10^5 B6	+	450	400	13	6
6	5×10^5 B10	+	1,400	1,000	70	76

* 5×10^6 unprimed spleen cells.

‡ (−) without and (+) with culture supernate from in vivo primed anti-Ly-1 plus C′-treated B6 spleen cells.

§ Mean PFC of triplicate cultures.

‖ 100 minus the ratio of the percentage of suppression (87 for experiment I and 71 for experiment II) of standard to percent suppression of test groups.

¶ Not determined.

Experiment J shows that cells from strain B10.A(3R) (I-J^b) but not B10.A(5R) (I-J^k) absorb anti-I-J^b antibody reactive with I-J^b Ly-2 TcsiF-producing cells, verifying the specificity of our reagent. Thus, by eliminating the I-J^+;Ly-2 T cell that produces Ly-2 TcsiF, only Ly-2 TsF is produced. B10 strain responses are readily suppressed when the contrasuppression system is not activated.

Spleen Cells from B10 Strains Can Be Suppressed by Supernates from Cultured Immune Ly-2

TABLE V

Cells Responsible for Blocking the Suppressive Activity of Ly-2 Factor Preparations Exhibit the ly-1$^+$,2$^+$;I-J$^+$;Qa-1$^+$ Phenotype

Mouse strain	T cells in culture*	T cell sets or subsets deleted	Ly-2 factor preparation‡	PFC/culture§		Suppression	
						%	
B10	Group 1						
	Unfractionated	None	−	1,600			
			+ (B6)	1,900		0	
	Group 2						
	Mixture of Ly-1$^-$ + Ly-2$^-$	Ly-1,2	−	1,200			
			+ (B6)	600		50	
	Group 3						
	Mixture of Ly-2$^-$ + I-J$^-$	I-J$^+$	−	1,500			
			+ (B6)	600		60	
	Group 4						
	Ly-2$^-$ + I-J$^-$ (control)‖	None	−	1,400			
			+ (B6)	1,400		0	
				Exp. I	Exp. II	Exp. I	Exp. II
B10.A	Group 1						
	Unfractionated	None	−	1,600	1,400		
			+ (B10.A)	1,500	1,400	6	0
	Group 2						
	Mixture of Ly-1$^-$ + Ly-2$^-$	Ly-1,2	−	1,000	1,100		
			+ (B10.A)	300	500	70	55
	Group 2a						
	Mixture of Ly-1$^-$ + Qa-1$^-$	Qa-1$^+$	−	1,100	1,000		
			+ (B10.A)	400	400	64	60
	Group 3						
	Mixture of Ly-2$^-$ + I-J$^-$	I-J$^+$	−	1,100	1,000		
			+ (B10.A)	500	350	55	65
	Group 4						
	Mixture of Ly-2$^-$ + I-J$^-$ (control)‖	None	−	700	900		
			+ (B10.A)	600	900	15	0

* All cultures contained 3 × 10^6 unprimed B cells and 2 × 10^6 total T cells. All cell mixture ratios were 50:50.

‡ (−) without or (+) with culture supernate from in vivo primed anti-Ly-1 plus C′-treated spleen cells. Strain source of factor is in parentheses.

§ Mean PFC of triplicate cultures.

‖ Cells treated with inappropriate I-J antiserum plus C′, i.e., serum of the wrong haplotype. Thus, the studies with the H-2^a and the H-2^b mice act as reciprocals for one another.

T Cells If I-J$^+$ Material Is Removed from the Supernates (Table VII). Results in Table VII show that factor preparations made in the presence of both I-J$^+$ and I-J$^-$; Ly-2 cells (I-J$^+$;Ly-2 T cells produce Ly-2 TcsiF;I-J$^-$;Ly-2 T cells produce Ly-2 TsF) can

TABLE VI

Spleen Cells from B10 Strains Can Be Suppressed by Factor Preparations Made by Cultured I-J$^-$ Ly-2 T Cells

Exp.	Spleen cells in culture*	Treatment of cells making Ly-2 factor preparation‡	PFC/culture§	Suppression
				%
D	B10.A	No factor added	1,500	Standard
	B10.A	NMS + C′	1,400	7
	B10.A	Anti-I-J^k (ASM-46) + C′	800	47
E	B10	No factor added	1,700	Standard
	B10	NMS + C′	1,700	0
	B10	Anti-I-J^b (ASM-5) + C′	340	80
F	B10.A	No factor added	1,150	Standard
	B10.A	NMS + C′	1,300	0
	B10.A	Anti-I-J^k (ASM-46) + C′	300	77
G	B10	No factor added	2,100	Standard
	B10	NMS + C′	2,400	0
	B10	Anti-I-J^b (ASM-5) + C′	700	67
J	B10	No factor added	1,200	Standard
	B10	NMS + C′	1,100	8
	B10	Anti-I-J^b (ASM-5) + C′	100	93
	B10	Anti-I-J^b abs. 3R + C′‖	1,200	0
	B10	Anti-I-J^b abs. 5R + C′¶	300	75

* 5×10^6 unprimed spleen cells.

‡ Culture supernate from in vivo primed anti-Ly-1 plus C′-treated spleen cells. Treatment was done after in vivo immunization and before starting factor preparation. Source of factor preparation was B10.A for experiments using B10.A mice and B6 for experiments using B10 mice.

§ Mean PFC of triplicate cultures.

¶ Anti-I-J^b abs. 3R, sera ASM-5 absorbed with B10.A(3R) lymphocytes, i.e., specific absorption.

‖ Anti-I-J^b abs. 5R, sera ASM-5 absorbed with B10.A(5R) lymphocytes, i.e., specificity control.

suppress unfractionated B10 spleen cell cultures if the biologically active materials are filtered through anti-I-J columns (experiments B, C, H, K, and L). Results in experiments H and K also show that the acid eluate obtained after filtration of the crude supernate through the anti-I-J columns renders the suppressor factor in the filtrate functionally inactive (In >20 experiments, we have not found any biological activity in these eluates unless TsF was also present [see Table IX for a typical result].) Experiment K shows additionally that absorption of the anti-I-J^b serum with I-J^k cells does not remove the ability of serum to bind (and release) I-J^{b+} Ly-2 TcsiF (specificity control). Normal mouse serum (NMS) control columns did not remove TcsiF activity (experiments B and L). Thus, removal of I-J$^+$ material (Ly-2 TcsiF) from Ly-2 factor preparations permits I-J$^-$ material (Ly-2 TsF) to suppress strain B10 responses, presumably as a result of a failure to activate the contrasuppression system. The results in experiment L of Table VII confirm the results presented in Table III demonstrating that B10 cells are as easily suppressed as B6 spleen cells when the

TABLE VII

Spleen Cells from B10 Strains Can Be Suppressed by Supernates from Cultured Immune Ly-2 T Cells If I-J$^+$ Material is Removed from the Factor Preparations

Exp.	Spleen cells in culture*	Ly-2 factor preparations‡	PFC/culture§	Suppression
				%
B	B10	−	1,000	Standard
	B10	+ (NMS [5R] filtrate)	900	10
	B10	+ (I-J eluate)‖	240	76
C	B10	−	850	Standard
	B10	+	800	6
	B10	+ (I-J filtrate)¶	250	71
H	B10	−	1,400	Standard
	B10	+	1,200	14
	B10	+ (I-J filtrate)¶	500	64
	B10	+ (I-J filtrate + I-J eluate)‖	1,400	0
K	B10	−	1,200	Standard
	B10	+	900	25
	B10	+ (I-J filtrate)**	300	75
	B10	+ (I-J filtrate + I-J eluate)‖	1,100	8
L	B10	+	1,000	Standard
	B10	+ (NMS [5R] filtrate)	900	10
	B10	+ (I-J filtrate)	250	81
	B6	−	1,300	Standard
	B6	+ (NMS [5R] filtrate)	450	65
	B6	+ (I-J filtrate)	250	81

* 5×10^6 unprimed spleen cells.

‡ (−) without or (+) with culture supernate from in vivo primed anti-Ly-1 plus C′-treated spleen cells. B6 mice were the strain source for all factors in experiment.

§ Mean of triplicate cultures.

‖ Acid eluate from anti-I-J^b immunoabsorbent column.

** Ly-2 factor preparation passed over anti-I-J^b immunoabsorbent coated with serum that had been absorbed with I-J^k (B10.A) lymphocytes before column preparation.

contrasuppressor circuit is quenched by removal of acceptor cells (Table III), as well as by removal of inducer signals (Table VII).

Spleen Cells from B10 Mice Can Be Suppressed by Supernates from Unfractionated Cultured Immune Ly-2 T Cells If the Supernates Are Absorbed with Cross-reacting Heterologous Erythrocytes (Table VIII). From previous studies, we knew that the anti-SRBC Ly-2 TsF could be absorbed with SRBC but not with HRBC.[2] We also had found that TcsiF could be absorbed with both SRBC and HRBC (4). We therefore tried to determine whether Ly-2 TcsiF in the B10 system had similar cross-reactive antigenic specificity. The results in Table VIII show that the material in the Ly-2 factor preparations responsible for inhibiting suppressor activity (Ly-2 TcsiF) in B10 mice is more cross-reactive than is Ly-2 TsF, i.e., Ly-2 TcsiF could be absorbed with HRBC in three experiments, whereas Ly-2 TsF could not. The absorption had some specificity, because burro erythrocytes (BRBC) failed to absorb either activity from two factor preparations,

TABLE VIII

Spleen Cells from B10 Mice Can Be Suppressed by Supernates from Cultured Immune Ly-2 T Cells If HRBC-binding Material Is Removed

Exp.	Spleen cells in culture*	Ly-2 factor preparation‡	PFC/culture§	Suppression
				%
H	B10	−	1,400	Standard
	B10	+	1,200	14
	B10	(SRBC)‖	1,100	21
	B10	(HRBC)	400	71
	B10	(BRBC)	1,200	14
Ia	B10	−	740	Standard
	B10	+	760	0
	B10	(SRBC)	850	0
	B10	(HRBC)	300	59
	B10	(BRBC)	850	0
Ib	B6	−	2,400	Standard
	B6	+	1,200	50
	B6	(SRBC)	2,200	8
	B6	(HRBC)	1,100	54
	B6	(BRBC)	1,100	54

* 5×10^6 unprimed spleen cells.

‡ (−) without or (+) with culture supernatant from in vivo primed anti-Ly-1 plus C′-treated B6 spleen cells.

§ Mean of triplicate cultures.

‖ Ly-2 factor preparation absorbed with cells in parentheses.

but did from a third. (Note: Table VIII, part b of experiment I, where factors were also tested on B6 whole spleen. This demonstrates the inability of HRBC and BRBC to absorb out suppressor factor under conditions where SRBC can.) Thus, removal of HRBC-binding material (Ly-2 TcsiF) from Ly-2 factor preparations permits Ly-2 TsF to suppress strain B10 responses.

Anti-I-J and Cross-reactive Erythrocytes Have Similar Capacities to Absorb Contrasuppressor-inducing Activity (Table IX). The results presented in Table IX show that HRBC and an anti-I-J immunoabsorbent have similar abilities to absorb out the contrasuppressor-inducing factor, which obscures the suppression when unfractionated Ly-2 factor preparations are added to unfractionated B10 spleen cells. In addition, they show that the eluate from an anti-I-J column has no significant helper activity (compare group 6 with group 1) unless mixed with contrasuppressor depleted suppressor factor (compare group 7 with group 5).

Taken together, these data demonstrate that the failure of B10 strains to be suppressed by Ly-2 factor preparations is a result of contrasuppression. Thus, Ly-2 factor preparations contain two activities: (*a*) Ly-2 TsF, which suppresses responses of Ly-1 T cells and B cells, is SRBC specific, I-J^-, and is produced by I-J^-;Ly-2 cells; (*b*) Ly-2 TcsiF, which induces Ly-1,2; I-J^+ T cells to express contrasuppressive activity, cross-reacts with related heterologous erythrocytes, is I-J^+, and is produced by I-J^+; Ly-2 cells.

TABLE IX

Comparison of the Ability of a Cross-reacting Erythrocyte (HRBC) and an Anti-I-J Immunoabsorbent to Absorb Contrasuppressive-inducing Activity and the Ability of I-J$^+$ Material to Reconstitute Such Activity

Group	Ly-2 factor preparation*	PFC/culture‡	Suppression
			%
1	−	2,900	Standard
2	+	3,200	0
3	(SRBC)§	3,400	0
4	(HRBC)§	1,300	55
5	(anti-I-J)‖	1,100	62
6	Eluate from anti-I-J¶	3,700	0
7	Mixture of groups 5 and 6	4,000	0

* (−) without or (+) with culture supernate from in vivo primed anti-Ly-1 plus C′-treated B6 spleen cells.

‡ Mean of triplicate cultures of 5×10^6 B6 spleen cells.

§ Ly-2 factor preparation absorbed with cells in parentheses.

‖ Ly-2 factor preparation passed over an anti-I-J^b immunoabsorbent.

¶ Acid eluate from column used in group 5.

Discussion

Results presented here show that the apparent failure of B10 mice to respond to the Ly-2 suppressor factor preparation we have produced is not a result of an inability of this factor to act on its acceptor cell. The lack of response is a result of an active process, induced by I-J$^+$ material (Ly-2 TcsiF), which serves to functionally block the biological activity of I-J$^-$ suppressor material (Ly-2 TsF). The blocking activity (contrasuppression) results from the activation of I-J$^+$;Qa-1$^+$;Ly-1,2 contrasuppressor cells by Ly-2 TcsiF. Spleen cells of B10 strains will exhibit the same sensitivity to Ly-2 suppressor factor preparations as do cells of other mouse strains if: (*a*) the Ly-2 cells that are producing the factor preparations are treated with an anti-I-J serum and C′ (eliminating I-J$^+$ cells producing Ly-2 TcsiF, but not I-J$^-$ cells producing Ly-2 TsF); (*b*) the supernates from cultured unfractionated Ly-2 cells are passed over anti-I-J immunoabsorbent columns (removing I-J$^+$ by Ly-2 TcsiF but not I-J$^-$;Ly-2 TsF); (*c*) the supernates are absorbed with HRBC (Ly-2 TcsiF binds HRBC, Ly-2 TsF does not), or (*d*) the acceptor cells that are induced by Ly-2 TcsiF are removed by treatment with any of the following antisera: Ly-1, Ly-2, I-J, or Qa-1.

The I-J$^+$ cell-free material (TcsiF) that activates the contrasuppressor cell is probably composed of an antigen-recognizing moiety (Tables VIII and IX), and an I-J gene-controlled alloantigen (Tables VII and IX). That the antigen-recognizing portion of the Ly-2 TcsiF differs from the antigen-specific portion of the Ly-2 TsF (in that the former cross-reacts with other heterologous erythrocytes[5] whereas the latter does not[2]) is of some interest. Why (and how) the Ly-2 cell that produces TcsiF should (or can) express a different variable region gene product than does the Ly-2 cell that produces TsF are important questions. Perhaps the regulatory activities of suppression and contrasuppression had different phylogenetic origins. It is known that high

[5] The pattern of the cross-reactions of TcsiF with different types of heterologous mammalian erythrocytes shows variation from batch to batch.

affinity antibodies exhibit more cross-reactions than do low affinity antibodies (10–15) and that high affinity antibodies appeared relatively late in phylogeny (16). Thus it is possible that the phylogenetic appearance of the ability to make high affinity antibodies coevolved with the development of the contrasuppressor circuit and that this is reflected in their cross-reactivity patterns. High affinity antibodies are particularly thymus dependent (15, 17) and appear late in the immune response (18). Thus, inhibition of the suppression produced by antibody feedback (19) (which is affinity dependent [20]; i.e., the higher the affinity, the greater the feedback) may be required for the continued progression of the immune response to levels of high affinity. Contrasuppression could be the mechanism by which this is brought about.

Whether the I-J gene product on the Ly-2 TcsiF is the same as the I-J gene products on other biologically active factors is now under investigation. However, this seems unlikely, based on theoretical grounds, as well as on preliminary experimental data that suggest that the I-J gene product expressed on cells in the contrasuppressor circuit is different from the I-J product expressed on cells in the suppressor circuit.

The observation that the Ly-2 TsF and the cell that produces it cannot be removed with any of the anti-I-J serum we have used is a consistent one (4)[2, 4] even though the Ly-1 inducer cell and Ly-1,2 amplifier cells in the suppressor circuit are I-J$^+$ and can be removed with these reagents (8).[6] Thus, although there may be numerous cells and cell products in the suppressor circuit that are I-J$^+$, the final Ly-2 effector cell may express too little of an I-J determinant to be detected by our reagents, or may express a unique I-J determinant that our anti-I-J sera fail to recognize. None of the previous reports on I-J$^+$ TsF have shown that these materials express suppressive activity in the absence of Ly-2$^+$ cells. In fact, those experiments that have been done to determine the acceptor cell for I-J$^+$ factors have found that these factors act as amplifiers or inducers of suppression rather than as molecules that suppress directly (2, 21).

Whereas our results show that the in vitro immune response of B10 mice is an excellent example for demonstrating the activity of the contrasuppressor circuit and for investigating the cellular interactions involved in the generation of contrasuppressor activity, the biological significance of the results from these studies is less clear. There is no reason to suspect that mice with B10 background genes have significantly abnormal immunoregulatory mechanisms. Thus, the results we have presented may be either trivial or in vitro artifacts. This is unlikely, however, because in vivo inoculation of in vitro generated contrasuppressor cells can turn a tolerogenic signal into an immunogenic one.[7] Nevertheless, a great deal of work is required before the precise role of modulation by cells of the contrasuppressor circuit in the in vivo immune response can be ascertained.

The exact importance of any of the immunoregulatory circuits or interactions described by us or others in the overall scheme of in vivo immunoregulation is uncertain. However, one cannot do the experiments to determine the in vivo importance of immunoregulation unless one recognizes the existence of the various interactions that take place within these immunoregulatory circuits. In considering the

[6] Yamauchi, K., D. B. Murphy, H. Cantor, and R. K. Gershon. Analysis of antigen specific, Ig restricted cell free material made by I-J$^+$ Ly-1 cells (Ly-1 TsiF) that induces Ly-2$^+$ cells to express suppressive activity. Manuscript submitted for publication.

[7] Ptak, W., D. R. Green, S. K. Durum, A. Kimura, D. B. Murphy, and R. K. Gershon. Immunoregulatory circuits that modulate responsiveness to suppressor cell signals: contrasuppressor cells can convert an *in vivo* tolerogenic signal into an immunogenic one. Manuscript submitted for publication.

potential physiological importance of the cell interactions we have described, it is important to note that we have found similar activities in all mice strains studied ([5, 6]; and our unpublished observations). The B10 mouse strain, which acts as a phenotypically exaggerated example when studied in vitro, makes it useful for study, but the circuit we have described is by no means confined to mice expressing B10 background genes. In fact, our preliminary results suggest that the expression of long-term memory by T cells requires that they develop increased resistance to suppressor mechanisms, and that the part of the contrasuppressor circuit we have analyzed in B10 mice plays an essential role in that development.

Thus, if we assume future work will establish the importance of the contrasuppressor circuit in vivo, the main question raised by our results (which requires an answer) is: Why do B10 T cells respond more actively to contrasuppressor inducer factors than do T cells from mice of other strains? There are two general classes of explanations for this phenomenon. One is that the B10 cells that are activated by the contrasuppressor-inducing factor (or other cells with which these acceptor cells communicate) are truly hyperactive when put into tissue culture. The second class of explanation is that cultured B10 spleen cells lack the activity of another type of Ly-2^+ modulatory cell, which has the job of helping suppressor factors work. This help may be necessary to counteract or balance the activity of cells in the contrasuppressor circuit. The notion of auxiliary cells that may help suppressors work has been previously suggested (2, 21–23). It is possible the TsF of Taniguchi et al. (1) and Tada et al. (2), which fails to work on B10 spleen cells, may be the product of these auxiliary cells. If this is the case, because the TsF of Tada et al. fails to bind to B10 acceptor cells (1), it is likely that an absence of auxiliary suppressor cell activity is a major factor in the expression of an apparent in vitro hyperactivity of contrasuppression in B10 mice. Obviously, if this is true, the need for auxiliary cells to make suppression manifest should disappear when the contrasuppression system is quenched by any of the techniques listed above. The notion that auxiliary, or perhaps more aptly termed "modulatory" cells, determine in part the sensitivity of effector cells to suppressor cell signals has been noted in circuits other than those which exert immunoregulatory effects (e.g., the nervous system [24]).

Whatever the lesion(s) is, that makes the contrasuppression system of B10 strain mice so dominant, it presents an ideal system for obtaining information that will be helpful in deciphering the complex series of interactions between the numerous subsets of T cells that are responsible for the finely tuned process of immune regulation.

Summary

The in vitro antibody response of spleen cells from B10 strain mice is not suppressed by factor preparations made by primed Ly-2 T cells, although these preparations can suppress the in vitro antibody response of spleen cells from other mouse strains (1–3).[2] The factor preparations from Ly-2 cells contain at least two separable activities: one that acts as a suppressor moiety (Ly-2 T cell suppressor factor [Ly-2 TsF]) and a second factor that acts as an inducer of contrasuppression (Ly-2 TcsiF); the latter initiates a series of cellular interactions that leads to the inhibition of suppression that we refer to as contrasuppression.

Removal of components (either cellular or humoral) of the contrasuppressor circuit makes spleen cells from B10 strain mice as easily suppressible as are those of other

mouse strains. Thus, removal of the contrasuppressor inducer cell and/or its biologically active product with the use of an anti-I-J serum, or removal of the functional acceptor cell of the inducer cell with the same or other (Ly-2;Qa-1) antisera breaks the B10 suppressor barrier. Contrasuppressive activity, but not helper activity can be eluted from anti-I-J immunoabsorbents. The addition of B10 T cells to either B6 or B10 spleen cell cultures deprived of acceptor cells for the TcsiF reconstitutes contrasuppression more efficiently than does the addition of C57BL/6 T cells. Ly-2 TcsiF is more cross-reactive than is Ly-2 TsF so that absorption of factor preparations from sheep erythrocyte-primed Ly-2 cells with horse erythrocytes also breaks the B10 suppressor barrier.

The hyperresponsiveness of splenic T cells from B10 strains to Ly-2 TcsiF may be an in vitro exaggeration of a normal in vivo process. Thus it is possible that one can take advantage of this unusual situation to help dissect out the cellular and subcellular components of T cell circuits that modulate sensitivity to immunoregulatory signals.

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