

MECHANISMS OF LY2 SUPPRESSOR CELL ACTIVITY
Activation of an Ly1 I-J⁺ Cell Is Required to Transduce the Suppressive
Signal

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The generation of suppression to sheep erythrocytes (SRBC)¹ in vitro involves the interaction of T cell subsets in a well-defined regulatory circuit. The effector of this circuit is an Ly1⁻,2⁺ T cell (Ly2 cell) that has the ability to suppress Ly1⁺,2⁻ T helper cells (Ly1 cells) to make primary anti-SE plaque-forming cell (PFC) responses in culture. We have described biologically active antigen-specific cell-free products of Ly2 T cells (Ly2 TsF) that can functionally mimic the action of the T suppressor effector cell in vitro (1). This cell-free material, like the T suppressor effector cell, (a) can exert its suppressive effects early in the course of an in vitro immune response, in contrast to inducers of suppression, which require a long latency period before suppressive activity is expressed (2), and (b) does not express any I-J or other I region major histocompatibility complex (MHC)-controlled specificities that can be detected using a large battery of antisera. This second finding is especially important in light of the fact that the biological activity of the molecule is restricted by genes coded for by MHC polymorphisms expressed by suppressor acceptor cell partners (1).

Previously we had thought that this molecule was produced by the terminal cell in the suppressor circuit, namely the Ly2 cell. Recently we found that by treating the Ly1 T acceptor cell population with anti-I-J serum, we completely abrogated the ability of the Ly2 TsF to suppress the helper activity of these Ly1 T cells, while not affecting their ability to deliver help to T cell-depleted spleen cells (B cells). The suppressive ability of the Ly2 TsF can be returned to cultures lacking I-J⁺ Ly1 cells by adding I-J⁺ molecules recovered from immune Ly1 cell-supernatants (3). In order for this I-J⁺ material used to return suppressive activity to the Ly2 TsF, the donor of the I-J⁺ molecule had to match the I-J haplotype

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¹ *Abbreviations used in this paper:* APC, antigen-presenting cells; BRBC, burro erythrocytes; BSS, balanced salt solution; FCS, fetal calf serum; HRBC, horse erythrocytes; Igh, immunoglobulin heavy chain gene loci; Igh-V, variable region of the Igh gene complex; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SE, sheep erythrocytes; T_H, T helper cells; TsF, suppressor factor from T cells; TsiF, suppressor inducer-factor from T cells.

of the cell that produced the Ly2 TsF and also had to match the Igh haplotype of the Ly1 T helper cell.

These results suggested to us that two separate macromolecules are required for effecting suppression in this system. The first molecule was supplied by an Ly2 cell, and can act alone to suppress Ly1 T cell populations by interacting with an Ly1 I-J⁺ cell that "transduces" the signal to its final target cell, an Ly1 I-J⁻ T helper cell, presumably by secreting an I-J⁺ molecule that can interact with the Ly2 TsF. We tested this hypothesis in a 48-h intermediate culture system by adding Ly2 TsF to cultures of spleen cells and subsequently assaying this supernatant for the presence of the I-J⁺ molecule required by the Ly2 TsF to effect suppression on I-J⁻ Ly1 T cells. We report here that this I-J⁺ molecule can be directly induced by the action of the Ly2 TsF and antigen on I-J⁺, Ly1 T cells in vitro. Further, the molecule induced shares all the characteristics of the I-J⁺ molecule found earlier in Ly1 TsiF: (a) it does not bind to antigen or to anti-immunoglobulin reagents; (b) it does not depend on the immunizing antigen; (c) cells that produce this I-J⁺ material must match the cells that produce the Ly2 TsF at MHC and the target cell at Igh for activity; and (d) it requires for production an Ly1, I-J⁺ T cell. We also found that successful induction of the I-J⁺ molecules by Ly2 TsF requires (a) an Ia⁺, Ig⁻ accessory cell, (b) antigen, and (c) homology at MHC between the cells that produce the Ly2 TsF and the cells that produce the I-J⁺ molecule.

These results indicate that the Ly2 TsF can function as a one-chain suppressor molecule only insofar as it can induce the production of a "partner" molecule, a molecule that works in tandem with the Ly2 TsF to effect suppression on the Ly1⁺ T helper cell. It also raises the possibility that a single molecular entity can be both an inducer molecule and a suppressor molecule, depending on the cell with which it interacts.

Materials and Methods

Mice. C57BL/6, BALB/c, B10.D2, B10.BR, B10.A, and C57BL/10J mice, 6–10 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, ME. C.B20, B.C9, BAB.14, B10.A(2R), B10.A(3R), B10.A(4R), and B10.A(5R) were bred and maintained at Yale University School of Medicine.

Antisera and Antigens. Monoclonal anti-Ly 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. Jonathan Sprent, University of Pennsylvania, Philadelphia, PA. Monoclonal anti-I-A^b reagents were provided by Dr. C. A. Janeway, Yale University School of Medicine. 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 (antisera No. ASM-5 and ASM-20). Anti-I-J^t was prepared by hyperimmunizing B10.A(3R) recipients with a mixture of B10.A(5R) spleen and lymph node cells (antiserum ASM-18). (We thank Dr. D. B. Murphy for preparing these antisera). Goat anti-mouse Ig sera was prepared by immunization with 1 mg of an equal mixture of five different myeloma proteins (MOPC 104E (IgM,K), TEPC15 (IgA,K), MOPC 141 (IgG2b,K), MOPC 21 (IgG1,K), and RPC5 (IgG2a,K) in a total of 2 ml complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37Ra (Difco Laboratories Inc., Detroit, MI) in two sites subcutaneously. Goats were boosted with the same mixed myeloma solution emulsified in incomplete Freund's adjuvant and bled starting 1 wk later. Depletion of cells bearing Lyt or I-J markers was achieved by incubating 1×10^7 cells/ml of antibody appropriately diluted in balanced salt solution (BSS) (1:100 for anti-Ly monoclonal antibody, 1:5 for

anti-Thy-1 monoclonal antibody hybridoma supernatant, or 1:10 for anti-I-J serum), washing, and incubating with complement for 45 min at 37°C (1×10^7 cells/ml of rabbit serum diluted 1:10 for anti-Ly, anti-I-A, or anti-I-J antibody or 1×10^7 cell/ml of guinea pig complement for anti-Thy-1 antibody). Complement used in these experiments was serum from animals selected for low natural cytotoxicity to mouse spleen cells. Sheep, horse, and burro erythrocytes were obtained from Colorado Serum Company Laboratories, Denver, CO.

Method of Cell Preparation. Spleen cells were washed in BSS and suspended in RPMI 1640 supplemented with antibiotics, 10% fetal calf serum (FCS), 100 mM glutamine, 25 mM Hepes, and 5×10^{-5} M 2-mercaptoethanol for tissue culture. T cells were prepared by the glass bead method of Wigzell (5). B cells were prepared by treating the cells with monoclonal anti-Thy-1 and complement.

Preparation of Lyt-1⁺-Derived and Lyt-2⁺-Derived Suppressor Materials. Preparation of Ly1 TsiF and Ly2 TsF has been previously described (1, 2). Briefly, a suspension of spleen cells from mice hyperimmunized with RBC was treated with anti-Lyt-2 (for Ly1 TsiF) or anti-Lyt-1 (for Ly2 TsF) and rabbit complement, and subsequently cultivated in vitro for 48 h in RPMI 1640 plus 10% FCS at a concentration of 10^7 cells/ml. After 48 h, supernatant fluids were cleared and passed through Millipore filters (Milex filters, Millipore Corp., Bedford, MA). In primary cultures, cells at 10^7 cells/ml were incubated for 48 h with antigen and Ly2 TsF added at a final dilution of 1:5. Supernatants were then cleared and passed through a Millipore filter before subsequent treatment.

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

Assay Cultures. Suppressor activity of the Ly1 TsiF or the Ly2 TsF was determined by adding these materials to cultures of unprimed spleen cells that had been treated with various test reagents. All cells were suspended in culture medium, at a concentration of 2×10^6 T cells and 2×10^6 B cells in 1 ml, and cultured with 0.05 ml of a 1% SRBC suspension in Falcon 3008 plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) in a 5% CO₂-95% air incubator at 37°C. The number of plaque-forming cells (PFC) were determined on day 5 by using the Cunningham modification of the Jerne-Nordin plaque assay as previously described (6). Results are given as the mean of three individual calculations from each culture condition.

Isolation of I-J⁺ Material. Supernatants containing Ly1 TsiF or from primary Ly1 T cell cultures were passed over the appropriate immunosorbent column made from anti-I-J antisera coupled to Sepharose 4B. After extensive washing, the column was eluted with 0.2 M sodium carbonate (Na₂CO₃) pH 11.0. The eluted material was concentrated to original volume, and dialyzed overnight against first phosphate-buffered saline (PBS), then RPMI 1640.

Results

An Ly1 I-J⁺ Cell Needed for Ly2 TsF Activity Can Be Replaced by Supernatant from a 48-h Culture of Spleen Cells, Ly2 TsF, and Antigen. Addition of supernatants from immune Ly2 cells had significant suppressive activity when added to cultures of Ly1 T cells and B cells (Table I, line 1). Removal of I-J⁺ Ly1 T cells from the assay culture, while not affecting the PFC response, eliminated the ability of Ly2 TsF to suppress these cultures (line 2). Suppressive activity is returned by the addition of an I-J⁺ molecule secreted by SRBC-primed Ly1 T cells (line 3). We investigated whether we could generate this I-J⁺ molecule in vitro in 48-h primary cultures of spleen cells. Supernatants of primary cultures to which nothing was added did not restore the suppressive activity of Ly2 TsF in the assay culture (line 4). Addition of Ly2 TsF to the primary culture did not

TABLE I

Addition of Supernatant from B6 Ly-1 T Cells Incubated with B6 Ly-2 TsF and SRBC Allows Ly-2 TsF to Suppress B6 Assay Cultures Depleted of I-J⁺ Ly-1 T Cells

Group no.	Assay cells*	Cells added	Primary culture [‡]		Anti-SRBC PFC/culture [†]	
			Ly2TsF added [§]	SRBC added	Without Ly2TsF	With Ly2TsF
1	Ly1 T + B	—	—	—	2,800	700
2	Ly1I-J ⁻ T + B	—	—	—	3,200	2,600
3	Ly1I-J ⁻ T + B	Primed	—	—	3,500	500
4	Ly1I-J ⁻ T + B	Naive	—	—	2,800	3,400
5	Ly1I-J ⁻ T + B	Naive	+	—	3,100	2,800
6	Ly1I-J ⁻ T + B	Naive	—	+	2,800	2,900
7	Ly1I-J ⁻ T + B	Naive	+	+	2,200	900

* Assay cultures contained Wigzell column-purified cells as a source of T cells and anti-Thy-1-treated spleen cells as a source of B cells as detailed in Materials and Methods. Culture supernatant from primary cultures were added at a final concentration of 1:2 to assay cultures on day 0.

[‡] Primary cultures contained 10⁷ SRBC-primed or naive B6 spleen cells.

[§] SRBC-specific Ly2 TsF was added at a final concentration of 1:5 at the initiation of primary culture.

^{||} 2 × 10⁶ SRBC were added to the initiation of primary in vitro cultures.

[†] Primary in vitro response to SRBC. Numbers given in boldface type indicate cultures that showed >50% suppression upon addition of TsF.

significantly increase the ability of this supernatant to replace the need for an I-J⁺ cell in the assay culture (line 5). Likewise, addition of antigen to these cultures had no effect on the ability to generate this I-J⁺ cell-replacing activity (line 6). However, addition of both Ly2 TsF and SRBC to primary cultures of normal B6 spleen cells generated a supernatant that, when added to assay cultures of Ly1 I-J⁻ T + B cells and Ly2 TsF, resulted in suppression (line 7). Therefore, it appears that the Ly2 TsF can induce the production of a molecule that functionally substitutes for an Ly1 I-J⁺ cell, and that this induction requires the presence of antigen in the primary culture.

Co-suppressive Activity in Primary Culture Supernatants (Which Allows Ly2 TsF to Suppress Assay Cultures Depleted of I-J⁺ Ly1 Cells) Is Mediated by a Molecule That Is Ig⁻, I-J⁺, and Antigen Nonspecific. We investigated the supernatant that replaces the need for an Ly1 I-J⁺ cell in Ly2 cell-mediated suppression to determine the molecular nature of this activity. Supernatants from normal B6 spleen cells induced by Ly2 TsF and antigen were passed over a goat anti-mouse Ig column and the filtrate and eluate were assayed for their ability to complement Ly2 TsF in the suppression of Ly1 I-J⁻ T plus B cells (Table II). Our results clearly show that while Ly2 TsF in secondary culture failed to suppress the response of Ly1 I-J⁻ T plus B cells (lines 1 and 2), addition of primary supernatant induced with Ly2 TsF replaces suppressive activity (line 3). It is the Ig⁻ column filtrate, and not the Ig⁺ column eluate, that contains the relevant activity (lines 4 + 5).

This supernatant was then passed over an anti-I-J immunosorbent to determine whether the molecule induced by the Ly2 TsF is I-J⁺. Passage of supernatant over an anti-I-J^b immunosorbent indicates that the molecule that replaces the need for the I-J⁺ cell in the assay culture exhibits the I-J^b haplotype marker (lines 6 and 7). Passage of the same supernatant over an anti-I-J^k immunosorbent resulted in no activity being retained in the column (lines 8 and 9). In addition,

TABLE II
Activity in Supernatants of Ly-1 T Cells Induced by Ly-2 TsF and SRBC That Assists Ly-2 TsF in Suppressing Cultures Depleted of I-J⁻ Ly-1 Cells is I-J⁺ and Antigen Non-binding

Group	Donor of Ly-2 TsF in primary culture*	Treatment of primary culture supernatant	Ly-2 TsF in assay culture [‡]	Anti-SRBC PFC/culture [†]	Suppression
1	—	—	—	2,800	Standard
2	—	—	B6	3,200	—
3	B6	—	B6	600	+
4	B6	G-anti MIg filtrate [‡]	B6	1,200	+
5	B6	G-anti MIg eluate	B6	3,400	—
6	B6	Anti-I-J ^b filtrate	B6	2,800	—
7	B6	Anti-I-J ^b eluate	B6	900	+
8	B6	Anti-I-J ^k filtrate	B6	1,200	+
9	B6	Anti-I-J ^k eluate	B6	3,100	—
10	B6	SRBC absorbed	B6	800	+

* See footnote[‡], table I.

[‡] G anti-MIg columns have previously been shown to remove >95% of the Ig⁺ material from normal mouse serum by ELISA assay. Anti-I-J^b and anti I-J^k columns have previously been shown to remove 95% of suppressive activity of an I-J⁺ Ly1 TsiF from B6 or B10.BR mice, respectively.

[§] Ly2 TsF was added at a final concentration of 1:5.

[†] Assay cultures were all B6 Thy-1-depleted spleen cells, B6 Ly1⁺, 2⁻ I-J⁻ T cells, and SRBC.

absorption of supernatant with the antigen used to induce the I-J⁺ chain that removes the Ly2 TsF from the primary culture supernatant, reveals that the I-J⁺ molecule does not bind antigen (line 10).

Our results indicate that while the I-J⁺ molecule induced by the Ly2 TsF does not bind antigen, antigen is required in the primary culture in order for Ly2 TsF to induce this molecule. Therefore, we determined whether the I-J⁺ molecule induced was restricted in its activity to the antigenic specificity of the TsF used to induce it. We did a series of experiments making hybrid complexes of Ly2 TsF and I-J⁺ molecules that were raised against different heterologous erythrocyte specificities. The I-J⁺ molecule was induced by either SRBC-, HRBC-, or BRBC-specific TsF in the primary culture and subsequently absorbed with the appropriate antigen. The results of these experiments are presented in Table III. Whether the I-J⁺ molecule was induced by SRBC-, HRBC-, or BRBC-specific TsF, this supernatant could work with either the SRBC- or the HRBC-specific Ly2 TsF to effect suppression. Such immunosuppressive complexes exhibited the antigen specificity of the Ly2 TsF used in the assay culture, not the one used to induce this molecule in the primary culture.

Cellular Requirements That Allow Induction of I-J⁺ Material by Ly2 TsF: Successful Induction Requires Both an I-A⁺ and an Ly1 I-J⁺ Cell in the Primary Culture. We investigated the nature of the cells required for Ly2 TsF and antigen to induce the production of I-J⁺ molecule (Table IV). Normal B6 spleen cells were depleted of various subsets of cells and assayed for the ability to produce I-J⁺ material in a primary culture after exposure to Ly2 TsF and antigen. Our results indicate that while Ig⁺ and Ly2⁺ cells are not required for the induction of I-J⁺ material, cells bearing I-A, I-J, Thy-1, and Lyt-1 markers are required for the induction

TABLE III
I-J⁺ Material Induced by Ly-2 TsF is Antigen Nonspecific

Ly-2 TsF in primary culture*	Ly-2 TsF in assay culture [‡]	Anti-SRBC PFC/culture [§]	Anti-HRBC PFC/culture [¶]
—	—	3,600	1,000
—	SRBC [†]	3,400	900
—	HRBC	3,100	950
SRBC	—	3,400	1,200
	SRBC	1,200	800
	HRBC	2,900	400
HRBC	—	3,500	1,100
	SRBC	1,700	900
	HRBC	3,000	300
BRBC	—	4,300	1,400
	SRBC	2,100	1,100
	HRBC	3,900	500

* Primary cultures were naive B6 Ly1⁺,2⁻ T cells incubated with appropriate Ly2 TsF diluted 1:5 specificity, and homologous erythrocytes for 48 h. Supernatant was then harvested, absorbed with the immunizing antigen, and added to assay cultures at a final dilution of 1:2.

[‡] Assay cultures were B6 spleen cells depleted of Thy-1 cells, B6 Ly1⁺,2⁻ I-J⁻ T cells, Ly2 TsF diluted 1:10, and the appropriate antigen.

[§] Primary in vitro response of B6 mice to SRBC.

[¶] Primary in vitro response of B6 mice to HRBC.

[†] Antigen used to immunize B6 mice to produce factor.

TABLE IV
Cellular Requirements That Allow the Induction of I-J⁺ Material by Ly2 TsF

Group	Treatment of cellular source of I-J ⁺ material*	Anti SRBC PFC/culture [‡]	
		Without Ly2 TsF	With Ly2 TsF
1	No I-J ⁺ material	1,900	1,600
2	None	1,700	300
3	Ig ⁺ cell-depleted	1,700	600
4	Anti-Thy-1 treated	1,800	1,700
5	Anti-Ly1 treated	2,000	1,800
6	Anti-Ly2 treated	2,200	500
7	Anti-I-J ^b treated	1,800	2,000
8	Anti-I-A ^b treated	1,700	1,700
9	Groups 5 and 7 [§]	1,800	2,000
10	Groups 5 and 8	2,200	500
11	Groups 7 and 8	1,700	600

* 1.5×10^7 cells were treated with various antisera and incubated for 48 h with SRBC and Ly2 TsF diluted 1:5. Supernatants were then harvested, cleared, and absorbed with SRBC before being added to assay cultures.

[‡] See footnote *, Table I.

[§] Cells from groups 5 and 7, 5 and 8, or 7 and 8 were treated with antisera and complement and mixed before primary culture with Ly2 TsF and antigen.

event. We then mixed various cell populations after negative selection to determine whether all these markers were on the same set of cells. Groups 8–10 indicate that while Ly1 and I-J markers are on the same cell types, IA markers define a different cell subset that is also required for the induction event to take place.

Genetic Requirements That Allow Induction of I-J⁺ Material by Ly2 TsF: Ly2 TsF Must Match Cells That Produce the I-J⁺ Material at the IE Subregion of the MHC-linked Polymorphisms for Induction to Occur. Since Ly2 TsF must match the cell that produced the I-J⁺ molecule at the I-J subregion of the MHC in order for the functional molecular complex of Ly2 TsF and I-J⁺ material to suppress Ly1 I-J⁻ T plus B cells, we investigated the genetic restrictions involved in the induction of the I-J⁺ molecule by Ly2 TsF. To do this Ly1 cells from B6 mice were cocultured with a battery of Ly2 TsF produced in MHC and Igh congenic mice. The resulting supernatants were then absorbed with antigen and assayed for the presence of I-J⁺ material on B6 cultures of Ly1 I-J⁻ T plus B cells by the addition of syngeneic Ly2 TsF. The results in Table V show that successful induction of the I-J⁺ molecule by Ly2 TsF requires MHC homology between the cells that produce the Ly2 TsF, and cells that produce the I-J⁺ material.

We went further to localize the genetic homology requirements between the Ly2 TsF and the induced cell population within the MHC. Ly2 TsF from a variety of MHC recombinant mice were used to induce I-J⁺ material from B10.BR Ly1 T cells (Table VI). It was found that successful induction required genetic

TABLE V
Genetic Requirements That Allow the Induction of I-J⁺ Material by Ly2 TsF: Ly2 TsF Must Match Cells That Produce the I-J⁺ Material at MHC for Functional Activity

Ly2 TsF in primary culture**	Identity of Ly2 TsF with B6 cells in primary culture		Ly2 TsF in assay culture†	Anti-SRBC PFC/culture‡	
	H-2	Igh		I	II
—			—	3,300	3,400
—			B6	2,600	4,200
B6	+	+	B6	1,100	1,100
B.C9	+	—	B6	1,000	1,200
C.B20	—	+	B6	2,800	3,500
BALB/c	—	—	B6	2,800	3,600
BALB.B	+	—	B6	1,400	ND [†]
B10.D2	—	+	B6	3,000	ND
BALB.K	—	—	B6	2,600	ND
B10.BR	—	+	B6	2,700	ND

* The I-J⁺ material was obtained by incubating Ly1⁺,2⁻ T cells from B6 mice with the indicated Ly2 TsF diluted 1:5 for 48 h as described and subsequently absorbing the supernatant with antigen before adding to the assay culture.

‡ SRBC-specific.

† Results are from assay cultures that contained 2×10^6 Ly1⁺,2⁻ I-J⁻ T cells, 2×10^6 anti-Thy-treated spleen cells, Ly2 TsF diluted 1:10, supernatant from primary cultures diluted 1:2, and SRBC. Boldface numbers represent cultures that showed significant suppression (>50%) of the PFC response. I and II represent results from independent experiments.

† ND, not done.

TABLE VI

Genetic Requirements That Allow the Induction of I-J⁺ Material by Ly2TsF: Ly2TsF Must Match Cells That Produce the I-J⁺ Material at the IE Subregion of the MHC for Functional Activity

Ly2TsF in primary culture* [‡]	MHC Identity of Ly2TsF with B10.BR cells in primary culture						Ly2TsF in assay culture	Anti-SRBC PFC/culture [§]	Suppression [†]
	K	A	J	E	C	D			
—							—	4,300	
—							B10.BR	4,100	—
B10.BR	+	+	+	+	+	+	B10.BR	1,100	+
B10	—	—	—	—	—	—	B10.BR	3,200	—
B10.A	+	+	+	+	—	—	B10.BR	1,400	+
B10.A(2R)	+	+	+	+	—	—	B10.BR	700	+
B10.A(3R)	—	—	—	+	—	—	B10.BR	1,800	+
B10.A(4R)	+	+	—	—	—	—	B10.BR	3,400	—
B10.A(5R)	—	—	+	+	—	—	B10.BR	800	+

* The I-J⁺ material was obtained by incubating Ly1⁺, 2⁺ T cells from B10.BR mice with the indicated Ly2TsF diluted 1:5 for 48 h as described and subsequently absorbing the supernatant with antigen before adding to assay culture.

[‡] SRBC-specific.

[§] Results are from B6 assay cultures that contained T cells, B cells, B10.BR Ly2TsF diluted 1:10, supernatants from primary cultures diluted 1:2, and SRBC as described. Boldface numbers represent cultures that showed significant suppression.

[†] Cultures that showed >50% reduction in the PFC response were deemed positive for suppression.

homology between the Ly2 TsF and the cells producing the I-J⁺ material at the IE subregion of the MHC.

In every case where this criteria is not met, there is no detectable biologically active I-J⁺ material found in the 48-h culture supernatant.

Genetic Requirements That Allow Ly1 I-J⁺ Material Induced by Ly2 TsF to Assist Ly2 TsF in Suppressing B6 Assay Cells Depleted of I-J⁺ Ly1 Cells; I-J⁺ Material Must Match Ly2 TsF at H-2 and Acceptor Cells at Igh-linked Polymorphisms for Functional Activity. Previous results (3) have shown that in order for the I-J⁺ molecule from Ly1 TsF to work in consort with the Ly2 TsF to suppress Ly1 I-J⁻ T plus B cells, the cell that secretes the I-J⁺ molecule had to: (a) match the cell that secreted the Ly2 TsF at MHC-linked polymorphisms; and (b) match the acceptor cell population at Igh-linked polymorphisms. We investigated whether the I-J⁺ molecule induced by the Ly2 TsF in the preculture behaved in a similar fashion. The results of two separate experiments of this type are given in Table VII. These results indicate that only three combinations of Ly2 TsF and I-J⁺ material were able to suppress B6 Ly1 T cells that lacked I-J⁺ cells. The first was the syngeneic mixture (B6 (H-2^b, Igh^b) I-J⁺ piece together with B6 Ly2 TsF on B6 cells). In keeping with our earlier findings, C.B20 (H-2^d, Igh^b) I-J⁺ pieced together with BALB/c (H-2^d, Igh^a) Ly2 TsF could suppress B6 cells while the reverse, B.C9 I-J⁺ pieced with B6 Ly2 TsF, could not. In addition, B10.D2 (H-2^d, Igh^b) I-J⁺ material plus BALB/c Ly2 TsF, derived from strains that match each other only at the MHC, worked perfectly well in suppressing B6 spleen cells, which matches B10.D2 at Igh-V. The mapping of the Igh restriction to the interaction of acceptor cells and the I-J⁺ material was strengthened by the observation that C.B20 I-J⁺ material plus BALB/c Ly2 TsF worked in restoring suppression on

TABLE VII
*I-J⁺ Molecule Induced by Ly-2 TsF Must Match Ly-2 TsF at H-2 and Acceptor Cells
 at Igh-linked Polymorphisms for Functional Activity*

Strain source of I-J ⁺ molecules*†	Ly-2 TsF in assay culture	Anti-SRBC PFC/culture‡	
		B6	B.C9
—	—	4,300	1,800
—	B6	3,900	1,900
—	BALB/c	3,800	2,100
B6(H-2 ^b , Igh ^b)	—	4,900	2,100
	B6	1,200	1,800
	BALB/c	3,700	1,700
B.C9(H-2 ^b , Igh ^a)	—	4,700	2,000
	B6	5,000	300
	BALB/c	5,600	1,800
BAB.14(H-2 ^d , Igh-V ^a)	—	4,000	1,900
	B6	3,700	2,200
	BALB/c	4,900	400
C.B20(H-2 ^d , Igh ^b)	—	4,700	1,700
	B6	4,100	1,600
	BALB/c	800	1,800
BALB/c(H-2 ^d , Igh ^a)	—	4,700	1,800
	B6	3,200	1,600
	BALB/c	4,000	200
B10.D2(H-2 ^d , Igh ^b)	—	4,000	2,100
	B6	3,700	1,900
	BALB/c	1,100	1,800

* I-J molecules were obtained from primary cultures of Ly1 T cells, syngeneic Ly2 TsF, and antigen from the given strains. Haplotype of strains used: B6 (H-2^b, Igh^b); Balb/c (H-2^d, Igh^a); B.C9 (H-2^b, Igh^a); C.B20 (H-2^d, Igh^b); Bab.14 (H-2^d, Igh-C^b Igh-V^a); B10.D2 (H-2^d, Igh^b). Supernatants were absorbed with SRBC before being added to assay culture.

† See footnote †, Table I.

‡ Assay cultures contained 2×10^6 Ly1⁺ I-J⁻ T cells, 2×10^6 B cells, Ly2 TsF diluted 1:5, and SRBC. Boldfaced numbers indicate cultures in which significant (50%) suppression was seen.

B6 but not B.C9 assay cultures, while B.C9 (H-2^b, Igh^a) I-J⁺ material with B6 Ly2 TsF did not.

The use of B.C.9 mice (H-2^b, Igh^a) confirmed the requirement for Igh matching in the interaction of the Ly2 TsF I-J⁺ suppressor complex with its T_H cell target. Combinations of suppressor factors unable to suppress B6 cells (Igh^b) but fully capable of suppressing the Igh congenic B.C9 (Igh^a) cells were: B6 Ly2 TsF with B.C9 I-J⁺ chain, BALB/c Ly2 TsF with BAB.14 I-J⁺ chain, and BALB/c Ly2 TsF with BALB/c I-J⁺ chain. These results are consistent with the idea that the I-J⁺ molecule found in Ly1 cell supernatant that restores suppressive activities to

the Ly2 TsF in cultures depleted of the I-J⁺ cells (3) is similar to the molecule we find induced by Ly2 TsF in the intermediate culture.

The I-J⁺ Molecule Induced by Ly2 TsF Cannot Functionally Replace the I-J⁺ Molecule in Ly1 TsiF. Since previous results have shown that a I-J⁺ molecule from Ly1 cell supernatants could mimic the functional activity of I-J⁺ molecules induced by Ly2 TsF (3), it remained to be determined whether the I-J⁺ molecule needed for Ly1 TsiF activity was functionally identical to the I-J⁺ molecule induced by the Ly2 TsF in vitro. To solve this dilemma, we reasoned that while immune Ly1 cell supernatants may contain a great number of I-J⁺ molecules, I-J⁺ molecules induced by Ly2 TsF in vitro may be much more restricted in their activity, i.e., may only work with Ly2 TsF to effect suppression on Ly1 T cells. To test this hypothesis, we asked whether I-J⁺ molecules induced by Ly2 TsF could mimic the I-J⁺ molecule needed for Ly1 TsiF activity. Ly1 TsiF was fractionated into antigen-binding and I-J⁺ chains by passage over an anti-I-J immunosorbent, and the eluted I-J⁺ molecules were added to either Ly1 TsiF I-J⁻ chain or Ly2 TsF to test for its ability to reconstitute suppressor activity. Likewise, I-J⁺ material induced in primary culture by syngeneic Ly2 TsF was used after absorption with antigen to reconstitute Ly1 I-J⁻ chain or Ly2 TsF activity. Ly1 TsiF activity was measured on whole T plus B cultures, while Ly2 TsF activity was measured on Ly1⁺,2⁻ I-J⁻ T plus B cultures. The results, in Table VIII, shows that I-J⁺ material from either Ly1 cell supernatant or Ly2 TsF-induced cultures can restore Ly2 TsF activity (lines 7-9), but only I-J⁺ material from Ly1 cell

TABLE VIII
I-J⁺ Molecule Induced by Ly-2 TsF Does Not Substitute for the I-J⁺ Molecule Needed for Ly1 TsiF Activity

Source of I-J ⁺ molecule*	Source of Ag binding chain [‡]	Anti-SRBC PFC/culture	
		Whole T + B [§]	Ly1 I-J ⁻ T + B [¶]
—	—	3,100	3,300
Ly1 CS	—	2,700	3,400
Ly-2 TsF induced	—	2,600	2,800
—	Ly-1-CS	3,300**	ND [†]
Ly1 CS	Ly-1-CS	1,100	ND
Ly-2 TsF induced	Ly-1-CS	3,000 ^{‡‡}	ND
—	Ly-2 TsF	800	3,300
Ly1 CS	Ly-2 TsF	600	1,500
Ly-2 TsF induced	Ly-2 TsF	500	1,000

* I-J⁺ material was either isolated from Ly1 cell supernatant (CS) by absorption with SRBC or was made from primary cultures of B6 Ly1 T cells, SRBC-specific Ly2 TsF, and SRBC as detailed in Materials and Methods.

[‡] SRBC-specific. Ly1 antigen (Ag) binding chain was obtained by passage of Ly1 TsiF over an anti-I-J immunosorbent.

[§] Assay for Ly1 TsiF activity.

[¶] Assay for Ly2 TsF-I-J⁺ chain activity.

[†] ND, not done; no suppression expected in these groups.

** Important negative result showing Ly1 antigen-binding chain does *not* induce I-J⁺ material needed for Ly1 TsiF activity.

^{‡‡} Important negative result showing I-J⁺ chain induced by Ly2 TsF does not work with Ly1 TsiF antigen-binding chain.

supernatants could restore the suppressive activity of Ly1 I-J⁻ antigen-binding chain (lines 4-6). Therefore, it appears that while Ly1 TsiF contains I-J⁺ material that has more than one activity, the I-J⁺ material induced by Ly2 TsF can only complement with Ly2 TsF for suppressive activity, and does not contain the activity necessary to replace the I-J⁺ material normally involved in the induction of suppression of Ly1 TsiF.

Discussion

We have previously found a product of hyperimmune Ly2 T cells (Ly2 TsF) to be a one-chain suppressor factor that is antigen-specific and can directly suppress Ly1 T cells (1), so long as these cells share MHC polymorphisms with the cells producing the Ly2 TsF. However, the action of this molecule was found to be dependent on an intermediary cell, an Ly1 I-J⁺ T cell that was not the final target of suppression. The activity of this cell could be functionally replaced by the addition of I-J⁺ material secreted by Ly1 T cells from hyperimmune mice (3). The cellular source of this I-J⁺ material had to share I-J-linked polymorphisms with the cellular source of the Ly2 TsF, and Igh-V-linked polymorphisms with the target cells in order to form a suppressive molecular complex. Therefore, while Ly2 TsF is a one-chain molecule, the ultimate molecular complex that effects suppression is made up of two chains: the Ly2 TsF and an I-J⁺ molecule supplied by the assay population.

In the present set of experiments, we investigated the mechanism by which Ly2 TsF induces production of an I-J⁺ molecule from the assay population, and how these two molecules interact to effect suppression. We found that (a) Ly2 TsF, presumably with the help of antigen and an I-A⁺ APC, induces an I-J⁺ antigen nonspecific molecule from an Ly1 I-J⁺ T cell in the assay population that appears to act selectively with the Ly2 TsF molecule; (b) this induction requires homology in the IE region of the MHC between cells producing Ly2 TsF and those producing the I-J⁺ molecule; (c) the interaction of the two molecules requires MHC homology and (d) the suppressive complex acts on Ly1⁺,2⁻ I-J⁻ T+B cells but changes the genetic homology requirements from the need for the cellular source of Ly2 TsF to match the target cells at MHC-linked polymorphisms to the need for target cells to match the cellular source of the I-J⁺ chain at Igh-V linked genes. Therefore, the MHC restriction observed using Ly2 TsF reflects a requirement for interaction with the cells providing the I-J⁺ partner molecule Ly2 TsF requires to effect suppression, and not a restriction in its ability to interact with the final target cell of suppression. The ultimate genetic restriction of the suppressor complex of Ly2 TsF and the I-J⁺ molecule resides strictly in the I-J⁺ molecule, and not the Ly2 TsF.

These results complement and extend our earlier findings that the Ly2 TsF requires two separate macromolecules to mediate suppressive activity (3). We have been studying two different suppressor factors in our laboratory that also require two separate molecules for activity: Ly1 TsiF (4), an antigen-specific T cell factor secreted by Ly1 T cells that induces suppressor activity, and TNP-TsF (7), a factor that acts to inhibit the activity of immune Ly1 T cells that transfer contact sensitivity. A number of other laboratories have also reported

two-chain antigen-specific suppressor factors, including Taniguchi et al. (8), and Taussig and Holliman (9).

Since it is clear that the Ly2 TsF molecular complex is composed of two chains, there is some question as to which molecule contains the suppressive information. We believe that the functional suppressor information is on the antigen-binding molecule of the Ly2 TsF. There are two main reasons for this assignment: (a) Fresno et al. (10, 11) have shown that function suppressor activity resides in the C terminal piece of the antigen-binding chain of a clonal T suppressor effector factor; and (b) we have shown that an artificially constructed molecular hybrid complex consisting of antigen-binding Ly2 TsF and the B chain of cholera toxin (the cholera toxin molecule consists of two chains with the A chain containing the functionally active site while B chain serves as the schlepper molecule (12)) can suppress lymphoid cells that express GM-1, the cell surface receptor for cholera toxin B chain (Wang et al., manuscript in preparation). This functional similarity between cholera toxin B chain and the I-J⁺ molecule is especially intriguing since it assumes a focusing function for the I-J⁺ molecule. This similarity may help to explain the mechanism of recognition and internalization of the suppressive moiety of this suppressor complex and help us to form a model for suppressor factor activity that may be a biological generalization.

The initial event is the antigen-dependent induction of I-J⁺ molecules by the antigen-binding piece of the Ly2 TsF. The induction of factors in suppressor circuits by other factors has been previously described (13). In these experiments, TsF factors can be induced by in vitro immunization with Ts1 factors in the absence of antigen. The fact that antigen is required in our system suggests that antigen may play an important role in "activating" the TsF to initiate the cellular interaction. The antigen-activated TsF then induces an I-J⁺ cell to secrete a cell-free product that functionally replaces the activity of this cell. This induction process also requires the presence of a cell that is I-A⁺, Ig⁻, and Thy-1⁻. Previously, investigators have found that I-J (14-17) and I-C (18) regions restrict the induction of various cell types in suppressor cell circuits, while I-A and I-E gene products have been primarily in the induction of T helper cells (19). The finding of I-A⁺ and I-E⁺ cells involved in the induction of certain cells in the suppressor circuit may represent the involvement of these certain cell types both in the induction of helper and suppressor T cell subsets. Recently, Habu et al. (20), found an I-J⁺, I-A⁺, I-E⁺ non-T, non-B cell was required for the in vitro response of normal spleen cells to SRBC. It is quite possible that this cell, presumably an APC, may also be required for transducing suppressor cell signals. Recently Minami et al. reported that I-J⁺ cells, which they call FPC (factor-presenting cells), are involved in the induction of various cells in their suppressor circuit (21). Experiments are currently underway to delineate the involvement of different cell types in the induction of the I-J⁺ molecule by Ly2 TsF.

The nature of the interactions involved in the induction of the I-J⁺ molecule is currently unknown, but almost certainly involves the recognition of a cell surface molecule on the surface of the Ly1 I-J⁺ cell. This recognition, presumably by the Ly2 TsF, results in the activation of this cell to begin secreting a molecule that now works in consort with the Ly2 TsF to mediate antigen-specific suppression. This molecular interaction may involve an I-J and anti-I-J like immune

reaction. In any case, the functionally active molecular complex can then interact with its target cell and deliver the signal carried by the functionally suppressive antigen-binding chain. The focusing event mediated by the I-J⁺ chain and the target cell receptor is influenced by Igh-V-linked genes. This places the H-2 restriction of the Ly2 TsF on the induction and interaction with the I-J⁺ molecule, and the subsequent Igh-V restriction on the interaction of the schlepper molecule with its target cell.

It therefore appears that while the Ly2 TsF under the appropriate conditions has potent suppressor activity, it also has the ability to induce a cell activity as well, namely the production of I-J⁺ chain production, depending on which cell this factor interacts with. Although it remains to be determined whether it is the same molecule that induces the I-J⁺ chain production that later works in tandem with such molecules to effect suppression, an interesting mechanism may be at work here: a single molecular entity may have both an inducing and suppressing activity depending upon the target cell. It remains to be seen whether other suppressor-effector molecules work in a fashion similar to the one described here.

While these experiments raise a number of important questions about the nature of cellular interactions in the suppressor circuit, even more perplexing problems are raised about the nature of the genetic restrictions exhibited by cell-free suppressor factors: (a) what is the nature of the genetic restriction of the induction of the I-J⁺ molecule by Ly2 TsF, while subsequent functional interaction between these two molecules is I-J restricted? Our results suggest that the genetic restriction maps to the I-E subregion of the MHC. What is the relationship between I-J and IE determinants, if any?; (b) where is the structural gene for the Ly2 TsF coded, and how does a single molecular entity contain specificity for antigen and MHC region restrictions? and (c) how does a product of the 17th chromosome get onto a molecule with structural gene products from the 12th chromosome (22), as is the case with the I-J⁺ schlepper under investigation?

In conclusion, we would like to put forth the notion that the molecular complex that effects suppression in this system has seven functionally active molecular sites (Fig. 1): (a) the MHC restricting element on Ly2 TsF that interacts with the IA⁺ APC; (b) the inducing site on Ly2 TsF for the I-J⁺ molecule; (c) the suppressive moiety on the Ly2 antigen-binding TsF; (d) the I-J⁺ determinant on the schlepper molecule; (e) the anti-I-J receptor on the Ly2 TsF, which imparts an I-J subregion genetic restriction on the interaction of Ly2 TsF and the I-J⁺ molecule; (f) the Igh-V restricting element on the I-J⁺ schlepper chain; and (g) the antigen-binding receptor on the Ly2 TsF, which imparts antigen specificity to the factor. These seven sites must work in consort to form an antigen-specific suppressor effector molecule.

Summary

A cell-free product secreted by Ly1⁻2⁺ T cells (Ly2 TsF) can suppress the *in vitro* response to sheep erythrocytes (SRBC) of spleen cells depleted of Ly2⁺ T cells. This suppressor factor expresses biological activity only when the acceptor cells share major histocompatibility complex (MHC)-linked polymorphic genes with the cells that made the Ly2 TsF. Removal of Ly1 I-J⁺ cells from the assay

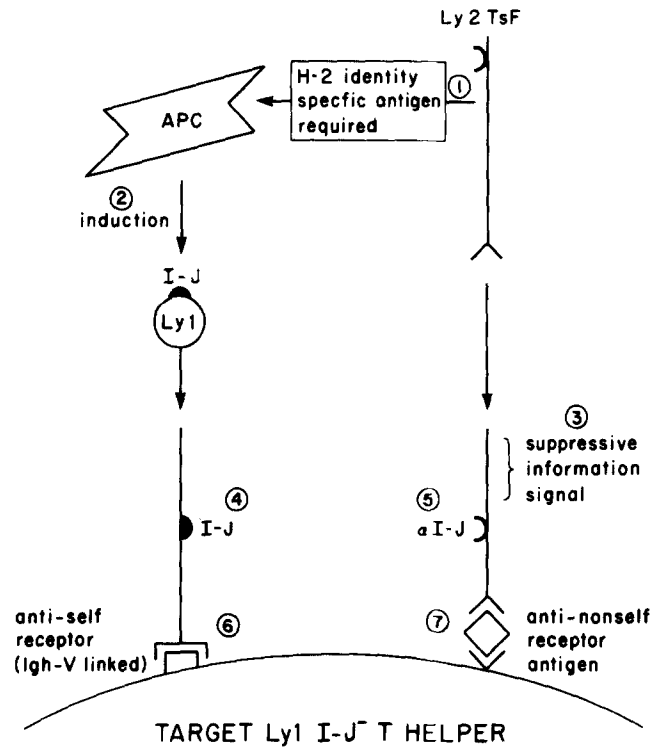


FIGURE 1. Seven active sites involved in the suppressive effects of Ly2TsF (see Discussion).

culture abrogates the ability of Ly2 TsF to suppress these cultures, but we can replace the need for the I-J⁺ cells in the assay culture with an I-J⁺ soluble factor derived from them. We investigated the cellular interactions involved in the activation of I-J⁺ cells by Ly2 TsF *in vitro*. We have been able to induce the production of an I-J⁺ molecule needed for Ly2 TsF activity in a 48-h intermediate culture of B cell-depleted Ly1 spleen cells, Ly2 TsF, and antigen. This molecule not only fails to bind antigen, but is also antigen nonspecific in that it can be induced by Ly2 TsF of irrelevant specificities. In order to replace the activity of the Ly1 I-J⁺ cell in the assay culture, the cell induced by Ly2 TsF to produce the I-J⁺ molecule *in vitro* must share genetic polymorphisms linked to the MHC with the Ly2 TsF, and genetic polymorphisms linked to the Igh-V gene complex with the target cell. In order for Ly2 TsF to induce cells of the primary culture to produce the I-J⁺ molecule, Ly2 TsF must share genetic polymorphisms linked to the IE region of the MHC with the Ly1 I-J⁺ cell producing the I-J⁺ molecule.

These results indicate that the suppressive mechanism of Ly2 TsF involves the interaction with an Ly1 I-J⁺ molecule. This I-J⁺ molecule serves to focus the antigen-specific suppressor molecule on the target cell. The recognition event of this suppressive complex on the surface of the acceptor cell is controlled by Igh-V-linked genes restricted by the I-J⁺ molecule of the suppressor complex. This suppressor interaction is confined to the suppressor effector phase of the suppressor circuit since the I-J⁺ molecules needed for by Ly2 TsF activity do not substitute for the I-J⁺ molecules needed for the activity of Ly1 TsiF, a T cell

factor that initiates the suppressor cell circuit. Thus, two distinct I-J⁺ Ly1 cell-derived molecules, each carrying V_H-linked genetic restrictions, must exist in the suppressor circuit.

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