

# HAPTEN-SPECIFIC T CELL RESPONSES TO 4-HYDROXY-3-NITROPHENYL ACETYL

## XI. Pseudogenetic Restrictions of Hybridoma Suppressor Factors\*

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The sequence of cellular interactions resulting in the suppression of cellular and humoral immune responses to the hapten 4-hydroxy-3-nitrophenyl acetyl (NP)<sup>1</sup> has been well characterized (1-3). At least three distinct subpopulations of NP-induced T lymphocytes have been identified in this suppressor cell circuit (1, 2). The first cell population, termed Ts<sup>i</sup> or Ts<sub>1</sub>, functions during the induction phase of the immune response and bears an antigen-specific idiotypic receptor (1, 4). The Ts<sup>i</sup> population induces a second population of suppressor cells, known as Ts<sup>e</sup> or Ts<sub>2</sub> (1, 5). This Ts<sup>e</sup> population functions during the effector phase of the immune response (after antigen priming) and bears an anti-idiotypic receptor (1, 2). The target of the Ts<sup>e</sup> cells are an antigen-primed Ts<sub>3</sub> population, which is thought to represent the final effector suppressor cells (2). The conclusions derived from the NP system are consistent with most other observations relating to the cellular interactions involved in immune suppression (6). Thus, the NP system may represent a general model that elucidates the overall pathway of immunoregulation.

To analyze the Ts antigen receptors and the mechanisms of cellular communication between the various cell populations, we have prepared a series of monoclonal T cell hybridomas (7). These hybrids correspond to the Ts<sup>i</sup> population and produce a biologically active T cell-derived suppressor factor (TsF) that can substitute for the Ts<sup>i</sup> population. This report characterizes the TsF, the genetic restrictions of this factor, and the second-order suppressor cells induced by the factor. The results extend previous work in the NP system and yield a more complete model of the mechanism of immune suppression. The data further demonstrate that hybridoma TsF can suppress both cellular and humoral immune responses.

### Materials and Methods

*Mice.* All mice were either purchased from The Jackson Laboratory, Bar Harbor, Maine, or were bred in the animal facilities at Harvard Medical School, Boston, Mass. Mice were used

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<sup>1</sup> *Abbreviations used in this paper:* CS, cutaneous sensitivity; DMSO, dimethyl sulfoxide; DNFB, 2,4-dinitrofluorobenzene; FCS, fetal calf serum; GAT, polypeptide of L-glutamic acid, L-alanine and L-tyrosine; HBSS, Hanks' balanced salt solution; KLH, keyhole limpet hemocyanin; NP, 4-hydroxy-3-nitrophenyl acetyl hapten; NP<sup>b</sup>, common idio type on C57BL anti-NP antibodies; NP-O-Su, NP-O-succinimide ester; PFC, plaque-forming cells; SRBC, sheep erythrocytes; Ts<sup>i</sup>, first order suppressor T cells; Ts<sup>e</sup>, second order suppressor T cells; Ts<sub>3</sub>, third order suppressor T cells; TsF, T cell-derived suppressor factor.

at 3–12 mo of age, and were maintained on laboratory chow and acidified, chlorinated water *ad libitum*.

**Antigens.** NP-O-succinimide (NP-O-Su) was purchased from Biosearch Co., San Rafael, Calif. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific Co., Pittsburgh, Pa. NP-keyhole limpet hemocyanin (KLH) and NP-Ficoll were conjugated by the reaction of NP-O-Su with AECM<sup>7b</sup>-Ficoll (Biosearch Co.) or KLH (Sigma Chemical Co., St. Louis, Mo.) as described previously (3). The NP-KLH and NP-Ficoll had an average of 30 and 17 NP groups per 100,000 mol wt, respectively.

**Antigen-specific T Cell Hybridomas and TsF.** The methods for preparation of antigen-specific T cell hybridomas have been described previously (7). Briefly, antigen-specific suppressor T cells were hybridized with BW5147 tumor cells. The hybrids with I-J and NP<sup>b</sup> idiotypic cell surface markers, i.e., CKB-17, CKB-39, and B6-29, were selected for further analysis. The B6-29 cells were cloned. The cell lines and clones derived from the parent lines were periodically passed over antigen plates and the NP-adherent cells were used to maintain the cell line. All of the hybridomas were cultured in RPMI-1640 containing 8% fetal calf serum (FCS). The TsF employed in the present experiments was collected from the cultured supernate of the hybridomas, when their cell numbers were  $\sim 7 \times 10^5$  cells/ml in the above medium. Our previous results (7) indicated that the CKB (Igh<sup>b</sup>, H-2<sup>k</sup>)-derived hybrids, designated CKB-17 and CKB-39, and the C57BL/6 hybrid, B6-29, possess I-J and NP<sup>b</sup> idiotypic determinants.

**Adsorption and Elution of TsF.** The methods of adsorption and elution of TsF using protein-conjugated Sepharose 4B columns were described in detail previously (7).

**Assay for the Ability of TsF to Induce Ts<sup>e</sup>.** To make the Ts<sup>e</sup> cells for *in vivo* studies, the TsF (0.3 ml) from each hybridoma was injected into naive mice intravenously for 4 d. On day 6, spleens from these mice were removed, and a single cell suspension was prepared in Hanks' balanced salt solution (HBSS).  $4 \times 10^7$  spleen cells were washed with HBSS three times and injected into NP- or dinitrofluorobenzene (DNFB)-primed mice. These recipient mice were then challenged with NP or DNFB antigen and the swelling responses were measured 24 h later. To make the Ts<sup>e</sup> suppressor cells for *in vitro* studies, 0.2 ml TsF was injected into normal C57BL/6 mice intravenously for 5 d. 1 d after the last injection, spleen cells from these mice were removed under sterile conditions, teased, and washed extensively, and  $5 \times 10^5$  viable cells were added to responder cultures, which had been challenged 4 d earlier with NP-Ficoll or sheep erythrocytes (SRBC). 1 d later, responder cells were assayed for NP and SRBC-specific plaque-forming cells (PFC).

**Assay for Suppressive Activity of TsF on NP-mediated Cutaneous Sensitivity (CS) Responses.** The assay of CS responses was described elsewhere (8). Briefly, each animal was primed subcutaneously with 7 mg of NP-O-Su in DMSO. Soon after priming, 0.3–0.4 ml of each hybridoma supernate, BW5147-cultured supernate, or fractionated samples were injected intravenously. Unless indicated otherwise, these intravenous injections were given for 4 d beginning on the day of antigen priming.

6 d after immunization, 24  $\mu$ g of NP-O-Su in 0.025 ml of phosphate-buffered saline, pH 7.2, was injected into the left footpad. Footpad swelling was measured 24 h after challenge. Swelling was determined as the difference, in units of  $10^{-3}$  cm, between the left and right footpad thickness.

**In Vitro Responder Cultures.** C57BL/6 (B6) mice were immunized intraperitoneally with 150  $\mu$ g NP-KLH with 25% pertussis vaccine. 4 wk later, their spleens were removed and teased into a single cell suspension under sterile conditions. Cells were washed and resuspended in Mishell-Dutton media containing 10% FCS.  $7.5 \times 10^6$  viable NP-primed cells were added to each well of Linbro culture plates. Cultures were challenged with 100 ng NP-Ficoll in 20  $\mu$ l. A primary SRBC response was obtained by the addition of  $2 \times 10^6$  washed SRBC to culture wells containing  $7.5 \times 10^6$  normal, unprimed spleen cells. All cultures were incubated in rocking boxes at 37°C in a 10% CO<sub>2</sub> atmosphere and were fed daily with 0.1 ml supplementary medium. 4 d after challenge,  $5 \times 10^5$  viable control or suppressor cells were added. 5 d after challenge, duplicate wells were pooled and assayed for total NP- or SRBC-specific PFC response in triplicate slides.

**PFC Assay.** NP- or SRBC-specific PFC were assayed as reported previously (3).

**DNFB Responses.** CS was induced by two daily paintings on the shaved abdomen with 25

$\mu\text{l}$  of 0.5% DNFB solution (Sigma Chemical Co) in a 4:1 dilution of acetone:olive oil (2). 6 d after the last painting, 20  $\mu\text{l}$  of 0.2% DNFB in the same vehicle was applied to the left ear, and the ear swelling was measured as the difference between the left and right ear thicknesses. When the specificity of TsF or Ts<sup>e</sup> cells were determined on DNFB responses, the injections were done as for CS responses to NP antigen.

*Percent Suppression.* The percent suppression in the present study was calculated by the following formula: percent suppression =  $100 \times (\text{swelling of BW tumor supernate-injected group} - \text{swelling of TsF injected group}) / (\text{swelling of BW tumor supernate-injected group} - \text{swelling of unprimed group})$ .

*Data Analysis.* Statistical analysis of the experimental data with respect to controls was performed by the two-tailed Student's *t* test.

## Results

*Specificity of Hybridoma TsF.* To assess the potency of the hybridoma-derived factors, 0.3 ml of various dilutions of TsF were injected daily into mice from the day of priming with NP-O-Su to the day before antigen challenge. The supernates from the BW5147 cell line, which was the parental tumor used for the hybridization, were used as a control. The C57BL/6-derived B6-29 factor was injected into C57BL/6 mice, and the CKB-derived factors were injected into H-2- and Igh-compatible B10.BR mice. As shown in Table I, TsF derived from the B6-29, CKB-17, and CKB-39 hybrid cell lines significantly suppressed the NP response when used undiluted or diluted at 1:10. At 1:100 dilutions, these factors demonstrated variable levels of suppression and the suppressive activity was no longer observed when the supernates were further

TABLE I  
*Specificity of Hybridoma Suppressor Factors*

Source of TsF	Strain tested	TsF dilution	Swelling Response $\pm$ SE*	
			NP-O-Su	DNFB
BW5147	C57BL/6	Undiluted	15.3 $\pm$ 1.6 (8)	17 $\pm$ 1 (4)
B6-29	C57BL/6	Undiluted	2.9 $\pm$ 1.2 (8)‡	16 $\pm$ 3 (4)
		1:10	3.4 $\pm$ 1.8 (7)‡	NT§
		1:100	8.0 $\pm$ 2.0 (7)	NT
		1:1,000	15.2 $\pm$ 0.5 (7)	NT
BW5147	B10.BR	Undiluted	20.2 $\pm$ 2.1 (8)	21.5 $\pm$ 2.2 (4)
CKB-17	B10.BR	Undiluted	9.8 $\pm$ 2.0 (5)‡	20.5 $\pm$ 3.2 (4)
		1:10	11.8 $\pm$ 1.7 (5)‡	NT
		1:100	20.5 $\pm$ 1.8 (4)	NT
		1:1,000	17.5 $\pm$ 1.0 (4)	NT
CKB-39	B10.BR	Undiluted	8.5 $\pm$ 3.0 (4)	19.5 $\pm$ 3.8 (4)
		1:10	7.0 $\pm$ 2.3 (5)‡	NT
		1:100	14.0 $\pm$ 2.9 (4)	NT
		1:1,000	21.8 $\pm$ 2.7 (4)	NT

\* Each group of mice was primed with NP-O-Su or DNFB, followed by daily intravenous injections of TsF until the day before antigen challenge. The results are expressed as the mean increment of footpad swelling  $\pm$  SE. The number of mice per group is indicated in parentheses. Background swelling in C57BL/6 mice was 3.2  $\pm$  1.7 (9) for NP-O-Su and 4  $\pm$  1 (4) for DNFB. Background swelling in B10.BR mice was 8.3  $\pm$  1.7 (8) for NP-O-Su and 3  $\pm$  1.2 (4) for DNFB.

‡  $P < 0.01$ .

§ Not tested.

||  $P < 0.05$ .

TABLE II  
*Immunochemical Characterization of Hybridoma Suppressor Factors*

Immunoabsorbent column	B6-29 fractions		CKB-17 fractions	
	Unbound	Eluate	Unbound	Eluate
None	86*		61	
Anti-I-J <sup>b</sup>	-22	81	59	9
Anti-I-J <sup>k</sup>	89	18	3	71
Anti-Ig	94	-20	87	9
Anti-NP <sup>b</sup>	12	46	ND‡	ND
NP-bovine serum albumin	-13	46	17	62

\* The data are expressed as average percent suppression. The results were obtained from three separate experiments.

‡ Not determined.

diluted to 1:1,000. The inability of these hybridoma-derived factors to suppress DNFB-induced CS responses demonstrates their antigen specificity (Table I).

*Characterization of TsF.* To characterize the biologically active factors produced by these cells, the B6-29 and CKB-17 hybrids were selected for further analysis. Culture supernates from these cells were passed over immunoabsorbent columns and the adsorbed fractions were eluted with glycine-HCl buffer, pH 3.2. The data in Table II demonstrate that the suppressive activity was absorbed by anti-idiotypic, anti-I-J, and antigen columns. Furthermore, suppressive activity could be specifically recovered by elution from these columns. Controls for these experiments include the ability of allele-specific anti-I-J alloantisera to specifically absorb these factors. Thus, anti-I-J<sup>b</sup> absorbed the B6-derived factor, whereas anti-I-J<sup>k</sup> absorbed the CKB-derived factor. The suppressor factor lacked conventional immunoglobulin determinants because polyvalent guinea pig anti-immunoglobulin antisera failed to absorb the suppressive activity (Table II). Furthermore, the activity could not be absorbed on an NP<sup>b</sup> idiotype-coupled column (data not shown). Thus, the TsF bear antigen-specific idiotypic receptors and I-J alloantigenic determinants.

*Suppression in the Induction vs. Effector Phase.* The Ts<sup>i</sup> and Ts<sup>e</sup> suppressor cell populations were initially designated based on their ability to suppress during the induction or effector phases of the immune response, respectively (1, 4, 5). To determine in which phase of the immune response the hybridoma suppressor factors were functional, they were injected either at the time of antigen priming (induction phase) or at the time of antigen challenge (effector phase). BW5147 supernates were used as controls. As shown in Table III, TsF obtained from the B6-29, CKB-17, and CKB-39 cell lines significantly suppressed NP responses during the induction phase, but not the effector phase, of the response. We have recently identified another I-J-bearing CKB-derived hybridoma cell line (#59) that suppresses NP-specific immune responses, but unlike the other hybrids, is not lysed by anti-NP<sup>b</sup> idiotypic antisera (7). In contrast to the B6-29, CKB-17, and CKB-39 TsF, the supernates from CKB-59 cells failed to suppress during the induction phase but demonstrated significant suppression during the effector phase of the NP CS response (Table III). Further characterization of the CKB-59 factor will be reported elsewhere.

*Ability of Suppressor Factor to Generate a Ts<sup>e</sup> Population.* The ability of B6-29, CKB-17, and CKB-39 hybridoma suppressor factors to function only during the induction

TABLE III  
*Comparison of the Ability of TsF to Suppress in the Induction vs. Effector Phases of the CS Response\**

TsF source	Strain tested	NP-O-Su-induced foodpad swelling $\pm$ SE	
		Induction phase	Effector phase
BW5147	C57BL/6	30.0 $\pm$ 1.8 (4)	30.0 $\pm$ 1.8 (4)
B6-29	C57BL/6	13.8 $\pm$ 0.6 (4)‡	31.0 $\pm$ 3.5 (4)
BW5147	B10.BR	15.2 $\pm$ 1.3 (6)	19.3 $\pm$ 1.3 (6)
CKB-17	B10.BR	4.0 $\pm$ 1.9 (5)‡	15.3 $\pm$ 1.1 (4)
CKB-39	B10.BR	9.3 $\pm$ 1.1 (10)§	21.7 $\pm$ 2.4 (6)
CKB-59	B10.BR	13.8 $\pm$ 0.5 (6)	5.2 $\pm$ 3.0 (6)‡

\* 0.4 ml TsF was injected either on the day of and the day after antigen priming (induction phase) or the day before and the day of antigen challenge (effector phase). Background swelling was <10 units.

‡ Significant suppression,  $P < 0.01$ .

§ Significant suppression,  $P < 0.05$ .

TABLE IV  
*Ability of TsF to Generate Antigen-specific Effector Phase Ts In Vivo\**

TsF source	Strain for Ts <sup>e</sup> generation	NP-O-Su responses		DNFB responses	
		NP-O-Su priming	Footpad swelling $\pm$ SE	DNFB priming	Ear swelling $\pm$ SE
BW5147	C57BL/6	+	14.3 $\pm$ 1.5 (4)	+	23.7 $\pm$ 1.8 (4)
B6-29	C57BL/6	+	4.8 $\pm$ 2.3 (5)‡	+	24.4 $\pm$ 1.7 (5)
—	C57BL/6	—	2.2 $\pm$ 2.3 (5)	—	3.6 $\pm$ 0.7 (8)
BW5147	B10.BR	+	20.4 $\pm$ 2.2 (8)	+	14.5 $\pm$ 3.1 (4)
CKB-39	B10.BR	+	10.7 $\pm$ 1.8 (9)‡	+	17.8 $\pm$ 1.4 (4)
—	B10.BR	—	4.7 $\pm$ 1.3 (9)	—	1.8 $\pm$ 0.9 (4)
BW5147	B10.A (2R)	+	21.8 $\pm$ 4.3 (4)	+	17.8 $\pm$ 1.4 (4)
CKB-17	B10.A (2R)	+	9.6 $\pm$ 1.9 (5)‡	+	18.7 $\pm$ 1.7 (3)
—	B10.A (2R)	—	2.4 $\pm$ 1.6 (5)	—	1.8 $\pm$ 0.9 (4)

\* 0.3 ml TsF was injected into each mouse on days 0, 1, 2, and 3. On day 6,  $4 \times 10^7$  spleen cells from these mice were transferred intravenously into syngeneic recipients, which had previously been primed with NP-O-Su or DNFB.

‡ Significant suppression,  $P < 0.05$ .

phase of the immune response suggested that these factors may be capable of inducing the production of Ts<sup>e</sup>. To test this prediction, 0.3 ml of suppressor factor was injected intravenously into Igh-matched naive recipients on days 0, 1, 2, and 3. On day 6,  $4 \times 10^7$  washed spleen cells were adoptively transferred intravenously into syngeneic recipients that had previously been primed with NP-O-Su or DNFB. The recipients were challenged ~1 h after cell transfer. As shown in Table IV, the B6-29-, CKB-17-, and CKB-39-derived suppressor factors generated a second population of suppressor cells that specifically suppressed NP responses. Thus, the Ts<sup>e</sup> generated by intravenous administration of hybridoma suppressor factor demonstrated 62–79% suppression of NP-induced CS responses and no suppression of DNFB responses. It is unlikely that the minute amounts of suppressor factor that may have been passively carried over into the transfer recipients caused this suppression, because the adoptive transfers were performed just before antigen challenge (effector phase) and previous

data (Table III) demonstrated that these suppressor factors would not function in the effector phase. Furthermore, complete H-2 compatibility between the cell producing the suppressor factor and the strain used to generate the effector phase suppressor cells is not required. Thus, the CKB-17-derived factor stimulated Ts<sup>e</sup> in B10.A(2R) mice. The CKB and B10.A(2R) strains share the H-2K through I-E regions of the H-2 complex but differ at the alleles for the I-C, S, and D regions.

*Suppression of In Vitro PFC Responses.* The previous series of experiments demonstrated the ability of hybridoma suppressor factor to modulate cellular immune responses. Because we have also demonstrated the specific suppression of NP-specific PFC responses (3, 9), we sought to determine whether hybridoma suppressor factor was also active on these in vitro responses. For these experiments, B6-29 or control hybridoma suppressor factors were administered intravenously into normal C57BL/6 recipients over a 5-d period. On day 6, spleen cells from these donors were washed and  $5 \times 10^5$  cells were added to NP-Ficoll or SRBC-stimulated Mishell-Dutton cultures. Duplicate wells were harvested 1 d later. As shown in Table V, the B6-29-generated suppressor cells specifically suppressed the in vitro NP response and showed no suppression of the in vitro SRBC response. The control hybridoma factors used in these experiments were derived from the same fusion as the B6-29 cell line but lacked

TABLE V  
Ability of B6-29 TsF to Induce Ts<sup>e</sup> That Suppress In Vitro NP-specific PFC Responses\*

Experiment	TsF source	Strain for Ts <sup>e</sup> induction	PFC responses‡	
			NP	SRBC
1	B6-29	C57BL/6	350 (70)	1,300 (-42)
	Control	C57BL/6	1,200	950
2	B6-29	C57BL/6	325 (64)	800 (-28)
	Control	C57BL/6	1,250	625

\* B6-29 or control hybridoma suppressor factors were given intravenously to normal C57BL/6 mice for 5 consecutive d. On day 6,  $5 \times 10^5$  spleen cells from these mice were added to  $7.5 \times 10^6$  responder cells challenged 4 d earlier with NP-Ficoll or SRBC. Duplicate wells were harvested on day 7 and NP- or SRBC-specific PFC were assayed.

‡ Percent suppression is indicated in parentheses.

TABLE VI  
Genetic Restriction of TsF Factors\*

TsF source	Percent suppression of NP-O-Su CS responses				
	C57BL/6 (H-2 <sup>b</sup> , Igh <sup>b</sup> )	B.C-8 (H-2 <sup>b</sup> , Igh <sup>a</sup> )	C3H (H-2 <sup>k</sup> , Igh <sup>j</sup> )	B10.BR (H-2 <sup>b</sup> , Igh <sup>b</sup> )	CWB (H-2 <sup>b</sup> , Igh <sup>b</sup> )
B6-29	96‡	7	16	101	ND
CKB-17	87	ND§	2	94	91
CKB-39	108	ND	6	93	117

\* Each group of mice was primed with NP-O-Su, followed by intravenous injection of 0.3 ml of undiluted TsF until the day before antigen challenge.

‡ The results are expressed as average percent suppression. The results were collected from several independent experiments.

§ Not determined.

functional activity. BW5147 supernates have also been used as controls in some experiments.

*Genetic Restriction of Hybridoma Suppressor Factors.* We were previously unable to determine the genetic restrictions of the Ts<sup>i</sup> cell population because of the potential complicating allogeneic effects between the suppressor cells and the histoincompatible recipients. Hybridoma suppressor factors derived from Ts<sup>i</sup> cells should not induce potential allogeneic effects and consequently these materials were tested in H-2- and/or Igh-incompatible strains. As summarized in Table VI, the B6-29 supernates induced nearly complete suppression of the syngeneic C57BL/6 (H-2<sup>b</sup>, Igh<sup>b</sup>) and H-2-incompatible, Igh-matched B10.BR (H-2<sup>k</sup>, Igh<sup>b</sup>) NP-induced CS responses. This indicated that H-2 identity was not a requirement for suppressor factor function. In contrast, the B6-29 suppressor factor failed to induce suppression in Igh congenic B.C-8 (H-2<sup>b</sup>, Igh<sup>a</sup>) mice. This indicates that homology between the suppressor factor and the recipient strain for genes linked to the heavy-chain allotype complex (Igh) is required to observe immune suppression. Similarly, the CKB-derived CKB-17 and CKB-39 hybridoma suppressor factors suppressed NP responses in H-2- and Igh-matched B10.BR mice and in B6 and CWB mice (H-2<sup>b</sup>, Igh<sup>b</sup>), which differ at the H-2 complex, but failed to suppress Igh congenic C3H (H-2<sup>k</sup>, Igh<sup>i</sup>) mice (Table VI).

*Igh-V Restriction of TsF.* To determine which region of the Igh complex controls the restriction of TsF, the Igh-congenic BALB/c, C.B-20, and BAB/14 strains were tested. These strains differ with respect to their Igh-C and Igh-V alleles. As indicated in Table VII, the B6-29 and CKB-17 hybridoma suppressor factors inhibited NP-O-Su CS responses in the C.B-20 strain (Igh-V<sup>b</sup>, Igh-C<sup>b</sup>) but not in BALB/c mice (Igh-V<sup>a</sup>, Igh-C<sup>a</sup>). To determine which region of the Igh complex controls this genetic restriction, BAB/14 mice were tested. The BAB/14 strain is also Igh congenic and bears a recombinant haplotype in which the Igh-V alleles were derived from the BALB/c strain and the Igh-C alleles are identical to those of the C.B-20 strain. As indicated in Table VII, the hybridoma suppressor factors failed to suppress BAB/14 mice, indicating that homology of the Igh-C region is not sufficient to obtain suppression. These results suggest that the Igh-V genes control the apparent genetic restrictions of these hybridoma suppressor factors.

*Genetic Restriction of Cells Generated by Hybridoma Suppressor Factor.* The inability of Igh mismatched recipients of hybridoma suppressor factor to generate suppression

TABLE VII  
*Igh-V Restriction of Hybridoma Suppressor Factor\**

TsF source	NP-O-Su priming	Strains tested		
		BALB/c (Igh-V <sup>a</sup> -Igh-C <sup>a</sup> )	C.B-20 (Igh-V <sup>b</sup> -Igh-C <sup>b</sup> )	BAB/14 (Igh-V <sup>a</sup> -Igh-C <sup>b</sup> )
BW5147	+	19.2 ± 1.9 (5)‡	13.6 ± 2.0 (5)	18.0 ± 2.4 (4)
B6-29	+	21.6 ± 2.6 (5)	4.8 ± 1.4 (6)‡	18.2 ± 3.4 (5)
CKB-17	+	19.7 ± 3.0 (6)	5.8 ± 2.1 (5)‡	17.3 ± 3.2 (3)
BW5147	-	3.6 ± 0.5 (5)	2.2 ± 1.2 (5)	4.0 ± 0.6 (5)

\* Mice were given 0.3 ml of TsF from the day of NP-O-Su priming until the day before antigen challenge.

‡ The data are expressed as mean footpad swelling response ± SE in units of 10<sup>-3</sup> cm. The number of mice tested is indicated in parentheses. Significant suppression, *P* < 0.05.

TABLE VIII  
Genetic Restrictions of Ts<sup>e</sup> Cells Induced by Hybridoma Suppressor Factor\*

TsF source	Strain for Ts <sup>e</sup> generation	Strains used to test Ts <sup>e</sup> activity				
		CKB (H-2 <sup>k</sup> , Igh <sup>b</sup> )	B10.BR (H-2 <sup>k</sup> , Igh <sup>b</sup> )	C3H (H-2 <sup>k</sup> , Igh <sup>b</sup> )	C57BL/6 (H-2 <sup>b</sup> , Igh <sup>b</sup> )	CWB (H-2 <sup>b</sup> , Igh <sup>b</sup> )
BW5147	C3H	22.6 ± 2.8 (5)‡	21.0 ± 1.4 (4)	20.0 ± 3.8 (5)	18.3 ± 2.2 (3)	ND§
CKB-17	C3H	8.5 ± 2.7 (4)‡	9.5 ± 1.0 (4)‡	20.5 ± 1.0 (4)	16.8 ± 1.3 (4)	ND
BW5147	C3H	23.6 ± 1.7 (5)	18.8 ± 1.5 (6)	20.0 ± 1.5 (6)	20.4 ± 2.3 (5)	20.3 ± 2.8 (4)
CKB-39	C3H	12.5 ± 0.6 (4)‡	7.0 ± 2.4 (5)‡	22.5 ± 2.1 (6)	19.4 ± 2.4 (5)	23.0 ± 2.8 (4)
BW5147	B10.BR	ND	19.0 ± 1.1 (4)	22.2 ± 2.6 (5)	26.0 ± 4.8 (6)	ND
CKB-39	B10.BR	ND	12.5 ± 0.9 (4)‡	22.7 ± 1.9 (5)	26.6 ± 2.3 (5)	ND
BW5147	C57BL/6	ND	23.8 ± 1.8 (5)	14.8 ± 1.0 (6)	29.5 ± 1.1 (4)	ND
CKB-17	C57BL/6	ND	25.0 ± 1.8 (5)	15.6 ± 0.9 (6)	8.7 ± 1.4 (4)‡	ND
CKB-39	C57BL/6	ND	25.2 ± 1.2 (5)	14.6 ± 1.2 (6)	10.6 ± 1.4 (5)‡	ND
BW5147	(B10 × B10.BR)F <sub>1</sub>	ND	21.0 ± 2.9 (5)	ND	18.0 ± 2.1 (5)	ND
B6-29	(B10 × B10.BR)F <sub>1</sub>	ND	7.6 ± 0.7 (5)‡	ND	6.0 ± 2.6 (5)‡	ND
CKB-17	(B10 × B10.BR)F <sub>1</sub>	ND	8.2 ± 2.3 (5)‡	ND	12.0 ± 2.8 (4)	ND

\* Normal mice were given 4 daily injections of 0.3 ml TsF. After 2 d, these Ts<sup>e</sup> donors were killed and 4 × 10<sup>7</sup> splenocytes were transferred on the day of antigen challenge to previously NP-O-Su primed recipients.

‡ The data are expressed as the mean footpad swelling responses ± SE in units of 10<sup>-3</sup> in. The number of mice is indicated in parentheses. Background responses were <10 units of swelling.

§ Not determined.

could be attributed either to: (a) a requirement for Igh homology between the suppressor factor and the Ts<sup>e</sup> cell populations; or (b) a requirement for homology between suppressor factor or the cells it generates, with another cell population functioning further along the suppressor pathway, such as the Ts<sub>3</sub> or the target cell of the suppression. Therefore, the following experiment was performed to distinguish these alternatives. CKB-17 and CKB-39 supernates were given to Igh-incompatible C3H mice. As indicated previously, CKB (H-2<sup>k</sup>, Igh<sup>b</sup>) mice are congenic with C3H (H-2<sup>k</sup>, Igh<sup>j</sup>) at the Igh complex. From Table VI it was noted that these suppressor factors would not function in the C3H strain. Furthermore, when the splenocytes from C3H mice that had received TsF were transferred into previously NP-primed C3H recipients, they also failed to induce suppression (Table VIII). However, when spleen cells from C3H mice that had received CKB suppressor factor were adoptively transferred into CKB or B10.BR recipients during the effector phase of the CS response, significant levels of suppression were noted (Table VIII). This indicates that the hybridoma suppressor factors generate Ts<sup>e</sup> in Igh-incompatible recipients, but these cells would only function in strains that have the same Igh genes as those used to induce the suppressor factor, i.e., the Ts<sup>e</sup> population is Igh restricted.

To determine whether the hybridoma suppressor factor-induced Ts<sup>e</sup> population was also H-2 restricted, the suppressor factor-induced C3H Ts<sup>e</sup> cells were also transferred into Igh-compatible, H-2-incompatible B6 or CWB recipients (H-2<sup>b</sup>, Igh<sup>b</sup>). As shown in Table VIII, these Ts<sup>e</sup> cells would not suppress the NP responses in either of these strains. Because the CKB and CWB strains are congenic and presumably only differ at the H-2 complex, the data suggest that the Ts<sup>e</sup> population is both H-2 and Igh restricted. To confirm these findings, a reciprocal experiment was performed in which CKB-39 supernates were given to B10.BR mice, H-2-compatible, Igh-incompatible C3H mice, or H-2-incompatible, Igh-compatible C57BL/6 mice. Again, only the B10.BR mice were suppressed, which suggests a dual requirement for both H-2 and Igh compatibility.

When CKB-17 or CKB-39 TsF was given to C57BL/6 mice to generate Ts<sup>e</sup>, the



suppressive activity was only noted when the  $T_s^e$  were transferred into H-2- and Igh-compatible C57BL/6 recipients. Finally, to demonstrate that the  $T_s^e$  cells were not rejected, B6-29 or CKB-17 TsF was given to (B10  $\times$  B10.BR) $F_1$  mice; these  $F_1$  animals were then used as  $T_s^e$  donors (Table VIII). The  $F_1$  cells could transfer suppression to either haploidentical parental recipient, but did not transfer suppression into H-2-incompatible B10.D2 mice (data not shown). Finally, we have also demonstrated that thymocytes from CKB-17 TsF-injected mice can adoptively transfer suppression, indicating that the  $T_s^e$  cells are of thymus-derived origin.

### Discussion

The development of functional suppressor T cell hybrids will permit analysis of the T cell antigen receptor, antigen-specific TsF, and the mechanisms by which TsF modulates cellular and/or humoral immunity. Such hybrids also represent a constant source of monoclonal material for cell biologists and molecular geneticists to probe the mechanisms of cellular activation and differentiation at the subcellular and DNA levels.

In the past few years, several TsF-producing suppressor cell hybrids have been prepared (7, 10–13). Some of these hybrids were used to produce TsF for immunochemical analysis (12, 14). The present study focused on the mechanisms of cellular interactions in the suppressor cell pathway. The NP model was chosen because it is one of the most thoroughly characterized suppressor cell systems. In the NP system, three distinct suppressor T cell populations have been defined and the suppression affects both humoral and cellular immunity (1–3).

The B6-29, CKB-17, and CKB-39 hybrids characterized in this report represent the  $T_s^i$  population. The evidence supporting this conclusion includes: (a) the cells used for fusion were purified by the same methods that are used to enrich the  $T_s^i$  cell population, and (b) the hybrids have the same cell surface phenotype as the  $T_s^i$  population. Furthermore, the factor produced by these hybridoma cells has all the properties of  $T_s^i$ -derived factor ( $T_sF_1$ ). Thus, the B6-29, CKB-17, and CKB-39 hybridoma factors function during the induction phase of the immune response, bear idiotypic and I-J determinants, and induce  $T_s^e$ , which function during the effector phase of the response. These properties resemble those of other  $T_s^i$  populations or  $T_s^i$ -derived factors described in other systems (6).

However, in contrast to other reports, the hybridoma suppressor factors can generate  $T_s^e$  cells in the absence of antigen (15, 16). In the NP system, we previously demonstrated that transfers of purified  $T_s^i$  cells into naive recipients would not generate  $T_s^e$  unless antigen was also added (1). The present observations can be reconciled with the previous results by assuming that antigen is required for the expansion or differentiation of the  $T_s^i$  population to generate sufficient factor to induce functional second order suppressor cells. However, in the poly(L-Glu,L-Ala,L-Tyr) (GAT) system,  $T_sF_1$  will not generate  $T_s^e$  in the absence of antigen (15, 16). It is not clear why this disparity exists, but it should be noted that in the NP system the second order  $T_s^e$  cells have an anti-idiotypic specificity (1), whereas the specificity of the  $T_s^e$  cells in the GAT system has not been defined. Preliminary data suggest that the cells generated with GAT-TsF may not possess anti-idiotypic receptors, but appear to carry idiotypic receptors (Waltenbaugh, personal communication).

When tested directly for suppressive activity, there is an apparent Igh restriction in

TsF function, without any indication of any H-2 restrictions (Table VI). This apparent Igh restriction involves genes in the Igh-V region of the Igh complex (Table VII). However, this is only a pseudogenetic restriction, because these factors can still function, as demonstrated by their ability to generate Ts<sup>e</sup> cells in Igh-incompatible recipients. But to observe the activity of these Ts<sup>e</sup> cells, they must be transferred back to an Igh-compatible strain (Table VIII). Thus, CKB-17 or CKB-39 factors would not suppress the NP-*O*-Su response of Igh-incompatible C3H mice, but cells from C3H mice that had been treated with these factors could suppress NP-*O*-Su responses when transferred back to CKB or B10.BR recipients (Table VIII). Thus, there appear to be no H-2 or Igh restrictions on the ability of TsF to generate Ts<sup>e</sup>, but the pseudogenetic restriction reflects the requirement for an idiotype-matched Igh-V-compatible target cell for expression of suppressive activity. Pseudogenetic restrictions have also been observed in two other suppressor cell systems. Sunday et al. (17) observed that anti-T cell receptor antibodies would only directly suppress Igh-compatible strains. However, suppressor cells were also generated in Igh-incompatible strains, but their activity could only be observed by adoptively transferring these cells back to an Igh-compatible strain. Similarly, Sy et al. (18) noted a pseudogenetic restriction in the ability of idiotype-coupled cells to directly induce suppression in an Igh-incompatible recipient. These authors also demonstrated that suppressor cells were generated by adoptively transferring cells into Igh-matched recipients. Thus, in all of these systems, the pseudogenetic restriction appears to reflect a requirement for homology of second order suppressor cells, which bear anti-idiotypic receptors, with another cell population (presumably bearing idiotypic receptors) that functions later in the suppressor pathway. Indeed, we have previously demonstrated a requirement for a third suppressor cell population (Ts<sub>3</sub>) in the NP system, which must be Igh compatible with the Ts<sup>e</sup> population (2). This Ts<sup>e</sup>-Ts<sub>3</sub> interaction was not only restricted by Igh genes but also by genes in the H-2 complex (2). In this study, CKB-39 TsF induced Ts<sup>e</sup> in C3H mice, which suppress CKB (H-2<sup>k</sup>) mice, but failed to suppress H-2 congenic CWB (H-2<sup>b</sup>) mice (Table VIII). Similarly, Ts<sup>e</sup> generated in C3H mice would transfer suppression to B10.BR (H-2<sup>k</sup>) mice but not to Igh-compatible, H-2-incompatible C57BL (H-2<sup>b</sup>) mice (Table VIII). Thus, the Ts<sup>e</sup> population induced with hybridoma suppressor factor demonstrated an apparent dual restriction for both Igh and H-2 genes. We have previously reported a similar dual restriction in the activity of the antigen-induced effector phase suppressor cell population (5). However, we must remain cautious when interpreting these observations, because one or both of these apparent genetic restrictions may reflect another pseudogenetic restriction between cells or factors functioning at latter stages in the suppressor pathway.

Another purpose of the present study was to determine whether a single Ts<sup>i</sup> cell could modulate both cellular and humoral responses. Thus, B6-29 hybridoma suppressor factor was used to generate Ts<sup>e</sup> cells, which suppressed PFC responses when added during the effector phase of the Mishell-Dutton culture (Table V). Products from a single suppressor T cell clone can modulate both cellular and humoral responses. Furthermore, it should be noted that the NP-*O*-Su CS responses are mediated by at least three distinct T cell populations. One of the effector populations is genetically restricted by genes in the I region of the H-2 complex, and the other two populations are restricted by genes in the H-2K and H-2D regions (8, 19). The latter

populations are considered "killer cell-like" because they bear the Lyt-2 phenotype and react with NP-coupled H-2K or H-2D compatible cells but not NP-conjugated proteins (20). In contrast, the NP-specific H-2I-restricted CS clones respond to NP protein conjugates and lack the Lyt-2 marker (20). Because in many experiments the hybridoma suppressor factor completely inhibited the CS response (e.g., Tables I and VI), these factors must suppress multiple subpopulations of T cells, including those mediating H-2I and those mediating H-2K/H-2D restricted NP-specific responses. We must still determine whether the hybrid cells are producing a single product that suppresses all of these immune responses (perhaps by activating a single  $T_s^e$  that indirectly inhibits a common helper cell required for all of these responses), or if several distinct but related products are produced by these hybridoma cells. Further analysis of these suppressor cell hybrids and their factors will clarify these issues and will help to further characterize the mechanisms of immune regulation.

### Summary

Suppressor factor derived from three different murine T cell hybridomas were characterized. They specifically inhibited 4-hydroxy-3-nitrophenyl acetyl cutaneous sensitivity responses. The factors bind antigen and bear I-J and idiotypic determinants, but lack conventional immunoglobulin constant-region determinants. The factors function during the induction phase of the immune response, by inducing a second population of suppressor cells ( $T_s^e$ ). Suppressor factor can inhibit both cellular and plaque-forming cell responses in appropriate strains of mice. These hybridoma suppressor factors directly suppress strains of mice that are Igh-V homologous with the strain producing the factor. Thus, there is an apparent Igh-V restriction in the activity of these factors. However, this is a pseudogenetic restriction because these factors generate second order suppressor cells ( $T_s^e$ ) in Igh-incompatible mice, but in order to express the suppressive activity, the cells must be adoptively transferred into recipients that are Igh compatible with the strain producing the suppressor factor. Finally, it was shown that the factor-induced  $T_s^e$  population is under an apparent dual genetic restriction. Thus, Igh and H-2 homology is required in order for the  $T_s^e$  population to express its suppressive activity.

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### References

1. Weinberger, J. Z., R. N. Germain, B. Benacerraf, and M. E. Dorf. 1980. Hapten-specific T-cell responses to 4-hydroxy-3-nitrophenyl acetyl. V. Role of idiotypes in the suppressor pathway. *J. Exp. Med.* **152**:161.
2. Sunday, M. E., B. Benacerraf, and M. E. Dorf. 1981. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl. VIII. Suppressor cell pathways in cutaneous sensitivity responses. *J. Exp. Med.* **153**:811.
3. Sherr, D. H., S.-T. Ju, J. Z. Weinberger, B. Benacerraf, and M. E. Dorf. 1981. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl. VII. Idiotypic-specific suppression of plaque-forming cell responses. *J. Exp. Med.* **153**:640.
4. Weinberger, J. Z., R. N. Germain, S.-T. Ju, M. I. Greene, B. Benacerraf, and M. E. Dorf.

1979. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl. II. Demonstration of idiotypic determinants on suppressor T cells. *J. Exp. Med.* **150**:761.
5. Weinberger, J. Z., B. Benacerraf, and M. E. Dorf. 1980. Hapten-specific T-cell responses to 4-hydroxy-3-nitrophenyl acetyl. III. Interactions of effector suppressor T cells are restricted by I-A and Igh-V genes. *J. Exp. Med.* **151**:1413.
  6. Germain, R. N., and B. Benacerraf. 1981. A single major pathway of T-lymphocyte interactions in antigen-specific immune suppression. *Scand. J. Immunol.* **13**:1.
  7. Okuda, K., M. Minami, S-T. Ju, and M. E. Dorf. 1981. Functional association of idiotypic and I-J determinants on the antigen receptor of suppressor T cells. *Proc. Natl. Acad. Sci.* In press.
  8. Sunday, M. E., J. Z. Weinberger, B. Benacerraf, and M. E. Dorf. 1980. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl. IV. Specificity of cutaneous sensitivity responses. *J. Immunol.* **125**:1601.
  9. Sherr, D. H., and M. E. Dorf. 1981. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl. IX. Characterization of idiotype-specific effector phase suppressor cells on plaque-forming cell responses in vitro. *J. Exp. Med.* **153**:1445.
  10. Taniguchi, M., T. Saito, and T. Tada. 1979. Antigen-specific suppressive factor produced by a transplantable I-J bearing T-cell hybridoma. *Nature (Lond.)*. **278**:555.
  11. Kapp, J. A., B. A. Araneo, and B. L. Clevinger. 1980. Suppression of antibody and T cell proliferative responses to L-glutamic acid<sup>60</sup>-L-alanine<sup>30</sup>-L-tyrosine<sup>10</sup> by a specific monoclonal T cell factor. *J. Exp. Med.* **152**:235.
  12. Taussig, M. J., J. R. F. Corvalan, R. M. Binns, and A. Holliman. 1979. Production of an H-2-related suppressor factor by a hybrid T-cell line. *Nature (Lond.)*. **277**:305.
  13. Hewitt, J., and F. Y. Liew. 1979. Antigen-specific suppressor factors produced by T cell hybridomas for delayed-type hypersensitivity. *Eur. J. Immunol.* **9**:572.
  14. Pacifico, A., and J. D. Capra. 1980. T cell hybrids with arsonate specificity. I. Initial characterization of antigen-specific T cell products that bear a cross-reactive idiotype and determinants encoded by the murine major histocompatibility complex. *J. Exp. Med.* **152**:1289.
  15. Germain, R. N., J. Theze, J. A. Kapp, and B. Benacerraf. 1978. Antigen-specific T-cell-mediated suppression. I. Induction of L-glutamic acid<sup>60</sup>-L-alanine<sup>30</sup>-L-tyrosine<sup>10</sup> specific suppressor T cells in vitro requires both antigen-specific T-cell-suppressor factor and antigen. *J. Exp. Med.* **147**:123.
  16. Germain, R. N., and B. Benacerraf. 1980. Helper and suppressor T cell factors. *Springer Sem. Immunopathol.* **3**:93.
  17. Sunday, M. E., J. Z. Weinberger, S. Wolff, and M. E. Dorf. 1981. Anti-receptor antibody-induced suppression of murine H-Y-specific delayed-type hypersensitivity responses. *Eur. J. Immunol.* In press.
  18. Sy, M-S., M. H. Dietz, A. Nisonoff, R. N. Germain, B. Benacerraf, and M. I. Greene. 1980. Antigen and receptor driven regulatory mechanisms. V. The failure of idiotype coupled spleen cells to induce unresponsiveness in animals lacking the appropriate V<sub>H</sub> gene is due to the lack of idiotype matched targets. *J. Exp. Med.* **142**:1226.
  19. Sunday, M. E., B. Benacerraf, and M. E. Dorf. 1980. Hapten-specific responses to 4-hydroxy-3-nitrophenyl acetyl. VI. Evidence for different T cell receptors in cells that mediate H-2I-restricted and H-2D-restricted cutaneous sensitivity responses. *J. Exp. Med.* **152**:1554.
  20. Sunday, M. E., and M. E. Dorf. 1981. Hapten-specific T-cell responses to 4-hydroxy-3-nitrophenyl acetyl. X. Characterization of distinct T cell subsets mediating cutaneous sensitivity responses. *J. Immunol.* In press.