ANALYSIS OF T CELL HYBRIDOMAS

I. Characterization of H-2- and Igh-restricted Monoclonal Suppressor Factors*

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The sequential interactions between T lymphocyte subsets have been well characterized in several systems of antigen-specific immune suppression (1-2). The immune response to the hapten 4-hydroxy-3-nitrophenyl acetyl (NP)[1] has become one of the most extensively characterized of these model systems (3-6). Previous studies from our laboratory have revealed that in this suppressor network system, at least three distinct sets of T cells are required for suppression. The first set of suppressor T cells, termed Ts_1, are induced by antigen, bear an idiotypic receptor, and function early in the immune response. These Ts_1 induce a second set of suppressor T cells, termed Ts_2 (3, 7). The Ts_2 bear anti-idiotypic receptors and function late in the immune response (3-5). Furthermore, the Ts_2 population or a soluble factor derived from these cells activates an antigen-primed Ts_3 population that is thought to mediate the additional suppressor signals (4). Most studies, including these in the NP system, appear to be fragmentary in that the entire sequence of cell interactions has not been fully defined. A serious limitation of these studies has been the lack of suppressor cell clones or hybridomas to further characterize the different suppressor cell populations and the molecules responsible for the communication between these cells.

For a better understanding of the precise nature and function of each participating suppressor T cell subset, we have recently prepared a series of monoclonal T cell hybridomas that are specific for NP (7-8). We previously (7, 8) characterized these hybrids and demonstrated that they correspond to the Ts_1 population. We now characterize another group of T cell hybrids that corresponds to the Ts_2 population. Furthermore, we characterize the biologically active suppressor factor (TsF_2) produced by these cells and compare it with the TsF_1 factor obtained from the Ts_1 hybrids.

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 at

[1] *Abbreviations used in this paper:*   C, complement; CS, cutaneous sensitivity; DMSO, dimethyl sulfoxide; DNFB, 2,4-dinitro-1-fluorobenzene; HBSS, Hanks' balanced salt solution; NP, 4-hydroxy-3-nitrophenyl acetyl hapten; NP-*O*-Su, NP-*O*-succinimide ester; RAMG, rabbit anti-mouse immunoglobulin; Ts_1, Ts_2, Ts_3, first, second, or third order suppressor T cells, respectively; TsF_1, TsF_2, suppressor factor derived from Ts_1 or Ts_2 cells, respectively.

3-12 mo of age and were maintained on laboratory chow and acidified, chlorinated water ad lib.

Antigens. NP-*O*-Succinimide (NP-*O*-Su) was purchased from Biosearch Co., San Rafael, Calif. Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific Co., Pittsburgh, Pa. 2,4-dinitro-1-fluorobenzene (DNFB) was obtained from the Sigma Chemical Co., St. Louis, Mo.

NP-specific T Cell Hybridoma and TsF. The methods for preparation of T cell hybridomas by polyethylene glycol-mediated fusion with the BW5147 tumor line were described previously (8). Our previous results (8) indicated that the CKB-Ts$_1$-17 (Igh^b, H-2^k) and B6-Ts$_1$-29 (Igh^b, H-2^b) hybridoma lines, which are also used in this report, possess I-J and NP^b idiotypic determinants.

In addition to these Ts$_1$ lines, some Ts$_2$ hybrids were also produced from the above fusions (7). This was not surprising because the Ts$_1$ and Ts$_2$ populations were shown to coexist in the spleen at the time these cells were hybridized (3, 4, 9). The Ts$_2$ lines isolated in this fashion include CKB-Ts$_2$-59, CKB-Ts$_2$-225, and B6-Ts$_2$-28. The other Ts$_2$ lines were obtained by hybridization of BW5147 cells with Ts$_2$-containing spleen cells. The enrichment of NP-specific Ts$_2$ was described previously (3). Briefly, NP-primed C57BL/6 spleen cells were first fractionated on rabbit anti-mouse immunoglobulin (RAMG)-coated polystyrene petri plates for 1 h at room temperature. The unbounded cells were then incubated on another set of polystyrene petri plates coated with purified C57BL/6 anti-NP antibodies for 1 h at room temperature. Nonadherent cells were removed, and adherent cells were incubated on ice for 30 min. The adherent cells were resuspended by vigorous pipetting. These enriched Ts$_2$ populations were fused by polyethylene glycol with the BW5147 tumor cell line, as described in detail elsewhere (8).

The hybridized Ts$_2$ candidates were screened using a cytotoxicity test (8) with anti-I-J alloantisera. All the Ts$_2$ hybrids (CKB-Ts$_2$-59, CKB-Ts$_2$-217, CKB-Ts$_2$-225, B6-Ts$_2$-28, and B6-Ts$_2$-14) were periodically passed over C57BL/6 anti-NP antibody-coated plates, and the idiotype-adherent cells were used to maintain the cell lines. The CKB-Ts$_2$-59 and B6-Ts$_2$-28 hybridoma lines were cloned by limiting dilution, and each of the five clones tested had the same phenotype and suppressive activity as the parental line.

All of the hybridomas were cultured in RPMI 1640 containing 8% fetal calf serum and 0.01 M Hepes buffer. The suppressor factors used in the present experiment were collected from the cultured supernates of cells at a density of ~7 × 10^6 cells/ml in the above medium.

Adsorption and Elution of TsF. The methods of adsorption and elution of TsF using protein-conjugated Sepharose 4B columns were described in detail previously (8).

Absorption of TsF with T Cells. T cells were enriched from immune lymph node cells using RAMG-coated plastic petri dishes as described above. 1 ml of Ts$_2$ factor was incubated with 4 × 10^7 immune lymph node T cells or 2 × 10^6 hybridoma T cells for 1 h on ice with intermittent mixing. Then the supernates were centrifuged and tested for suppressor activity at a 1:5 dilution.

Assay for the Ability of TsF to Induce Suppressor Cells. To make Ts$_2$ cells for in vivo studies, hybridoma TsF$_1$ (0.3 ml) was injected i.v. into naive mice for 4 successive days. On day 6, spleens from these mice were removed, and a single cell suspension was prepared in Hanks' balanced salt solution (HBSS). 4 × 10^7 spleen cells were washed three times with HBSS and injected into NP-primed mice (7). The recipient mice were then challenged with NP, and the swelling responses were measured 24 h later, as described elsewhere (4).

Assay for Suppressive Activity of TsF on NP-mediated Cutaneous Sensitivity (CS) Responses. The assay for NP-specific CS responses was described elsewhere (4). Briefly, each animal was primed subcutaneously with 7 mg of NP-*O*-Su in DMSO. Unless indicated otherwise, the hybridoma factors were tested in the effector phase, 5 or 6 d after priming. 0.4 ml of each hybridoma supernate or BW5147 control supernate was injected intravenously on the day before and the day of antigen challenge. 6 d after immunization, 25 μg 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, measured in units of 10^{-3} cm, between the left and right footpad thickness.

DNFB Responses. Contact sensitivity was induced by painting the shaved abdomen twice

daily with 25 μl of 0.5% DNFB solution (Sigma Chemical Co.) in acetone:olive oil (4:1) (7). 6 d after the last painting, 20 μl 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.

Percent Suppression. The percent suppression in the present study was calculated by the following formula:

percent suppression = 100 × [(swelling of BW5147 tumor supernate-injected group − swelling of TsF-injected group) / (swelling of BW5147 tumor supernate-injected group − swelling of unprimed group)].

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

Results

Screening of Hybridoma Suppressor Cells. Suppressor cells were initially induced by the intravenous injection of 4 × 10⁷ NP-coupled syngeneic spleen cells. The suppressor T cells were purified by successive passage over RAMG and NPᵇ idiotype-coated plates. The idiotype-adherent T cells were fused with the AKR BW1547 thymoma tumor line with polyethylene glycol; selection of hybrids seeded at a concentration of 10³ spleen cells per well was carried out with hypoxanthine-aminopterin-thymidine selective media. Hybridoma colonies were noted in ~25% of the wells seeded. When the colonies were large enough for passage, an aliquot of cells was tested for the presence of I-J alloantigenic determinants by microcytoxicity testing. 23% of the colonies tested could be specifically lysed with allele-specific anti-I-J alloantisera. Supernates from the I-J-positive colonies were then screened for in vivo suppression of NP-specific CS responses. 0.4 ml of supernatant fluid was administered intravenously on the day before and the day of antigen challenge. In addition to the above supernates, several other I-J-positive cell lines from previous fusions were also screened in this assay system. Five of the cell lines that manifested at least 50% suppression of the CS immune response were saved for further analysis.

The hybrid cell lines that produced suppressor factors were also screened for other cell surface markers by microcytotoxicity testing. All hybridoma cell lines were lysed with anti-Thy-1.1 antisera and with one exception (CKB-Ts₂-59) were also lysed with anti-Thy-1.2 antisera, which detected the Thy-1.2 gene products introduced by the parental suppressor cell donors. Preliminary analysis with the anti-Lyt-2 monoclonal antibody suggests that several of the Ts₂ hybrids may also carry this cell surface marker (data not shown). However, none of the cell lines could by lysed with anti-NPᵇ idiotype antiserum plus complement (C) (data not shown), which contrasts with the results noted with a previous series of Ts₁ hybrids that could by lysed by the latter reagent (8).

Finally, it should be noted that the ability to lyse these cell hybrids with anti-I-J alloantibody plus C has proven to be a variable feature of these cell lines. With time, there appeared to be a continual decrease in the level of surface I-J determinants expressed on these cells. We have recently noted similar decreases in the level of I-J determinants expressed on the surfaces of Ts₁ hybridoma cell lines.

Specificity of Suppressor Factors. To test the biological activity of these presumptive suppressor hybrids, we screened for the ability of culture supernates obtained from cells growing at a density of ~7 × 10⁵ cells/ml to suppress in vivo CS responses. As shown in Table I, intravenous injection of 0.4 ml of supernate from the B6-Ts₂-28,

B6-Ts$_2$-14, and CKB-Ts$_2$-225 cell lines specifically suppressed NP-induced CS responses. The supernates demonstrated maximum or plateau levels of activity when given either undiluted or diluted 1:10. In these experiments the percent of suppression ranged from 50–75%. After further dilution, these materials no longer demonstrated suppressive activity. The failure of these factors to induce complete suppression of the CS response, coupled with the plateau level of suppression, suggested that they might be maximally effective if the different monoclonal factors derived from each strain were mixed. In three separate experiments, we observed that the percent of suppression was consistently greater when mice were given a mixture of suppressor factors, but the differences were small and not statistically significant.

The antigen specificity of these suppressor factors was demonstrated by their inability to suppress DNFB-induced contact sensitivity responses (Table I). The NP specificity of the CKB-Ts$_2$-59 and CKB-Ts$_2$-217 suppressor factors was similarly established (data not shown).

Comparison of Ts$_1$ and Ts$_2$-derived Factors. To compare this series of NP-specific suppressor factors with the previously characterized set of Ts$_1$ hybridoma factors, we determined the phase of the immune response in which the various factors were active. 0.4 ml of factor was injected intravenously 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). In confirmation of our previous data, the B6-Ts$_1$-29 and CKB-Ts$_1$-17 factors only demonstrated suppressive activity when administered during the induction phase of the immune response (Table II). In contrast, the B6-Ts$_2$-28, B6-Ts$_2$-14, CKB-Ts$_2$-59, and CKB-Ts$_2$-225 factors only suppress the NP response when administered during the effector phase (Table II).

We have previously demonstrated (7) that Ts$_1$- and Ts$_1$-derived factors induce a

TABLE I
Specificity of Hybridoma Suppressor Factors*

Source of TsF	Strain tested	TsF dilution	Swelling response ($\times$ 10^{-3} cm) $\pm$ SE	
			NP-O-Su	DNFB
BW5147	C57BL/6	Undiluted	22.0 $\pm$ 3.4	12.6 $\pm$ 2.1
B6-Ts$_2$-28	C57BL/6	Undiluted	8.5 $\pm$ 1.5‡	19.3 $\pm$ 4.6
		1/10	8.0 $\pm$ 1.0‡	
		1/100	18.2 $\pm$ 2.4	
B6-Ts$_2$-14	C57BL/6	Undiluted	11.2 $\pm$ 1.3‡	19.6 $\pm$ 5.5
		1/10	12.0 $\pm$ 1.0‡	
		1/100	19.0 $\pm$ 2.2	
BW5147	B10.BR	Undiluted	23.5 $\pm$ 1.3	17.6 $\pm$ 1.8
CKB-Ts$_2$-225	B10.BR	Undiluted	14.4 $\pm$ 1.7‡	17.3 $\pm$ 2.8
		1/10	14.8 $\pm$ 1.4‡	
		1/100	23.5 $\pm$ 1.8	

* Groups of four to five mice were immunized with either NP-O-Su or DNFB. On the day before and the day of antigen challenge, mice were given an injection of 0.4 ml i.v. of either control BW5147 or suppressor factors. The background responses of nonimmunized C57BL/6 mice were 3.5 $\pm$ 0.9 and 0.5 $\pm$ 0.6 for NP-O-Su and DNFB, respectively. The background responses of B10.BR mice were 6.4 $\pm$ 1.1 and 2.4 $\pm$ 0.9 for NP-O-Su and DNFB, respectively.
‡ Significant suppression, $P < 0.05$.

Table II

Suppression of the Induction vs. Effector Phase of the Immune Response*

Source of TsF	Strain tested	NP-*O*-Su priming	Footpad swelling ($\times 10^{-3}$ cm) $\pm$ SE	
			Induction phase	Effector phase
None	C57BL/6	+	19.0 $\pm$ 0.9	19.0 $\pm$ 0.9
B6-Ts$_1$-29	C57BL/6	+	7.4 $\pm$ 0.7‡	18.6 $\pm$ 1.7
B6-Ts$_2$-28	C57BL/6	+	18.2 $\pm$ 1.3	12.2 $\pm$ 1.5‡
B6-Ts$_2$-14	C57BL/6	+	17.8 $\pm$ 1.1	12.2 $\pm$ 2.1‡
None	C57BL/6	−	5.4 $\pm$ 0.6	5.4 $\pm$ 0.6
None	B10.A	+	25.0 $\pm$ 2.5	25.0 $\pm$ 2.5
CKB-Ts$_1$-17	B10.A	+	8.6 $\pm$ 2.8‡	31.0 $\pm$ 4.5
CKB-Ts$_2$-59	B10.A	+	26.8 $\pm$ 4.3	12.4 $\pm$ 2.4‡
CKB-Ts$_2$-225	B10.A	+	25.4 $\pm$ 4.5	11.4 $\pm$ 2.7‡
None	B10.A	−	9.6 $\pm$ 1.4	9.6 $\pm$ 1.4

* Groups of four to five mice were immunized with NP-*O*-Su and were given TsF either at the time of antigen priming (induction phase) or antigen challenge (effector phase).

‡ Significant suppression, $P < 0.01$.

Table III

Comparison of the Ability of TsF$_1$ and TsF$_2$ to Generate Suppressor Cells*

Source of TsF	Strain for Ts generation	Transfer recipients	
		NP-*O*-Su priming	Footpad swelling $\pm$ SE
BW5147	B10.BR	+	31.2 $\pm$ 1.9
CKB-Ts$_1$-17	B10.BR	+	12.4 $\pm$ 2.2‡
CKB-Ts$_2$-59	B10.BR	+	33.8 $\pm$ 1.9
CKB-Ts$_2$-225	B10.BR	+	33.8 $\pm$ 3.1
None	None	−	8.2 $\pm$ 2.0

* Groups of four to five NP-*O*-Su-primed B10.BR mice received 4 $\times$ 10^7 spleen cells from donors that were treated with TsF. The mice were challenged 1–2 h. after cell transfer. Footpad swelling was measured 24 h after challenge in units of 10^{-3} cm.

‡ Significant suppression, $P < 0.01$.

second population of suppressor T cells. To determine whether this new series of factors could also induce suppressor cells, B10.BR mice were given four (day 0 through day 3) intravenous injections of 0.4 ml of TsF. 6 d later the animals were killed, and 4 $\times$ 10^7 splenocytes were adoptively transferred into NP-*O*-Su-primed syngeneic recipients that were challenged 1–2 h after the cell transfers. The data shown in Table III confirm that hybridoma CKB-Ts$_1$-17-derived factor can induce second-order suppressor cells. In contrast, the CKB-Ts$_2$-59 and CKB-Ts$_2$-225 factors fail to generate another population of suppressor cells. Thus, we can conclude that the factors characterized in the present report are different from the Ts$_1$-derived factors previously characterized, and this new group of hybridoma cell lines represents Ts$_2$ cells that produce a biologically active suppressor factor termed TsF$_2$.

Immunochemical Characterization of TsF$_2$. To further distinguish the CKB-Ts$_2$-225,

CKB-Ts$_2$-59, and B6-Ts$_2$-28 factors from the previously described Ts$_1$ factors, these materials were passed over a series of immunoadsorbent columns. The suppressive activity of the CKB (H-2^k)-derived factors could be depleted by passage over anti-I-J^k columns but not anti-I-J^b columns. In contrast, the C57BL/6 (H-2^b)-derived factor was specifically adsorbed by anti-I-J^b columns. The suppressive activity could be recovered by acid elution (Table IV). The suppressive activity of the B6-Ts$_2$-28, CKB-Ts$_2$-59 and CKB-Ts$_2$-225-derived suppressor factors were not adsorbed by polyvalent guinea pig anti-immunoglobulin columns, guinea pig anti-idiotype columns, or NP-KLH columns. This contrasts with the results obtained with B6-Ts$_1$-29-derived factor that could be specifically adsorbed by either anti-idiotype or NP-KLH immunoadsorbent columns (Table IV). However, the Ts$_2$ derived but not Ts$_1$-derived factors could be depleted on NP^b idiotype columns. Furthermore, the TsF$_2$ suppressive activity was recovered by acid elution from the NP^b idiotype columns. The specificity of the adsorption was controlled by the demonstration that the factors could not be adsorbed on columns containing nonimmune mouse immunoglobulin (Table IV). Thus, we conclude that these TsF$_2$ bear I-J determinants and have a specific receptor for NP^b idiotype.

Cellular Absorption of TsF$_2$. The binding of TsF$_2$ suppressive activity to NP^b idiotype-bearing immunoadsorbent columns indicated that these hybridoma factors bound to B cell-derived immunoglobulin products. In the next series of experiments, the ability of TsF$_2$ to bind to T cells was established. 1.0 ml of CKB-Ts$_2$-59-derived factor was absorbed with 4×10^7 B10.BR RAMG-purified lymph node T cells from mice that were primed 1 wk previously with either NP-*O*-Su or DNFB. Only the NP-*O*-Su-primed cells could absorb the TsF$_2$ suppressive activity (Table V). In addition, B6-Ts$_2$-14-derived factor was also specifically absorbed with NP-*O*-Su immune C57BL/6 T cells (Table V). To assess the influence of H-2 genotype on the ability of NP-*O*-Su immune T cells to absorb TsF$_2$ activity, CKB-Ts$_2$-59 factor (I-J^k) was absorbed with C57BL/6 (H-2^b) NP-*O*-Su-primed T cells. The data in Table V demonstrate that C57BL/6 T cells can absorb CKB-derived TsF$_2$, indicating that H-2 homology is not required between the strain producing the TsF$_2$ and the T cells

Table IV

*Immunochemical Characterization of Hybridoma Suppressor Factors**

Immunoad-sorbent columns	B6-Ts$_1$-29		B6-Ts$_2$-28		CKB-Ts$_2$-59		CKB-Ts$_2$-225	
	Unbound	Eluate	Unbound	Eluate	Unbound	Eluate	Unbound	Eluate
None	83		75		65		68	
Anti-IJ^k	89	−17	63	19	5	75	23	77
Anti-IJ^b	0	72	10	77	72	0	76	5
Anti-Ig	89	11	75	17	77	−1	50	1
Anti-NP^b	22	83	67	12	70	28	37	−1
NP^b	71	−1	9	86	4	68	0	104
Ig			69	5	48	−6		
NP-KLH	17	69	61	10	92	−11		

* Hybridoma-derived TsF containing supernatant fluids were adsorbed onto various columns and the unbound and acid-eluted fractions were tested for suppressive activity in either C57BL/6 or B10.BR recipients. Some of the results using the control B6-Ts$_1$-29-derived TsF were previously published (7, 8). The data are expressed as the percent suppression of the NP-*O*-Su CS response compared with BW5147 supernate controls. Less than 30% suppression is not considered significant.

TABLE V

*Absorption of TsF₂ Factors with T Cells**

Source of TsF	T cells used for absorption		Percent suppression ± SE
	Strain or hybridoma	Priming antigen	
BW5147	None	None	0 ± 8
CKB-Ts₂-59	None	None	71 ± 5‡
	B10.BR	NP-O-Su	−2 ± 9
	B10.BR	DNFB	61 ± 10‡
	C57BL/6	NP-O-Su	16 ± 5
	CKB	NP-O-Su	4 ± 8
	C3H	NP-O-Su	53 ± 8‡
	B6-Ts₁-3		−9 ± 12
	B6-Ts₁-29		1 ± 17
	CKB-Ts₁-17		14 ± 20
	CKB-Ts₁-19		14 ± 24
	CKB-Ts₁-39		8 ± 19
	AKR (BW5147 tumor)		76 ± 13‡
B6-Ts₂-14	None	None	76 ± 7‡
	C57BL/6	NP-O-Su	15 ± 12
	C57BL/6	DNFB	71 ± 13‡

* 1 ml of hybridoma-derived TsF₂ was absorbed with either 4×10^7 NP-O-Su- or DNFB-primed lymph node T cells from mice of the strains listed or with 5×10^6 Ts₁ hybridoma T cells. The factor was then diluted 1:5 and 0.4 ml was injected i.v. on the day before and the day of antigen challenge. The data represent the pooled results from three separate experiments (not all groups were included in each experiment). The data were normalized and the percent suppression ± SE was calculated.
‡ Significant suppression, $P < 0.01$.

used for absorption. To determine whether a requirement for Igh homology exists, CKB-Ts₂-59 factor was absorbed with CKB (Igh^b) or C3H (Igh^j) NP-primed lymph node T cells. The C3H cells failed to absorb significant levels of TsF₂ suppressive activity, whereas T cells from the Igh congenic CKB strain depleted suppressive activity (Table V). Finally, we attempted to absorb TsF₂ activity with 5×10^6 NP^b idiotype-bearing Ts₁ hybridoma cell lines derived from either C57BL/6 (H-2^b) or CKB (H-2^k) donors. As also shown in Table V, each of these Ts₁ hybrids absorbed TsF₂ activity, whereas two nonfunctional B6 T cell hybridoma lines (data not shown) and the BW5147 tumor line that was used to prepare the hybrids failed to absorb the suppressive activity. Thus, Ts₂-derived suppressor factor can specifically bind antigen-primed T cells or hybridoma cells that bear idiotypic determinants. Furthermore, the binding of TsF₂ to idiotypic T cells does not require H-2 homology.

Genetic Restriction of TsF₂. We have previously characterized (4, 7, 9) the genetic restrictions of the splenic Ts₂ cell population. The results of these past experiments indicated that this population had a dual restriction for both H-2 and Igh-V complex genes. Because these experiments always used a heterogeneous population of cells, it was never clear whether one group of Ts₂ cells was Igh restricted and another subset was H-2 restricted. The production of monoclonal Ts₂ hybridoma lines now permits definitive analysis of this issue. The data in Table VI demonstrate that monoclonal TsF₂ factors are restricted by both H-2 and Igh genes. Thus, CKB-Ts₂-59, CKB-Ts₂-225, and CKB-Ts₂-217 factors that are derived from cells that carry the H-2^k and

TABLE VI

*Genetic Restriction of TsF₂**

Source of TsF	NP-O-Su priming	Recipient strains					
		B6.Igh^e (H-2^b, Igh^e)	C57BL/6 (H-2^b, Igh^b)	B10.BR (H-2^k, Igh^b)	C3H (H-2^k, Igh^j)	CKB (H-2^k, Igh^b)	CWB (H-2^b, Igh^b)
BW5147	+		18.1 ± 2.5	20.5 ± 1.1	17.3 ± 1.7		
CKB-Ts₂-59	+		19.3 ± 1.7	12.2 ± 1.8‡	17.5 ± 1.3		
CKB-Ts₂-225	+		19.8 ± 1.6	13.2 ± 1.2‡	17.0 ± 2.0		
CKB-Ts₂-217	+		20.2 ± 0.9	11.4 ± 1.6‡	16.8 ± 2.3		
BW5147	−		5.9 ± 0.5	7.9 ± 1.2	2.2 ± 0.5		
BW5147	+	18.4 ± 1.1	19.6 ± 2.9	19.6 ± 1.7			
B6-Ts₂-28	+	17.3 ± 2.2	7.6 ± 1.2‡	18.4 ± 1.0			
B6-Ts₂-14	+	20.0 ± 0.9	7.2 ± 1.2‡	22.6 ± 0.8			
BW5147	−	2.0 ± 0.9	3.6 ± 1.0	9.0 ± 2.1			
BW5147	+				25.2 ± 2.8	22.0 ± 1.3	20.4 ± 2.6
CKB-Ts₂-225	+				27.2 ± 1.4	9.5 ± 3.5‡	18.4 ± 1.7
CKB-Ts₂-59	+				21.6 ± 2.1	8.6 ± 3.1‡	20.5 ± 4.5
BW5147	−				2.0 ± 1.7	4.3 ± 2.7	4.8 ± 1.8

* Groups of four to five mice were immunized with NP-O-Su and were given TsF during the effector phase of the immune response. The data are expressed as the increment of footpad swelling ± SE in units of 10^{-3} cm.
‡ Significant suppression, $P < 0.01$.

Igh^b haplotypes only supress NP-induced CS responses of CKB (H-2^k, Igh^b) or B10.BR (H-2^k, Igh^b) mice. These factors fail to suppress NP-induced CS responses in Igh congenic C3H (H-2^k, Igh^j) mice or H-2 congenic CWB (H-2^b, Igh^b) mice. Similarly, the C57BL/6 (H-2^b, Igh^b)-derived factors suppress the CS responses of C57BL/6 mice but fail to suppress NP-specific responses induced in Igh congenic B6.Igh^e or H-2 congenic B10.BR recipients (Table VI). These experiments were repeated using monoclonal TsF₂ derived from the cloned B6-Ts₂-28 and CKB-Ts₂-59 cell lines; comparable data were obtained (data not shown).

Intra-H-2 Mapping of TsF₂ Restrictions. To determine which subregions of the H-2 complex restricted TsF₂ activity, the B6-Ts₂-28, CKB-Ts₂-59, and CKB-Ts₂-225 factors were tested in B10.MBR (K^b, I^k, S^k, D^q), B10.A(3R) (K^b, IA^b, IB^b, IJ^b, IE^k, IC^d, S^d, D^d), B10.A(4R) (K^k, IA^k, IB^b, IJ^b, IE^b, IC^b, S^b, D^b), and B10.A(5R) (K^b, IA^b, IB^b, IJ^k, IE^k, IC^d, S^d, D^d) NP-O-Su-primed recipients. The results of three independent experiments were pooled (Table VII). The C57BL (H-2^b)-derived B6-Ts₂-28 factor suppressed NP responses in H-2K-, I-A-, I-B-, and I-J-compatibile B10.A(3R) and I-B-, I-J-, I-E-, I-C-, S-, and H-2D-compatible B10.A(4R) mice but failed to suppress H-2K-compatible B10.MBR or H-2K-, I-A-, and I-B-compatible B10.A(5R) recipients. In contrast, the CKB (H-2^k)-derived CKB-Ts₂-59 and CKB-Ts₂-225 factors suppressed I-A-, I-B-, I-J-, I-E-, I-C-, and S-compatible B10.MBR and I-J- and I-E-compatible B10.A(5R) recipients but failed to suppress I-E-compatible B10.A(3R) or H-2K- and I-A-compatible B10.A(4R) recipients. Thus, it appears that I-J homology is required between the TsF₂ donor and recipient strains for factor activity.

Discussion

We characterize five T cell hybridomas that correspond to the Ts^e or Ts₂ cells previously identified in the NP system. These hybrids differ from three other NP-specific suppressor T cell hybridomas previously characterized that represented Ts₁ cells. The differences between the hybridoma Ts₁ and Ts₂ surface phenotypes correlate

TABLE VII

Intra-H-2 Mapping of TsF₂ Restriction *

Source of TsF₂	NP-O-Su priming	Recipient strains			
		B10.MBR (bkkkkkq)	B10.A(3R) (bbbbkddd)	B10.A(4R) (kkbbbbbb)	B10.A(5R) (bbbkkddd)
BW5147	+	29.3 + 2.7	24.8 + 2.0	20.6 + 1.2	23.1 + 1.9
B6-Ts₂-28	+	31.6 ± 3.5	15.0 ± 1.6‡	11.1 ± 1.3‡	20.7 ± 1.4
CKB-Ts₂-59	+	16.3 + 1.6‡	23.5 + 2.0	19.6 + 2.8	16.4 + 1.6‡
BW5147	−	10.6 ± 1.8	8.5 ± 1.1	6.6 ± 1.1	10.3 ± 0.9
BW5147	+	29.8 + 2.8	25.2 + 2.2	20.8 ± 1.1	23.2 + 1.7
CKB-Ts₂-225	+	19.3 ± 2.5‡	23.6 ± 1.8	20.6 ± 2.9	14.8 ± 1.5‡
BW5147	−	10.1 + 1.9	8.4 + 1.2	7.6 + 0.8	9.7 + 1.2

* Groups of 12–15 mice were immunized with NP-O-Su and were given TsF during the effector phase of the CS response. The data are expressed as the increment of footpad swelling ± SE in unis of 10^{-3} cm. The alleles at the H-2K, IA, IB, IJ, IE, IC, S, and H-2D regions of the recipient strains are indicated in parentheses.

‡ Significant suppression, $P < 0.05$.

with those noted between the splenic Ts₁ and Ts₂ populations in the NP and other systems (1, 2, 4). Thus, Ts₁ cells bear I-J, Thy-1, and idiotypic determinants, whereas Ts₂ cells bear the I-J and Thy-1 markers but lack NPᵇ idiotypic determinants. The Ts₂ hybridoma cells generally display the same surface phenotype but occasionally some hybrids lose the parental Thy-1.2 marker, e.g., CKB-Ts₂-59, presumably due to loss of the parental chromosome coding for this marker (8).

The antigen-specific suppressor activity of the Ts₂ hybridoma culture supernates also has several features that distinguish it from the previously characterized Ts₁-derived factors. First, TsF₂ functions during the effector phase of the immune response, whereas TsF₁ only induces suppression when given during the induction phase. These differences in the kinetics of TsF activity reflect differences in the mechanism by which these factors function. Thus, TsF₁ induces a second population of suppressor cells (Table III and reference 8), whereas TsF₂ presumably activates an antigen-primed Ts₃ target population. Because TsF₂ does not function when given during the induction phase of the immune response, we assume that this material, in the absence of the appropriate target cells, has a very short biological half-life.

The second method used to distinguish these two I-J-bearing factors is to characterize the binding properties of each factor. As shown in Table IV, TsF₁ bears NPᵇ idiotypic determinants and binds to hapten-coupled immunoadsorbant columns. In contrast, TsF₂ lacks NPᵇ idiotypic determinants and hapten-binding specificity, but can specifically bind to NPᵇ idiotype or to T cells that bear NPᵇ idiotypic determinants, i.e., the Ts₂-derived factor is anti-idiotypic. The specificity of these factors correlates with the specificity of the Ts₁ and Ts₂ cell surface receptors. Thus, Ts₁ cells bear NPᵇ idiotypic determinants and can bind hapten, whereas the Ts₂ population bears anti-idiotypic receptors (3–5). These correlations between Ts and TsF specificity support the notion that TsF may represent a solubilized membrane receptor.

It is important to note the specificity of cellular absorption of TsF₂ (Table V). Thus, TsF₂ can be absorbed by H-2-compatible or H-2-incompatible NP immune T cells but not by T cells primed with another antigen. These findings contrast with those of

Taniguchi et al. (10), who demonstrated that in their system, suppressor-factor binding was H-2 restricted and their suppressor factor could be absorbed by normal T cells. These authors postulated (10) that TsF binding was controlled by an I-J acceptor site on a subpopulation of suppressor T cells. Because of these major differences between the two systems studied, we conclude that the factors and cellular interactions described in the latter report may relate to other suppressor products (perhaps Ts_3) rather than those described in this report.

The third method for distinguishing between the TsF_1 and TsF_2 factors is by the genetic restrictions required for factor activity. Thus, TsF_1 factor will suppress antigen-primed mice that are H-2 incompatible with the strain producing the factor (7). In contrast, TsF_2 can only suppress antigen-primed mice that share H-2 genes with the strain producing the TsF_2 factor. The nature of the TsF_2 H-2 restriction is not clear because NP^b idiotype columns that lack H-2 determinants and NP-immune T cells from H-2 incompatible mice can absorb the factor (Tables IV and V). To account for these paradoxical observations we propose the following possibilities: (a) the H-2 restriction reflects a pseudogenetic restriction in which TsF_2 activates an H-2-restricted Ts_3 cell population. Pseudogenetic restrictions involving Ts_1 cells and factors have been well documented in the NP and other systems (7, 11, 12). Alternatively, (b) the Ts_3 cells (which are considered to represent the cellular targets of TsF_2) might have a receptor that recognizes H-2 determinants on TsF_2. Activation of Ts_3 cells might therefore require the simultaneous stimulation of both idiotypic and H-2 receptors, resulting in an apparent dual genetic restriction. This could account for the ability of H-2-incompatible cells to absorb the H-2-restricted TsF_2. Further experiments are required to understand the nature of the dual H-2 and Igh restriction of the TsF_2 factor. However, the present results demonstrate that these dual restrictions are properties of monoclonal factors from a series of hybridoma T cell lines.

The genetic restrictions of antigen- or TsF_1-induced Ts_2 cells have been previously characterized. We and others (3, 4, 6) have demonstrated that this cell population is both Igh and H-2I restricted. One series of experiments attempted to dissect the H-2 restriction of Ts_2 cells and demonstrated that genes in the I-A subregion control this restriction. However, those previous experiments (9) involved the in vivo transfer of immunocompetent splenic Ts_2 cell populations into allogeneic recipients. We assumed that the potential allogeneic effects had no influence on suppressive activity. However, Bromberg et al. (13) recently demonstrated the activitation of T suppressor cells by an allogeneic effect. Thus, the conclusions from our previous experiment must be reinterpreted in view of this complicating factor. In the present experiment, three different monoclonal TsF_2 factors were used to investigate genetic restrictions. Because soluble factors were used in the present experiments instead of immunocompetent cells, we believe that the potential complications introduced by allogeneic effects have been eliminated. Therefore, the I-J and Igh restrictions observed in the current experiments probably reflect the restrictions on the Ts_2 cell population. Tada et al. (14) had previously demonstrated the involvement of I-J restrictions in a different suppressor cell system, but the restictions in still other systems have mapped to different regions of the H-2 complex (15–17). The reasons for these apparent discrepancies are not clear; they might reflect interactions between different suppressor cell subsets or alternative receptors on some Ts populations.

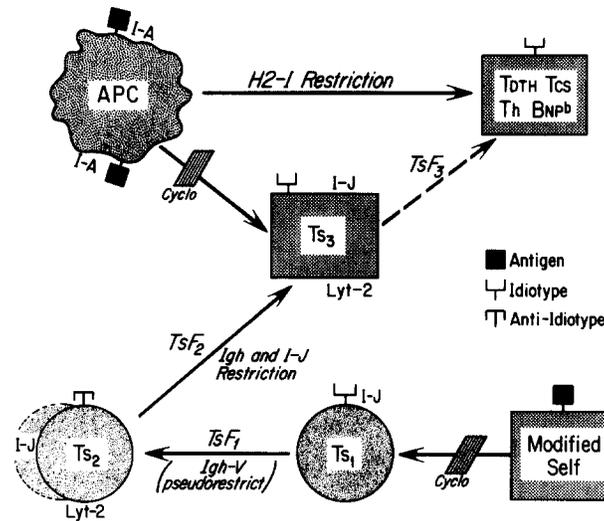The present results can be combined with previous data on the NP suppressor cell

FIG. 1. Immunoregulation of NP-induced immune responses. A schematic representation of the cellular populations in the suppressor pathway. See text for details.

system to describe an overall suppressor pathway. A similar pathway has been proposed (1) for suppression to a variety of other antigens. As summarized in Fig. 1, entry into the suppressor pathway can be obtained by the intravenous administration of antigen-modified syngeneic cells. In a different suppressor cell system, Sherr et al. (18) demonstrated that the antigen must be associated with Ia-positive adherent cells to induce immune suppression. The first cell observed in the suppressor pathway is a cyclophosphomide-sensitive, I-J-bearing Ts₁ cell that binds antigen. The antigen receptor on this cell was studied using a series of Ts₁ hybridoma cell lines, and the data indicated that both the I-J and idiotype determinants were functionally involved in the antigen receptors of Ts₁ cells (8). The culture supernates of the monoclonal Ts₁ hybrid lines contain a biologically active suppressor factor termed TsF₁. This factor bears I-J and idiotypic determinants. In the absence of antigen, TsF₁ induces a second population of suppressor cells termed Ts₂. The ability of Ts₁ and TsF₁ to induce Ts₂ cells is not restricted by either H-2- or Igh-linked genes. However, the Ts₂ that are generated will only function in strains of mice bearing the appropriate Igh alleles. This type of apparent Igh restriction was termed a pseudorestriction (7).

The Ts₂ cell population bears Lyt-2 and I-J determinants and has an anti-idiotypic receptor (3, 4). In contrast to inducer Ts₁ cells, Ts₂ cells suppress during the effector phase of the immune response. The hybridoma Ts₂ cells described in this report produce a suppressor factor termed TsF₂, which bears I-J determinants and has binding specificity for NP^b idiotype; this factor probably corresponds to the factor characterized by Dietz et al. (19) in another suppressor cell system. Monoclonal TsF₂ factor is restricted by both H-2 and Igh genes. There are suggestions that the Ts₂ population might be heterogeneous (20). The presumed target of TsF₂ is an antigen-primed, I-J$^+$, Lyt-2$^+$ cell population that bears idiotypic determinants (4).[2] This third

[2] Sherr, D. H., and M. E. Dorf. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl. XIII. Characterization of a third order T cells (Ts₃) involved in suppression of in vitro PFC responses. Manuscript submitted for publication.

cell population is termed Ts₃. NP-specific Ts₃ cells are poorly characterized but have been identified in cell preparations from antigen-primed lymph nodes (4). Hybridoma Ts₃ lines have not yet been described. Thus, some of the properties of Ts₃ cells may be attributable to distinct T cell subsets that are present in the mixed Ts₃ population. The Ts₃ population or a factor (TsF₃) produced by this population may interact directly or indirectly (via other cellular intermediates) with the T cells that mediate CS or helper activity for NP^b-specific B cells. The precise nature and mechanism of the final phase of immune suppression remains unresolved. Experiments are underway to prepare Ts₃ hybridomas to help clarify some of the outstanding questions concerning the final phases of the suppressor T cell pathway.

Summary

Five hybridoma T cell lines were prepared by fusion of second order suppressor T cells (Ts₂) with the BW5147 thymoma. The culture supernates from these T cell hybrids contained a factor, TsF₂, which specifically suppressed 4-hydroxy-3-nitrophenyl acetyl hapten (NP)-induced cutaneous sensitivity responses. TsF₂ activity was observed when the factor was administered during the effector phases of the immune response. TsF₂ bears I-J determinants and has binding specificity for NP^b idiotypic determinants. TsF₂ suppressor activity could be absorbed on antigen-primed H-2-incompatible T cells but cannot suppress H-2-incompatible mice. In addition to this H-2 restriction, which maps to the I-J subregion, monoclonal TsF₂ also has an Igh genetic restriction. The present results are combined with previous data to describe the cellular interactions leading to immune suppression.

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