ANALYSIS OF T CELL HYBRIDOMAS

II. Comparisons among Three Distinct Types of Monoclonal Suppressor Factors*

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Recent advances in cell hybridization techniques have permitted the fusion of functional T cell subpopulations with tumor cells to yield stable T cell hybridomas (1–9). Such hybrids immortalize the biological activity of an individual cell and permit analysis of monoclonal T cell-derived helper (5) and suppression factors (2, 4, 6, 9, 10). The preparation of monoclonal T cell hybridomas and isolation of biologically active factors derived from monoclonal hybridoma lines can provide a means of dissecting interacting T cell subsets that exist within heterogeneous T cell populations. Such an approach permits a more precise understanding of the mechanism of T cell interactions.

Immune suppression is one such T cell-mediated activity in which multiple T cell populations and soluble factors are required (11). Although initial characterization of the various suppressor T cell populations (Ts)[1] has been possible with heterogeneous Ts populations, it remains difficult to analyze the contributions of individual T cell subsets and to insure that a single subset instead of a mixture of subsets is being studied. Furthermore, the quantities of cells or factors obtained from conventional cell preparations are often insufficient for detailed analysis. Thus, the establishment of monoclonal T cell hybrids with specific suppressor function provides an ideal tool for the characterization of the distinct suppressor cell subsets, their products, and their interactions.

Regulation of the immune response to the hapten 4-hydroxy-3-nitrophenyl acetyl (NP) has been extensively characterized (12–16). Previous studies using conventional analysis and monoclonal T cell hybridomas have revealed that in the NP suppressor cell pathway at least three distinct subsets of T cells are required for suppression (13, 14). The first set of Ts, termed Ts_1, are induced by antigen, bear idiotypic (Id^+) receptors, and can function early in the immune response (10, 12). These Ts_1 cells, or a factor produced by these cells, can induce a second set of Ts, termed Ts_2 (10, 13,

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[1] *Abbreviations used in this paper:* C, complement; CGAT, common idiotype on anti-GAT antibodies; CS, cutaneous sensitivity; DMSO, dimethyl sulfoxide; DNFB, 2,4 dinitro-1-fluorobenzene; HBSS, Hanks' balanced salt solution; KLH, keyhole limpet hemocyanin; NP, 4-hydroxy-3-nitrophenyl acetyl hapten; NP^b, common idiotype on C57BL anti-NP antibodies; NP-O-Su, NP-O-succinimide ester; PBS, phosphate-buffered saline; RAMG, rabbit anti-mouse immunoglobulin; Ts_1, Ts_2, Ts_3, first-, second-, or third-order suppressor T cells, respectively; TsF_1, TsF_2, TsF_3, suppressor factor derived from Ts_1, Ts_2, or Ts_3 cells, respectively.

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14). The Ts_2 cells bear anti-idiotypic receptors and function late in the immune response (13–15). This Ts_2 cell population or a soluble factor derived from it activates an antigen-primed Ts_3 population that is thought to provide still another suppressor signal (14).

The Ts_3 population was first described by Sy et al. (17) in antigen-primed animals. Further analysis involving the NP system as well as the *p*-azobenezenearsonate suppressor system has shown that the Ts_3 population possessed idiotypic, Lyt-2, and I-J determinants (14, 18).[2] In addition, the induction of functional Ts_3 cells is sensitive to cyclophosphamide (14, 17). Despite these results, the exact mechanism by which a Ts_3 population manifests immune suppression as well as the nature of the Ts_3-derived factor remained unknown.

To obtain a better understanding of the precise nature and function of Ts_3 cells, we prepared T cell hybridomas that corresponded to the Ts_3 population. In this communication we characterize the biologically active suppressor factors (TsF_3) produced by these hybridoma cells and compare these factors with previously characterized NP-specific TsF_1 and TsF_2 factors.

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 3–12 mo of age and were maintained on laboratory chow and acidified, clorinated 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) and keyhole limpet hemocyanin (KLH) were obtained from Sigma Chemical Co., St. Louis, Mo.

Antisera. Both B10.A(3R) anti-B10.A(5R) (anti-I-J^k) and B10.A(5R) anti-B10.A(3R) (anti-I-J^b) were produced by immunization with spleen and lymph node cells, as described elsewhere (4). Both monoclonal anti-Thy 1.1 and Thy-1.2 antibodies were purchased from New England Nuclear, Boston, Mass. Guinea pig anti-CGAT and anti-NP^b anti-idiotype antisera were prepared as detailed elsewhere (12, 19). The characterization of these reagents that detect a common idiotype on anti-GAT and anti-NP antibodies, respectively, was described previously (4).

Preparation of Ts_3 Cells from Antigen Plates. The methods for the preparation and enrichment of NP-binding T cells were described in detail elsewhere (12).[2] In brief, 5×10^7 regional lymph node or spleen cells from CKB(H-2^k, Igh-1^b) and C57BL/6 (H-2^b, Igh-1^b) mice, which were immunized subcutaneously with 2 mg NP-O-Su or with 100 μg NP-KLH i.p. in Maalox Pertussis (15), were added to purified anti-mouse immunoglobulin (RAMG)-coated petri dishes to remove B cells. The nonadherent T cells were incubated on NP-bovine serum albumin-coated petri dishes for 45 min at room temperature. Nonadherent cells were removed by gentle swirling, and the plates were placed on ice for 20 min. The antigen-binding cells were collected from the plate by gentle pipetting and used as the source of Ts_3 cells.

Hybridization and Screening of Ts_3 Hybridoma Lines. Ts_3-enriched CKB or C57BL/6 lymphocytes were hybridized with BW5147 T lymphoma cells. The polyethylene glycol-mediated hybridizing method was exactly the same as previously reported (4). The hybridized Ts_3 candidates were screened using a cytotoxicity test (4) with allele-specific anti-I-J and anti-NP^b antisera. Three fusions were performed; the CKB-Ts_3-3 and CKB-Ts_3-9 lines were derived from one fusion using CKB NP-O-Su-primed lymph node cells. The B6-Ts_3-2 line was similarly derived from a fusion between NP-O-Su-primed lymph node cells from C57BL/6 mice and BW5147

[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 cell (Ts_3) involved in suppression of in vitro PFC responses. Manuscript in preparation.

tumor cells. The B6-Ts_3-19 line was obtained from a third fusion using antigen-adherent splenic T cells from an NP-KLH-primed C57BL/6 mouse. In addition, the B6-Ts_3-8 hybridoma line was derived from still another fusion in which we were screening for Ts_2 hybrids (Minami and Okuda, unpublished observations). All the Ts_3 hybrids were periodically passaged over the antigen-coated plates, and the idiotype-adherent cells were used to maintain the cell lines. The B6-Ts_3-2, CKB-Ts_3-3, and CKB-Ts_3-9 hybridoma lines were cloned by limiting dilution; 10 of the 11 clones tested had the same phenotype and suppressive activity as the parental line, but one CKB-Ts_3-3 clone lacked functional activity.

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 supernatants of cells at an approximate density of 7×10^5 cells/ml in the above medium.

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

Assay for Suppressive Activity of TsF on NP-mediated Cutaneous Sensitivity (CS) Responses. The assay for NP-specific CS responses was described elsewhere (10, 14). Briefly, each animal was primed subcutaneously with 2 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 supernatant or BW5147 control supernatant was injected i.v. on the day before and the day of antigen challenge. 6 d after immunization, mice were challenged in the left footpad with 0.025 ml phosphate-buffered saline (PBS) solution containing 30 μg of NP-O-Su (prepared by mixing 25 μl of a 2% NP-O-Su/DMSO solution in 0.4 ml PBS). 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.

Functional Analysis of TsF_2 and TsF_3 Factors in Cyclophosphamide-treated Antigen-primed Mice. Mice were primed subcutaneously with NP-O-Su on day 0, as described above. 24 h later, they were treated with an i.p. injection of either saline or 50 mg/kg cyclophosphamide in saline. On days 5 and 6, each mouse received 0.4 ml i.v. of control BW5147 tumor supernatant or Ts_2- or Ts_3-derived factors. On day 6, mice were challenge with NP-O-Su, and CS responses were measured 24 h later.

DNFB Contact Sensitivity Responses. Contact sensitivity was induced by two daily paintings on the shaved abdomen with 25 μl of 0.5% DNFB solution (Sigma Chemical Co., St. Louis, Mo.) in acetone:olive oil (4:1) (4). 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.

Double Antigen Ear Challenge. Individual mice were immunized with either DNFB alone or with DNFB plus NP-O-Su, as described above. Mice were challenged in the left ear by painting with 0.2% DNFB, injecting 0.015 ml containing 6.0 μg NP-O-Su (prepared by mixing 0.025 ml of 0.7% NP-O-Su in DMSO with 0.4 ml PBS, pH 7.7), or with both antigens. The incremental ear swelling was measured 24 and 48 h thereafter. The concentration and volume of NP-O-Su used to challenge was predetermined to elicit high specific ear swelling and low nonspecific backgrounds.

Percent Suppression. The percent suppression in the present study was calculated by the following formula: percent suppression = 100 × [(swelling of BW tumor supernatant-injected group − swelling of TsF-injected group)/(swelling of BW tumor supernatant-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. After fusion of antigen-adherent T cells from NP-primed mice with AKR-derived BW5147 tumor cells, 320 of 2,300 wells developed hybridoma colonies. 260 of these 320 lines were screened by the microcytotoxicity test using allele-specific anti-I-J alloantisera. 26% were specifically lysed with anti-I-J alloantisera. 54 of these lines were then screened with guinea pig anti-NP^b or control

anti-CGAT-anti-idiotypic antisera; 39% (21 of 54) of these lines were specifically lysed with anti-NP^b idiotype antisera. Supernatants from those lines that were specifically lysed with both anti-I-J and anti-NP^b were tested for suppressive activity by administrating 0.4 ml of supernatant the day before and the day of antigen challenge. At least seven lines demonstrated significant levels of supppessive activity. Five of those that demonstrated >50% suppressive activity of the NP-O-Su CS response were selected for further phenotyping and characterization. The five hybridoma lines that constitutively produced suppressor factor were B6-Ts_3-2, B6-Ts_3-8, B6-Ts_3-19, CKB-Ts_3-3, and CKB-Ts_3-9. Upon retesting, two of the lines (B6-Ts_3-8 and B6-Ts_3-19) demonstrated variable or no reaction with the anti-I-J^b or the anti-NP^b idiotypic antisera, although these hybridomas retained their ability to secrete a biologically active suppressor factor (see below). It is not clear why these lines were screened as initially positive, but after continual culture, they appeared to lose their ability to express I-J and idiotypic determinants on their cell surface. Clones of each of these lines were also tested for the presence of I-J and idiotypic determinants, and again the clones appeared to have little or no expression of these antigenic determinants. In contrast, the other three lines have maintained the ability to express cell surface I-J and idiotype determinants.

Specificity of Suppressor Factors. To test the biological activity of these presumptive suppressor hybridomas, we screened for the ability of culture supernatants obtained from cells growing at an approximate density of 7×10^5 cells/ml to suppress in vivo CS responses. As shown in Table I, injection of 0.4 ml of culture supernatant from the B6-Ts_3-2, B6-Ts_3-19, and CKB-Ts_3-9 cell lines specifically suppressed NP-O-Su-in-

TABLE I

*Specificity of Hybridoma Suppressor Factors**

Source of TsF	Strain tested	TsF dilution	Swelling response ($\times 10^{-3}$ cm) ± SE	
			NP-O-Su (footpad)	DNFB (ear)
BW5147	C57BL/6	Undiluted	27.3 ± 0.9	21.5 ± 0.6
B6-Ts_3-2	C57BL/6	Undiluted	13.0 ± 0.7‡	22.8 ± 1.7
		1/10	12.8 ± 1.3‡	
		1/100	21.0 ± 1.4	
		1/1,000	26.3 ± 0.9	
B6-Ts_3-19	C57BL/6	Undiluted	15.5 ± 0.6‡	26.4 ± 2.7
		1/10	14.8 ± 1.1‡	
		1/100	20.8 ± 1.9‡	
		1/1,1000	27.5 ± 1.1	
BW5147	CKB	Undiluted	25.5 ± 0.5	22.8 ± 2.7
CKB-Ts_3-9	CKB	Undiluted	13.0 ± 1.4‡	22.0 ± 0.7
		1/10	14.7 ± 0.9‡	
		1/100	25.8 ± 5.6	
		1/1,000	25.8 ± 1.1	

* Groups of four to five mice were immunized with either NP-O-Su or DNFB. The day before and the day of antigen challenge mice were given an i.v. injection of 0.4 ml of either control BW5147 or suppressor factor. The background responses of nonimmunized C57BL/6 mice were 4.1 ± 0.7 and 11.2 ± 1.2 for NP-O-Su and DNFB, respectively. The background responses of CKB mice were 3.0 ± 0.4 and 2.6 ± 0.7 for NP-O-Su and DNFB, respectively.

‡ Significant suppression, $P < 0.005$.

duced CS responses. The culture supernatants demonstrated maximum 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 significant levels of suppressive activity.

The antigen specificity of these suppressor factors was initially demonstrated by their inability to suppress the DNFB-induced contact sensitivity responses (Table I). The NP specificity of the B6-Ts_3-8 and CKB-Ts_3-3 suppressor factors was similarly established (data not shown).

Although the specificity of these suppressor factors was demonstrated by the above criteria, it could still be argued that specific antigen is only required to trigger the suppressive activity, and once triggered, the suppression would be nonspecific. Several examples of such antigen-dependent nonspecific suppression have been noted in the literature (22–24). To evaluate the latter possibility, groups of four to five C57BL/6 mice were immunized with either DNFB alone or DNFB plus NP-O-Su. After 5 d, the animals were given either control BW5147 supernatants or culture supernatants from B6-Ts_3-2 or B6-T_3-19 cells. The mice were then challenged in the left ear by painting with DNFB, injecting 7.0 μg NP-O-Su in 15 μl PBS (pH 7.7), or with both antigens. The incremental ear swelling was measured at 24 and 48 h. The data in Table II verify specificity of the B6-Ts_3-2 and the B6-Ts_3-19 factors. Thus, these latter factors fail to suppress ear swelling responses induced and elicited with DNFB or responses induced with NP-O-Su plus DNFB priming and elicited with DNFB alone. However, the activity of the B6-Ts_3-2 and B6-Ts_3-19-derived factors was confirmed by their ability to suppress ear swelling responses in mice primed with both NP-O-Su plus DNFB and challenged with NP-O-Su alone. To determine whether these

TABLE II

*Specificity of Suppressor Factor in Doubly-Primed or Challenged Recipients**

Antigen priming	TsF source	Antigen challenge	Ear swelling ($\times 10^{-3}$ cm) ± SE	
			24 h	*48 h*
DNFB	BW5147	DNFB	13.0 ± 1.7	13.3 ± 1.0
DNFB	B6-Ts_3-2	DNFB	16.0 ± 3.0	16.0 ± 2.3
DNFB	B6-Ts_3-19	DNFB	11.0 ± 1.8	14.0 ± 1.2
—	BW5147	DNFB	1.0 ± 0.4	2.0 ± 0.4
NP-O-Su + DNFB	BW5147	DNFB	15.0 ± 0.7	13.0 ± 1.8
NP-O-Su + DNFB	B6-Ts_3-2	DNFB	13.0 ± 1.4	12.5 ± 1.7
NP-O-Su + DNFB	B6-Ts_3-19	DNFB	16.0 ± 2.7	15.0 ± 1.1
—	BW5147	DNFB	1.0 ± 0.4	2.0 ± 0.4
NP-O-Su + DNFB	BW5147	NP-O-Su	18.5 ± 1.2	16.5 ± 1.3
NP-O-Su + DNFB	B6-Ts_3-2	NP-O-Su	8.3 ± 0.9‡	7.8 ± 1.4‡
NP-O-Su + DNFB	B6-Ts_3-19	NP-O-Su	8.3 ± 2.7‡	8.3 ± 1.1‡
—	BW5147	NP-O-Su	8.3 ± 1.9	4.3 ± 0.8
DNFB	BW547	NP-O-Su + DNFB	16.5 + 1.2	12.8 ± 1.0
DNFB	B6-Ts_3-2	NP-O-Su + DNFB	19.3 ± 2.5	12.8 ± 1.4
DNFB	B6-Ts_3-19	NP-O-Su + DNFB	19.0 ± 2.0	13.8 ± 1.7
—	BW5147	NP-O-Su + DNFB	10.5 ± 1.2	6.5 ± 1.0

* Groups of four to five mice were primed with DNFB alone or with DNFB and NP-O-Su. 5 and 6 d thereafter, they were given TsF or control factors, followed by ear challenge with DNFB, NP-O-Su, or both antigens. The specific ear swelling responses were measured 24 and 48 h after challenge. The data are expressed as the increment of ear swelling ($\times 10^{-3}$ cm) ± SE.

‡ Significant suppression, $P < 0.005$.

suppressor factors would nonspecifically suppress DNFB responses when the animals were challenged with both NP-O-Su plus DNFB, another series of animals were tested. As shown in Table II, the presence of NP-O-Su in the challenge site is not sufficient to suppress DNFB-induced contact sensitivity responses. Thus, the data demonstrate that these hybridoma-derived suppressor factors are antigen specific.

Effector-Phase Suppression. To compare this series of NP-specific suppressor factors with the previously characterized Ts_1- and Ts_2-derived hybridoma factors, we determined when in the course of the immune response the various factors were active. 0.4 ml of factor was injected i.v. 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). As previously reported (10), the B6-Ts_1-29- and CKB-Ts_1-17-derived control TsF_1 factors only demonstrated suppressive activity when administered during the induction phase of the immune response (Table III). In contrast, the previously characterized B6-Ts_2-28-derived TsF_2 factor (20) and the five new hybridoma factors were only active when administered in the effector phase of the NP-O-Su CS response (Table III).

Genetic Restrictions of Hybridoma Suppressor Factors. To further compare this new series of hybridoma suppressor factors with those previously characterized, the genetic restrictions of this new series of factors was determined in comparison with the monoclonal control Ts_1- and Ts_2-derived factors. The Ts_1 factor was administered during the induction phase of the immune response, whereas the Ts_2 and the new series of suppressor factors were administered during the effector phase. As shown in Table IV and in confirmation of previous data (10, 20), the C57BL/6-derived Ts_1 factor from the B6-Ts_1-29 cells suppressed NP-specific CS responses in both syngeneic

TABLE III

Comparison of the Ability of TsF to Suppress CS Responses in the Induction vs. Effector Phase

TsF source	Strain tested	NP-O-Su primed	NP-O-Su-induced swelling ($\times 10^{-3}$ cm) ± SE	
			Induction phase	Effector phase
None	C57BL/6	+	36.4 ± 1.4	36.5 ± 1.4
B6-Ts_1-29	C57BL/6	+	18.3 ± 1.7‡	35.3 ± 2.9
B6-Ts_2-28	C57BL/6	+	34.9 ± 1.3	23.5 ± 1.5‡
B6-Ts_3-2	C57BL/6	+	39.5 ± 4.2	13.8 ± 2.2‡
B6-Ts_3-8	C57BL/6	+	36.0 ± 5.4	21.1 ± 2.1‡
B6-Ts_3-19	C57BL/6	+	36.8 ± 3.3	19.5 ± 3.0‡
None	C57BL/6	−	10.6 ± 2.6	10.6 ± 2.6
None	B10.A	+	34.6 ± 3.6	34.6 ± 3.6
CKB-Ts_1-17	B10.A	+	12.8 ± 1.8‡	30.4 ± 2.9
CKB-Ts_3-3	B10.A	+	31.5 ± 2.6	18.3 ± 1.7‡
CKB-Ts_3-9	B10.A	+	32.3 ± 1.3	16.8 ± 2.0‡
None	B10.A	−	8.7 ± 1.2	8.7 ± 1.2

* 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). The results with the control TsF_1 and TsF_2 factors were obtained from separate experiments. The data were normalized for presentation.

‡ Significant suppression, $P < 0.001$.

TABLE IV

*Genetic Restrictions of Suppressor Factors**

TsF source	NP-O-Su priming	Footpad swelling ($\times 10^{-3}$ cm) in recipient strains ± SE				
		C57BL/6 ($H\text{-}2^b$, Igh^b)	B6.Igh^e ($H\text{-}2^b$, Igh^e)	B10.WB ($H\text{-}2^j$, Igh^b)	CWB ($H\text{-}2^b$, Igh^b)	C3H.SW ($H\text{-}2^b$, IgH^j)
BW5147	+	24.8 ± 1.9	17.4 ± 1.1	20.8 ± 0.7	31.2 ± 3.1	29.8 ± 2.3
B6-Ts_1-29	+	10.7 ± 1.3‡	NT§	9.7 ± 1.0‡	NT	NT
B6-Ts_2-28	+	12.6 ± 1.9‡	16.5 ± 1.9	19.3 ± 0.9	NT	NT
B6-Ts_3-2	+	12.2 ± 0.7‡	18.8 ± 0.3	21.5 ± 0.4	12.3 ± 1.9‡	29.5 ± 1.0
B6-Ts_3-8	+	14.8 ± 1.2‡	NT	22.5 ± 2.1	19.5 ± 2.9‡	26.0 ± 1.5
B6-Ts_3-19	+	9.1 ± 3.1‡	NT	25.1 ± 3.4	14.8 ± 2.6‡	32.5 ± 4.2
BW5147	−	5.2 ± 1.0	3.5 ± 1.2	5.5 ± 1.3	6.3 ± 1.3	7.8 ± 1.9

* Groups of four to five mice were immunized with NP-O-Su and were given TsF during the induction (TsF_1) or 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, $P < 0.05$.

§ Not tested.

C57BL/6 ($H\text{-}2^b$, Igh^b) and H-2-incompatible B10.WB ($H\text{-}2^j$, Igh^b) mice. In contrast, the C57BL/6-derived B6-Ts_2-28 factor would not suppress NP-specific CS responses in H-2-incompatible B10.WB mice. Furthermore, the Ts_2 factor also failed to suppress CS responses in Igh congenic B6.Igh^e ($H\text{-}2^b$, Igh^e) mice. Similarly, the B6-Ts_3-2, B6-Ts_3-8, and B6-Ts_3-19 factors also failed to suppress NP responses in H-2-incompatible B10.WB mice or in Igh-incompatible B6.Igh^e and C3H.SW ($H\text{-}2^b$, Igh^j) mice. Because the C57BL/6 and B6.Igh^e strains as well as the C3H.SW and CWB ($H\text{-}2^b$, Igh^b) strains are both congenic with respect to the Igh complex, the data suggest that the Ts_2 and the presumed Ts_3-derived factors are restricted by genes in both the Igh and H-2 complexes. It should be noted that the B6-Ts_3-2 cells that produced this factor were a cloned subline. In another series of experiments, factors derived from cloned sublines of CKB-Ts_3-3 and CKB-Ts_3-9 cells were also shown to be both H-2 and Igh restricted (data not shown). Variations in the magnitude of the NP-specific CS responses in different inbred strains were noted in the data shown in Table IV. This variation appears to correlate with the age of the recipient mice; older animals generally yield larger footpad swelling responses (unpublished observations).

To determine which subregion of the H-2 complex restricted suppressor factor activity, control B6-Ts_2-28-derived TsF_2, which was previously shown to be I-J^b restricted (20), and the new series of hybridoma-derived 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, 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 combined results of two independent experiments were normalized and presented in Table V. The C57BL/6 ($H\text{-}2^b$)-derived B6-Ts_2-28, B6-T_3-2, and B6-Ts_3-19 factors suppress NP responses in H-2K-, I-A-, I-B-, and I-J-compatible 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($H\text{-}2^k$)-derived factors that were derived from cloned CKB-Ts_3-3 and CKB-Ts_3-9 sublines 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-com-

TABLE V

*Intra-H-2 Mapping of TsF_3 Restriction**

Source of TsF_3	NP-O-Su Priming	Footpad swelling ($\times 10^{-3}$ cm) in recipient strains ± SE			
		B10.MBR (bkkkkkkq)	B10.A(3R) (bbbbkddd)	B10.A(4R) (kkbbbbbb)	B10.A(5R) (bbbkkddd)
BW5147	+	36.5 ± 3.6	3.8 ± 1.4	36.2 ± 2.0	31.6 ± 1.6
B6-Ts_2-28	+	39.9 ± 5.2	20.6 ± 3.0‡	19.3 ± 2.3‡	28.0 ± 2.1
B6-Ts_3-2	+	34.5 ± 2.1	16.8 ± 3.3‡	23.0 ± 2.0‡	31.0 ± 8.7
B6-Ts_3-19	+	39.8 ± 2.3	24.5 ± 3.0‡	20.5 ± 3.2‡	38.0 ± 4.5
CKB-Ts_3-3	+	21.2 ± 2.6‡	36.3 ± 1.7	32.8 ± 2.1	25.0 ± 1.3‡
CKB-Ts_3-9	+	23.4 ± 2.3‡	36.5 ± 1.4	31.5 ± 2.7	21.0 ± 2.5‡
BW5147	−	8.8 ± 2.2	8.5 ± 2.2	11.3 ± 2.3	12.3 ± 1.3

* Groups of four to five 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 units 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. The results with the control TsF_2 factors were obtained from separate experiments, and the data were normalized for presentation.

‡ Significant suppression, $P < 0.005$.

TABLE VI

*Immunochemical Characterization of Hybridoma Suppressor Factors**

Immuno-adsorbent columns	B6-Ts_1-29		B6-Ts_2-28		B6-Ts_3-2		B6-Ts_3-8		B6-Ts_3-19		CKB-Ts_3-9	
	Unbound	Eluate	Unbound	Eluate	Unbound	Eluate	Unbound	Eluate	Unbound	Eluate	Unbound	Eluate
None	83		5		67		65		69		68	
Anti-I-J^k	89	−17	63	19	68	−9	57	4	56	5	0	58
Anti-IJ^b	0	72	10	77	−9	87	6	64	11	73	50	3
Anti-Ig	89	11	75	17	75	5	65	−13	45	7	52	−8
Anti-NP^b	22	83	67	12	27	81	21	63	4	83	−3	62
NP^b	71	−1	9	86	92	5	69	9	74	10	59	6
NP-KLH	17	69	61	10	22	94	−5	70	0	42	−1	56

* 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. The results using the control B6-Ts_1-29 and B6-Ts_2-28-derived TsF were previously published (20). The data are expressed as the percent suppression of the NP-O-Su CS response compared with BW5147 supernatant controls. Less than 30% suppression is not significant.

patible B10.A(4R) recipients. Thus, it appears that I-J homology is required between both the factor donor and recipient strains for TsF_2 and TsF_3 functional activity.

Immunochemical Characterization of Suppresor Factors. In further attempts to distinguish the Ts_2- and the presumed Ts_3-derived factors from each other and from the previously described TsF_1 factors, selected factors were passed over a series of immunoadsorbent columns. The suppressive activity of the C57BL/6($H\text{-}2^b$)-derived factors could be depleted by passage over anti-IJ^b columns but not anti-IJ^k columns. In contrast, CKB-Ts_3-9 ($H\text{-}2^k$)-derived factor was specifically adsorbed by anti-IJ^k columns. The suppressive activity could be recovered by acid elution (Table VI). The suppressive activity of the Ts_1, Ts_2, and the new series of hybridoma factors were not adsorbed by polyvalent guinea pig anti-immunoglobulin columns that were reactive with μ, γ_1, γ_{2a}, γ_{2b}, κ, and λ chains. However, the Ts_1 and the new series of hybridoma factors were adsorbed by guinea pig anti-idiotype columns and NP-KLH columns. This contrasts the results obtained with B6-Ts_2-28-derived factor, which could not be adsorbed by either anti-idiotype or NP-KLH immunoabsorbent columns (Table VI and reference 20). However, B6-Ts_2-28-derived TsF_2 lacks an NP binding site and in

contrast to the other factors could be depleted on NP^b idiotype columns. The specificity of adsorption was controlled by demonstrating that suppressive activity could be recovered by acid elution from the appropriate columns. Thus, we conclude that this new series of hybridoma factors bear I-J and idiotypic determinants and have a specific receptor for NP hapten. These features distinguish the new series of effector-phase hybridoma-derived suppressor factors from the previously described TsF_2 suppressor factors (20).

Activity of TsF in Cyclophosphamide-treated Recipients. Previous reports (14, 17) demonstrated that the precursor Ts_3 cells were sensitive to cyclophosphamide treatment. To further compare Ts_2- and Ts_3-derived suppressor factors, hybridoma TsF_2 and TsF_3 were administered to animals that were treated with 50 mg/kg of cyclophosphamide 1 d after antigen priming. The hybridoma-derived suppressor factors were administered during the effector phase of the immune response. As shown in Table VII, cyclophosphamide pretreatment does not alter the magnitude of the NP-specific CS response. Furthermore, B6-Ts_2-28-derived factor failed to suppress cyclophosphamide-treated mice, whereas the B6-T_3-8- and B6-Ts_3-19-derived factors were active in cyclophosphamide-treated recipients. Similar results were noted with CKB-derived TsF_2 and TsF_3 factors (data not shown). These results suggest that TsF_2 activates a cyclophosphamide-sensitive Ts_3 target population and that TsF_3 can replace the requirement for this cyclophosphamide-sensitive antigen-primed suppressor cell population.

Discussion

This report characterizes a new series of suppressor cell hybridoma lines. Five T cell hybridoma lines have been characterized. These new hybridomas differ from the two

TABLE VII

Ability of TsF to Suppress Cyclophosphamide-pretreated Recipients *

Experiment number	TsF source	NP-O-Su priming	Cyclophosphamide treatment	Footpad swelling ($\times 10^{-3}$ cm) ± SE
1	BW5147	+	−	24.0 ± 1.6
	BW5147	+	+	23.6 ± 0.7
	B6-Ts_2-28	+	−	12.3 ± 1.4‡
	B6-Ts_2-28	+	+	23.2 ± 1.6
	B6-Ts_3-2	+	−	10.8 ± 1.9‡
	B6-Ts_3-2	+	+	10.8 ± 1.1‡
	B6-Ts_3-8	+	−	11.0 ± 1.5‡
	B6-Ts_3-8	+	+	13.6 ± 1.0‡
	BW5147	−	−	5.8 ± 1.1
2	BW5147	+	−	31.0 ± 1.0
	BW5147	+	+	29.3 ± 2.1
	B6-Ts_2-28	+	−	14.8 ± 1.0‡
	B6-Ts_2-28	+	+	31.3 ± 1.8
	B6-Ts_3-19	+	−	18.8 ± 1.4‡
	B6-Ts_3-19	+	+	21.8 ± 1.4‡
	None	−	−	11.6 ± 1.2

* Groups of four to five mice were immunized with NP-O-Su, and 24 h later some groups were given 50 mg/kg cyclophosphamide i.p. During the effector phase of the CS response the animals were given 0.4 ml of TsF/d. The data are expressed as the increment of footpad swelling ± SE in units of 10^{-3} cm.

‡ Significant suppression, $P < 0.001$.

other types of NP-specific suppressor T cell hybridoma lines previously characterized (10, 20). First, the cells used for fusion are primarily derived from mice that have been immunized with either NP-O-Su or NP-KLH. In the past, we have used spleen cells from animals that were tolerized by intravenously administering NP-coupled syngeneic cells. However, we obtained one Ts_3 hybridoma line from spleen cells of mice that were tolerized with NP-coupled syngeneic cells. Although this demonstrates that Ts_3 cells were also present in tolerized animals, the frequency of such cells must be very low. Thus, only one such hybridoma line has been discovered after the screening of several thousand hybridomas from animals that were tolerized with NP-coupled syngeneic cells.

This new series of T cell hybridomas bear allele-specific I-J cell surface determinants and generally bear NP^b-related idiotypic determinants. The presence of Thy-1.2 marker on most of the hybridoma lines and the Lyt-2.2 marker on the B6-Ts_3-19 hybridoma (data not shown) is consistent with the previously reported phenotype of the Ts_3 population. In a few cases, phenotyping for the Lyt-2.2 marker was not possible because two different monoclonal reagents gave disparate results.

The suppressor factors derived from these hybridoma cell lines were also characterized. The TsF_3 suppressor factors differed from the previously characterized TsF_1 and TsF_2 factors. TsF_3 factor acts during the effector phase in contrast to TsF_1 factor, which is active only during the induction phase of the CS response. It is not clear why TsF_2 or TsF_3 factors function only during the effector phase and do not mediate suppression when given during the induction phase of the CS response. One possibility is that these materials have a very short biological half-life. Alternatively, the timing of factor administration with respect to the occurrence of an appropriately differentiated target cell population might be critical.

The antigenic properties of TsF_3 were determined by passing these factors over a series of immunoadsorbent columns. Ts_1- and Ts_3-derived factors had similar characteristics. First, both factors bore I-J and NP^b idiotypic determinants and bound to antigen columns; all the suppressor factors lacked constant region immunoglobulin determinants. As reported elsewhere (20), TsF_2 factor bound to idiotype columns and apparently possessed an anti-idiotypic receptor (Table VI).

To further distinguish TsF_3 factor than TsF_1 and TsF_2 factors, the genetics of factor activity were analyzed. TsF_3 factor, like TsF_2 factor, only suppressed strains that were matched at both the Igh and H-2 gene complexes (Table IV). In contrast, TsF_1 factor lacks at least H-2 restrictions, but as described elsewhere (10), is pseudo-restricted by genes in the Igh complex. To further distinguish the TsF_3 factors from the TsF_2 effector-phase factors, the activity of these factors was tested in animals that had been treated with cyclophosphamide. TsF_2 factor failed to suppress cyclophosphamide-treated mice, whereas TsF_3 factor retained its activity in animals pretreated with cyclophosphamide (Table VII). Finally, the target cells of each type of suppressor factor are distinct. Thus, TsF_1 functions by inducing a Ts_2 population, whereas Ts_2 factor appears to trigger a cyclophosphamide-sensitive Ts_3 cell population. The target cell of the TsF_3 factor is unknown. Table VIII summarizes the similarities and differences between Ts_1, Ts_2, and Ts_3 cells and the factors derived from these cells.

The literature describes many suppressor cell systems, some of which appear to be antigen specific (24–29), whereas others demonstrate elements of nonspecificity (21–23, 28). The NP system has been shown to be specific in this and in previous reports

TABLE VIII

Characteristics of NP-specific Suppressor Factors

Properties of TsF	Phenotype of cell producing TsF	Type of suppressor factor		
		TsF_1	TsF_2	TsF_3
	I-J	+	+	+
	NP^b idiotype	+	−	+
	Cell type	Ts_1	Ts_2	Ts_3
MHC determinants		I-J	I-J	I-J
Igh-C determinants		None	None	None
Igh-V determinants		NP^b	?	NP^b
Binding specificity		NP	NP^b idiotype	NP
Phase of action		Induction	Effector	Effector
Cellular target		Pre-Ts_2	Ts_3	?
Genetic restriction		None	I-J and Igh	I-J and Igh
Activity in cyclophosphamide-treated recipients		?	None	Yes

(10, 14, 15). The experiments using animals primed or challenged with DNFB together with NP-O-Su strengthen the conclusion that the suppression mediated in the NP system is antigen specific (Table II). The fact that TsF_3 factor is Igh restricted differs from the observations in some suppressor cell systems (18, 22, 29). There are several possible explanations for these apparently disparate results. First, the systems and the methods used are quite different in the various suppressor models studied. The different methods for evaluating specificity might account for the results observed. It is noteworthy that Fresno et al. (26) have characterized a specific TsF_3-like factor, which upon cleavage or partial degradation becomes antigen nonspecific. We have not evaluated this possibility in our system. An alternative possibility is that there are several Ts_3-like cells, some of which mediate nonspecific suppression. If the latter is true, we must conclude that in the NP system the antigen-specific Igh-restricted Ts_3 population appears to predominate.

The Ts_3 factors show dual genetic restriction, requiring both I-J and Igh region homology for factor activity. Such findings of dual genetic restrictions are not unique to the NP system nor the TsF_3-derived factors. Thus, Suzuki et al. (16) and Dietz et al. (30) have recently also reported dual genetic restrictions of suppressor factors. The fact that Igh restrictions are observed suggests that idiotype-anti-idiotype interactions are required between TsF_3 and its target cells. Furthermore, these Igh restrictions argue against an antigen-bridging mechanism occurring between the TsF_3 and its target cells. The results imply, therefore, that the target of TsF_3 might be an anti-idiotypic suppressor or helper cell population. At the present time, we are attempting to characterize this target population. The I-J restrictions observed with TsF_3 factor might reflect a requirement for an acceptor site on the target cell population that is required for triggering or inactivating the target cell. Alternatively, they might reflect a pseudo-genetic restriction similar to that previously characterized for TsF_1 factors (10).

The Ts_3 cells described in this report constitutively secrete TsF_3 factor. This suggests that some of the Ts_3 cells are normally present in antigen-primed animals. These Ts_3 appear to be fully differentiated and might be involved in the autoregulation of the NP-specific immune response. The question of how these Ts_3 cells are activated

remains unanswered. One possibility is that a few Ts_2 cells also exist in antigen-primed mice and these Ts_2 cells release TsF_2 factor that activates a small percentage of the Ts_3 population. Another possibility is that Ts_3 cells might also be activated by other means, e.g., anti-idiotype antibody. Finally, we cannot exclude the possibility that in situ the Ts_3 cells were not actively secreting TsF_3 factor but that after fusion with the BW5147 tumor line the hybrids began to actively secrete TsF_3 factor. Preliminary experiments suggest that antigen-primed mice possess a population of mature Ts_3 cells that do not secrete TsF_3 unless activated with TsF_2 factor. This resting Ts_3 population presumably accounts for the bulk of the Ts_3 cells in antigen-primed mice. Thus, the NP responses of immune animals are not completely suppressed until sufficient Ts_3 suppressor cells are activated to regulate the immune response.

The characterizaton of three distinct series of suppressor factors specific for the CS response to the NP hapten demonstrates the intricacy of the immunoregulatory pathway. The precise differences between the immunoregulatory molecules responsible for the communications between suppressor T cells is unknown. However, the potential availability of large quantities of monoclonal TsF_1, TsF_2, and TsF_3 factors derived from both the C57BL/6 and CKB series of hybridoma lines should permit molecular comparisons among these functionally distinct T cell-derived products.

Summary

Five hybridoma T cell lines were prepared by fusion of Ts_3 cells with the BW5147 thymoma. The culture supernatants from these T cell hybrids contained a factor, TsF_3, which specifically suppressed 4-hydroxy-3-nitrophenyl acetyl hapten (NP)-hapten cutaneous sensitivity responses. The properties of this new series of hybridoma factors was compared with those of two previously characterized types of NP-specific suppressor factors (TsF_1 and TsF_2). TsF_3 activity was only observed if the factor was administered during the effector phases of the immune response. TsF_3 bears I-J and C57BL anti-NP antibody idiotypic determinants and has binding specificity for the NP hapten. Furthermore, TsF_3 does not suppress H-2 (I-J)-incompatible mice. In addition to this H-2 restriction, the monoclonal TsF_3 factors also demonstrated an Igh genetic restriction. Finally, the TsF_3 factors could be distinguished by their ability to suppress cyclophosphamide-treated recipients.

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