THE ROLE OF I-J AND Igh DETERMINANTS ON F_1-DERIVED SUPPRESSOR FACTOR IN CONTROLLING RESTRICTION SPECIFICITY*

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Over the past decade there have been numerous descriptions of suppressor T cells (Ts) and of factors derived from such cells. Several apparently related, but nonidentical, suppressor regulatory pathways consisting of a variety of T-T interactions have been described (1–4). Our laboratory has concentrated on one of these models of antigen-specific suppression involving the hapten 4-hydroxy-3-nitrophenyl acetyl (NP). In the NP model system, we have identified at least three distinct suppressor T cell subsets (Ts_1, Ts_2, Ts_3) (5–7). Furthermore, we have demonstrated a requirement for accessory cells at various points in the suppressor pathway (8, 9). All of these cellular elements are needed to modulate the contact sensitivity (CS) response to NP. Furthermore, the same T cell populations are required for suppression of NP-specific plaque-forming cell (PFC) responses (10, 11).

The final effector T cell population in the suppressor cell cascade appears to be an Lyt-2^+ subset that we have termed Ts_3 (6). Ts_3 cells are induced as a consequence of conventional immunization concomitant with the induction of T cells mediating helper or CS activity (8, 12). However, these primed Ts_3 cells appear to remain inactive until appropriately triggered by Ts_2 cells or a factor derived from Ts_2 cells (12, 13). The Ts_3 cells are antigen-specific and bind to NP-coated petri dishes (6, 7, 14). They react with anti-Thy-1, anti-Lyt-2, anti-I-J, and anti-NP^b (antiidiotype) antisera (6, 7, 14). The activity of Ts_3 cells can be demonstrated in cyclophosphamide-treated recipients and is restricted by I-J and Igh genes (6, 12).

Ts_3 hybridomas that constitutively secrete a suppressor factor, TsF_3, have been obtained in the NP system. Five hybridoma T cell lines were prepared by fusion of C57BL/6 (B6) or CKB derived Ts_3 cells with the BW5147 thymoma (7). The supernatants from these Ts_3 cell hybridomas contain factors (TsF_3) which, like Ts_3 cells have the capacity to specifically suppress NP-induced CS responses (7).

* *Abbreviations used in this paper:* B6, C57BL/6 mice; CS, contact sensitivity; CY, cyclophosphamide; DMSO, dimethylsulfoxide; DNFB, 2.4-dinitroflorobenzene; GAT, poly-(L-glu^{60}, L-ala^{30}, L-tyr^{10}); KLH, keyhole limpet hemocyanin; NP, 4-hydroxy-3-nitrophenyl acetyl hapten; NP-O-Su, NP-succinimide ester; PBS, phosphate-buffered saline; PFC, plaque-forming cell; RAMG, rabbit anti-mouse immunoglobulin; Ts_1, Ts_2, Ts_3 first-, second, and third-order suppressor T cells; TsF_1, TsF_2, TsF_3, suppressor factors derived from Ts_1, Ts_2, and Ts_3 cells.

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Suppression of CS responses is only observed if TsF_3 is administered during the effector phase of the immune response. TsF_3 reacts with allele-specific anti-I-J and anti-NP^b antisera and has binding specificity for the NP hapten (7). Furthermore, TsF_3 does not suppress I-J incompatible mice. In addition to this H-2 restriction, the monoclonal TsF_3 factors also demonstrate Igh genetic restrictions. Thus, TsF_3 displays dual genetic restrictions for both I-J and Igh linked genes (7).

Taniguchi et al. (15, 16) have shown that a KLH-specific TsF_3-like factor consists of a molecular dimer composed of a I-J–related piece (28,000 daltons) and an antigen-binding piece that bears determinants encoded by Igh-linked genes. The latter piece may have a membrane (45,000 daltons) and secretory (35,000 daltons) form (15). We have performed similar studies to analyze the molecular organization of the NP-specific TsF_3 (17). Thus, NP-specific TsF_3 was reduced and passed over antigen or anti-I-J immunoadsorbent columns. The activity of the reduced TsF_3 could not be recovered in the eluate or filtrate fractions from either column. However, suppressor activity was reconstituted by combining the appropriate filtrate and eluate fractions (17). These results are similar to those described by Taniguchi et al. for KLH-specific TsF (15, 16). The data imply a two-chain disulfide-linked structure for TsF_3, one chain binds antigen and the other bears I-J–related determinants. To determine if the same class of TsF_3 molecules were responsible for suppression of PFC responses, some of these fractions were also tested for suppressive activity in vitro, using NP-ficoll cultures (11). The data demonstrated that hybridoma-derived TsF_3 molecules with similar biophysical characteristics were responsible for suppression of both CS and PFC responses (11, 17).

Since TsF_3 is dual restricted and consists of a two-chain structure, we considered the possibility that each chain controlled one of the genetic restrictions. Efforts to physically separate and reassociate the chains from different TsF sources have failed in another system (18). Therefore, we used another approach to analyze this issue. The genetic restrictions of Ts_3 cells from NP-primed H-2 and Igh heterozygous (B6 × C3H)F_1 mice were analyzed. Four cloned F_1-derived Ts_3 hybridoma lines were established. The properties of these clones are the subject of the present report.

Materials and Methods

Mice. All mice were either purchased from The Jackson Laboratory, Bar Harbor, ME or bred in the animal facilities at Harvard Medical School, Boston, MA. Mice were used at 2–5 mo of age and were maintained on laboratory chow and acidified, chlorinated water ad lib.

Animals used in the study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. (NIH) 78-23).

Antigen. NP-O-Succinimide (NP-O-Su) was purchased from the Biosearch Co., San Rafael, CA, Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific Co., Pittsburgh, PA. 2,4-dinitro-1-fluorobenzene (DNFB) was obtained from Eastman Kodak Co., Rochester, NY.

Antisera. Both B10.A(3R) anti-B10.A(5R) (anti-$I\text{-}J^k$) and B10.A(5R) anti-B10.(3R) (anti-$I\text{-}J^b$) were produced by immunization with spleen and lymph node cells, as described

elsewhere (19). Monoclonal anti-I-J antibodies, WF8.C2.4 (anti-I-J^k), WF8.D2.3 (ant-I-J^k), and WF9.40.5 (anti-I-J^b) were characterized previously (20, 21). Both monoclonal anti-Thy-1.1 and Thy-1.2 antibodies were purchased from New England Nuclear, Boston, MA. Guinea pig anti-CGAT, anti-NP^b and anti-NP^j antiidiotype antisera were prepared as detailed elsewhere (19). These reagents detect a common idiotype on anti-GAT, C57BL/6 anti-NP, and C3H anti-NP antibodies, respectively, and have been described previously (22, 23).

In Vitro Activation of NP-primed Lymph Node Ts_3 Cells with TsF_2. Regional lymph node cells from mice that had been immunized subcutaneously with 2 mg NP-O-Su were used as the source of Ts_3 cells (12). B6-Ts_2-28, C3H.SW-Ts_2−7, and CKB-Ts_2-59-derived TsF_2, which have been previously characterized and described (12, 24), were used for activation of lymph node Ts_3 cells in vitro. 5×10^7 NP-primed lymph node cells were cultured for 2 h in 10 ml RMPI 1640 with 10% fetal calf serum and 0.1 mM Hepes plus 50 μl TsF_2 ascites fluid derived from B6-Ts_2-28, CKB-Ts_2-59 C3H.SW-Ts_2-7, or BW5147 cells that were grown in (AKR × B6)F_1, (AKR × CKB)F_1,(AKR × C3H.SW)F_1, or AKR mice, respectively. After culture, these activated lymph node cells were washed three times with Hanks' balanced salt solution and resuspended.

Functional Analysis of the Activated NP-primed Lymph Node Ts_3 Cells in Cyclophosphamide-treated Antigen-primed Mice. Mice were primed subcutaneously with 2 mg of NP-O-Su in DMSO on day 0, as described elsewhere (12). 24 h later, they were treated with an intraperitoneal injection of 20 mg/kg cyclophosphamide (CY) in saline. On day 6, each mouse received intravenously 1×10^7 NP-primed lymph node cells activated with TsF_2 or control BW5147 factors, as described above, or received 0.5 ml of TsF_2 or control BW5147 factors. Immediately after transfer, mice were challenged for CS responses. It should be noted that 1×10^7 immune lymph node cells are not sufficient to transfer immunity under these experimental conditions.

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 (7, 14). In brief, 5×10^7 regional lymph node cells from (C57BL/6 × C3H)F_1 mice that were immunized subcutaneously with 2 mg NP-O-Su 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 (C57BL/6 × C3H)F_1 lymphocytes were hybridized with BW5147 T lymphoma cells. The polyethylene glycol-mediated hybridizing method was exactly the same as previously reported (7, 19). The hybridized Ts_3 candidates were screened using a cytotoxicity test (19) with allele-specific anti-I-J reagents. Two fusions were performed and ~500 colonies were screened.

All of the hybridomas were cultured in RMPI 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 method of absorption and elution of TsF using protein-conjugated Sepharose-4B columns were described in detail previously (25).

Assay for Suppressive Activity of TsF on NP-mediated Cutaneous or Contact Sensitivity (CS) Responses. The assay for NP-specific CS responses was described elsewhere (26). Briefly, each animal was primed subcutaneously with 1 or 2 mg of NP-O-Su in DMSO. Unless indicated otherwise, the hybridoma factors were tested in the effector phase, 5 and/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.

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

Percent Suppression. The percent suppression in the present study was calculated by the following formula: percent suppression = 100 × [(swelling of BW5147 tumor supernatant injected group − swelling of TsF injected group)/(swelling of BW5147 tumor supernatant injected group − swelling of unprimed group)].

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

Results

Our initial experiments were designed to analyze the specificity of Ts_3 populations derived from H-2 and Igh heterozygous (B6 × C3H)F_1 mice. One purpose of these studies was to determine whether we could identify Ts_3 populations in which the H-2 restriction specificity was derived from one parent and the Igh restriction specificity was derived from the other parent. Ts_3 cells were generated in (B6 × C3H)F_1 donors (H-2^b; Igh^b/H-2^k; Igh^j) by s.c. priming with 2 mg NP-O-Su. After 6 d, 1 × 10^7 draining lymph node cells that had been activated in vitro with monoclonal TsF_2 from a variety of sources were adoptively transferred to NP-primed cyclophosphamide (CY)–treated C57BL/6 (H-2^b; Igh^b), C3H.SW (H-2^b; Igh^j), C3H (H-2^k; Igh^j), CKB (H-2^k; Igh^b), or (B6 × C3H)F_1 recipients. The CY treatment functionally eliminates Ts_3 generation in the recipients, as evidenced by the inability of TsF_2 to suppress CS responses in CY-treated animals (Table I). This reconstitution protocol allows direct analysis of Ts_3 activity by the adoptively transferred cell population. The recipients were challenged immediately after cell transfer. Footpad swelling was measured 24 h after challenge. The data in Table I summarize the results of seven experiments using this in vivo system. (B6 × C3H)F_1 Ts_3 cells that were transferred to CY-treated B6 recipients, were only activated following coculture with B6-derived TsF_2, suppression was not observed following activation with CKB, or C3H.SW-derived TsF_2 in these recipients. When the same Ts_3 cell population was adoptively transferred to C3H.SW recipients, only the C3H.SW-derived TsF_2 activated these cells to mediate their suppressive activity. The TsF_2 derived from H-2–compatible Igh-incompatible B6 donors, was functionally unable to activate (B6 × C3H)F_1 Ts_3 cells when assayed in C3H.SW recipients, although as shown above the same F1-derived Ts_3 population was functional when assayed in B6 recipients. Similarly, only CKB-derived TsF_2 was capable of activating the (B6 × C3H)F_1-derived Ts_3 population to express functional suppressive activity in CKB or B10.BR recipients. TsF_2 derived from H-2–incompatible B6 donors was unable to activate the (B6 × C3H)F_1 Ts_3 subpopulation to mediate suppression in CKB or B10.BR recipients. Furthermore, neither B6, C3H.SW, or CKB-derived TsF_2 could activate (B6 × C3H)F_1 Ts_3 cells that were adoptively transferred to C3H recipients. Unfortunately, we have not prepared a monoclonal C3H-derived TsF_2 to serve as positive control for the latter groups. Nonetheless, it appears that the (B6 × C3H)F_1 Ts_3 population, consists of at least three and probably four functionally distinct subsets; each Ts_3 subset was restricted to one

TABLE I
Activation of H-2 and Igh Heterozygous Ts_3 Cells with Monoclonal Suppressor Factors[‡]

CY-treated recipient	Ts_3 donor	TsF_2 source for Ts_3 activation	Footpad swelling ± SE[§]
C57BL/6	(B6 × C3H)F_1	BW5147	35.5 ± 2.2 (17)
	(B6 × C3H)F_1	B6-Ts_2-28	19.1 ± 2.0 (17)*
	(B6 × C3H)F_1	C3H.SW-Ts_2-7	33.7 ± 1.3 (16)
	(B6 × C3H)F_1	CKB-Ts_2-59	34.5 ± 2.4 (4)
	None	BW5147	33.8 ± 1.9 (9)
	None	B6-Ts_2-28	32.9 ± 2.8 (7)
C3H.SW	(B6 × C3H)F_1	BW5147	32.8 ± 1.4 (23)
	(B6 × C3H)F_1	B6-Ts_2-28	33.8 ± 2.3 (20)
	(B6 × C3H)F_1	C3H.SW-Ts_2-7	19.9 ± 1.5 (20)*
	(B6 × C3H)F_1	CKB-Ts_2-59	33.8 ± 2.1 (4)
	None	BW5147	34.9 ± 2.9 (9)
	None	C3W.SW-Ts_2-7	30.9 ± 2.5 (8)
C3H	(B6 × C3H)F_1	BW5147	36.2 ± 2.8 (12)
	(B6 × C3H)F_1	B6-Ts_2-28	35.4 ± 2.7 (11)
	(B6 × C3H)F_1	C3H.SW-Ts_2-7	34.2 ± 2.2 (13)
	(B6 × C3H)F_1	CKB-Ts_2-59	33.9 ± 3.6 (8)
CKB or B10.BR	(B6 × C3H)F_1	BW5147	37.4 ± 2.2 (8)
	(B6 × C3H)F_1	B6-Ts_2-28	37.8 ± 1.9 (9)
	(B6 × C3H)F_1	C3H.SW-Ts_2-7	38.1 ± 2.1 (8)
	(B6 × C3H)F_1	CKB-Ts_2-59	24.2 ± 1.8 (9)*
	None	BW5147	39.5 ± 4.2 (4)
	None	CKB-Ts_2-59	36.0 ± 2.3 (4)
(B6 × C3H)F_1	(B6 × C3H)F_1	BW5147	32.0 ± 1.8 (4)
	(B6 × C3H)F_1	B6-Ts_2-28	18.5 ± 1.3 (4)*
	(B6 × C3H)F_1	C3H.SW-Ts_2-7	22.7 ± 2.3 (3)*
	(B6 × C3H)F_1	CKB-Ts_2-59	18.3 ± 1.7 (4)*
	None	BW5147	30.0 ± 1.6 (5)
	None	B6-Ts_2-28	32.3 ± 2.6 (4)

‡ In vitro activation of regional lymph node Ts_3 cells from NP-O-Su-primed mice with TsF_2 was done as described in Materials and Methods. Activation was continued for 2 h. Recipient mice were primed with NP-O-Su; 24 h later all mice were given 20 mg/kg cyclophosphamide, and 6 d later received 1×10^7 activated Ts_3 before antigen challenge. The data represent the pooled results from seven separate experiments (not all groups were included in each experiment).

§ The data from seven experiments were pooled and expressed as the increment of footpad swelling ± SE in units of 10^{-3} cm. The background responses of nonimmunized mice ranged from 4.5 to 13.7. The number of mice tested is indicated in parentheses. An asterisk indicates significant suppression, $P<0.01$.

parental H-2 and Igh specificity. Furthermore, the data suggest that each Ts_3 subpopulation must be activated by a TsF_2, which must share the same H-2 and Igh restriction as the Ts_3 population. Finally, we noted that when (B6 × C3H)F_1 recipients were used, either TsF_2 could activate the Ts_3 subsets to express suppressive activity (Table I).

The present data extend previous results in this system by demonstrating that Ts_3 cells derived from H-2 and Igh heterozygous F_1 mice can contain four distinct populations of Ts_3 cells, each restricted to a particular combination of H-2 and Igh controlled specificities. However, to formally confirm these conclusions, which are based on analysis of heterogeneous cell populations, we undertook to demonstrate the same phenomenon at the clonal level. Therefore, NP-

specific Ts_3 cells from (B6 × C3H)F_1 mice were used for the preparation of a series of F_1-derived Ts_3 hybridomas.

Screening of Hybridoma Suppressor Cells

Ts_3 Suppressor cells were induced by priming (B6 × C3H)F_1 mice with 2 mg NP-O-Su s.c. Ts_3 cells were purified from regional lymph nodes by successive passage over RAMG- and NP-BSA-coated plates. The NP-BSA-adherent T cells were fused with the AKR-derived BW5147 thymoma line with polyethylene glycol; selection of hybridomas seeded at a concentration of 10^4 lymph node T cells per well was carried out with hypoxanthine-aminopterin-thymidine selective media. Hybridoma colonies were noted in ~50% 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 microcytotoxicity testing. 23% of the colonies tested demonstrated at least ± reactions with at least one allele-specific anti-I-J alloantiserum. Supernatants from these colonies were screened for in vivo suppression of NP-specific CS responses. 0.5 ml of supernatant fluid was given i.v. on the day before, and the day of, antigen challenge to (B6 × C3H)F_1 mice. Four cell lines that manifested significant levels of suppression were saved for further analysis.

The four hybridoma cell lines that produced suppressor factors were examined for other cell surface markers by microcytotoxicity testing (data not shown). All functional (B6 × C3H)F_1 hybridoma cell lines were lysed with anti-I-J^b and anti-I-J^k alloantisera. However, as noted with other Ts hybridoma cell lines (27), the percentage of lysis was usually weak (<40%) and with continued in vitro culture the ability to lyse these cells was lost or became highly variable. Antiidiotypic reagents (anti-NP^b) prepared against purified C57BL/6 anti-NP or anti-NIP serum antibodies were specifically lytic for the (B6 × C3H)F_1-Ts_3-1032 and (B6 × C3H)F_1-Ts_3-1114 hybridoma cells, but were not lytic for the (B6 × C3H)F_1-Ts_3-1127 and (B6 × C3H)F_1-Ts_3-1131 lines. In contrast, the antiidiotypic reagents (anti-NPj) prepared against purified C3H anti-NP antibodies were lytic for (B6 × C3H)F_1-Ts_3-1131, but not for the other three cell lines. Again, the percentage of hybridoma cells lysed was generally low (<30%) and after prolonged in vitro culture the cells were no longer susceptible to lysis with these reagents. In contrast, all these (B6 × C3H)F_1-derived hybridoma lines were readily lysed with either anti-Thy-1.1, anti-Thy-1.2, or anti-H-$2D^b$ antisera all of which yielded >80% lysis even after prolonged in vitro culture (data not shown).

Specificity of Suppressor Factors

To test the biological activity of these four (B6 × C3H)F_1 hybridoma-derived suppressor factors, we screened for the ability of culture supernatants obtained from cells growing at a density of ~7 × 10^5 cells/ml to suppress in vivo NP or DNFB CS responses. Table II shows that i.v. injection of 0.5 ml of culture supernatant from the (B6 × C3H)F_1-Ts_3-1032, (B6 × C3H)F_1-Ts_3-1114, (B6 × C3H)F_1-Ts_3-1127, and (B6 × C3H)F_1-Ts_3-1131 cell lines specifically suppressed the NP-induced CS response in (B6 × C3H)F_1 mice. In this experiment the percent suppression ranged from 60 to 75%. In contrast, these culture supernatants could not suppress DNFB-induced CS responses. Furthermore, the data (Table III) demonstrate that these factors were only active in the effector phase

TABLE II

Antigen Specificity of (B6 × C3H)F_1-Ts_3 Hybridoma Suppressor Factors‡

Recipient strain	Source of TsF	NP-O-Su footpad swelling ± SE§	DNFB ear swelling ± SE§
(B6 × C3H)F_1	BW5147	30.0 ± 2.4	12.3 ± 1.5
	F1-Ts_3-1032	16.3 ± 0.9*	12.3 ± 0.9
	F1-Ts_3-1114	14.0 ± 2.4*	13.8 ± 1.8
	F1-Ts_3-1131	14.0 ± 0.6*	13.3 ± 0.9
	F1-Ts_3-1127	15.0 ± 0.6*	13.5 ± 1.6

‡ Groups of four to five (B6 × C3H)F_1 mice were immunized with both NP-O-Su and DNFB. On the day before and the day of antigen challenge, mice were given 0.5 ml of control BW5147 or (B6 × C3H)F_1 hybridoma derived TsF_3, i.v.

§ The background responses of nonimmunized mice following antigen challenge were 7.8 ± 1.1 and 3.5 ± 0.3 × 10^{-3} cm for NP-O-Su and DNFB, respectively. An asterisk indicates significant suppression, $P<0.01$, compared with recipients which received control BW5147 factor.

TABLE III

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

Strain of mice	Source of TsF	Time of TsF administration	
		Induction phase	Effector phase
(B6 × C3H)F_1	BW5147	35.0 ± 0.3§	43.2 ± 2.6
	CKB-Ts_1-17	12.5 ± 1.6*	ND
	F_1-Ts_3-1032	35.0 ± 1.7	21.8 ± 3.1*
	F_1-Ts_3-1114	35.0 ± 1.3	26.8 ± 2.5*
	F_1-Ts_3-1127	36.0 ± 1.1	22.8 ± 2.0*
	F_1-Ts_3-1131	34.0 ± 2.0	27.5 ± 0.9*

‡ Groups of four to five (B6 × C3H)F_1 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).

§ Footpad swelling responses ± SE are indicated in units of 10^{-3} cm. The level of background swelling in unprimed mice that had received the challenge was 10.3 ± 1.2. An asterisk indicates significant suppression, $P<0.01$, compared to groups which received control BW5147-derived factor.

ND, not determined.

of the immune response. Thus, when 0.5 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), all the F_1-derived factors only functioned in the effector phase. In confirmation of our previous data (25), the control CKB-Ts_1-17 inducer factor (TsF_1) demonstrated suppressive activity when administered during the induction phase (Table III).

Genetic Restriction of (B6 × C3H)F_1-Ts_3 derived Factors

We had previously demonstrated that B6- or CKB-derived Ts_3 hybridoma factors have a dual genetic restriction for both H-2 and Igh-linked genes.

Furthermore, the previous data (Table I) indicated that at least three and probably four distinct populations of Ts_3 cells restricted by either parental H-2 and Igh haplotype exist in H-2 and Igh heterozygous F_1 animals. However, in the latter experiments heterogeneous populations of T cells were used and the cells were tested in semi-allogeneic combinations in which allogeneic effects are difficult to exclude. Therefore, the production of monoclonal Ts_3 hybridomas from H-2 and Igh heterozygous F_1 mice permitted a more definitive analysis of the specificity of F_1-derived Ts_3 cells and their factors. TsF_3 was administered i.v. during the effector phase of the NP-O-Su–induced CS response. Each F_1-derived TsF_3 was tested in (B6 × C3H)F_1, C57BL/6 (H-2^b; Igh^b), B10.BR (H-2^k; Igh^b), C3H/HeJ (H-2^k; Igh^j), and C3H.SW (H-2^b; Igh^j) recipients. As shown in Table IV each of the monoclonal (B6 × C3H)F_1–derived TsF_3 factors are restricted to one parental H-2 and one parental Igh haplotype. Thus, (B6 × C3H)F_1-Ts_3-1032 and (B6 × C3H)F_1-Ts_3-1114 factors only suppress NP-induced CS responses of C57BL/6 (H-2^b, Igh^b) mice. These factors fail to suppress NP-induced CS responses in Igh disparate C3H.SW (H-2^b, Igh^j) recipients or H-2 congenic B10.BR (H-2^k, Igh^b) mice. Similarly, (B6 × C3H)F_1 Ts-1127 factor only suppressed NP-responses of C3H.SW (H-2^b, Igh^j) mice and (B6 × C3H)F_1-Ts_3-1131 factor only suppressed NP-CS responses of C3H (H-2^k, Igh^j) mice (Table IV).

To determine which subregions of the H-2 complex restricted the activity of these factors, one hybridoma (B6 × C3H)F_1-Ts_3-1032 was selected for further

TABLE IV

Genetic Restrictions of (B6 × C3H)F_1 Hybridoma-derived TsF_3 Factors‡

TsF_3 source	Normalized Percent Suppression in Various Recipient Strains ± SE§				
	(B6 × C3H)F_1 (H-2^b/H-2^k; Igh^b/Igh^j)	C57BL/6 (H-2^b; Igh^b)	B10.BR (H-2^k; Igh^b)	C3H (H-2^k; Igh^j)	C3H.SW (H-2^b; Igh^j)
BW5147	0 ± 4 (21)	0 ± 2 (21)	0 ± 4 (14)	0 ± 4 (17)	0 ± 4 (20)
(B6 × C3H)F_1-Ts_3-1032	52 ± 6 (8)*	67 ± 5 (12)*	22 ± 12 (11)	11 ± 6 (7)	−1 ± 5 (8)
(B6 × C3H)F_1-Ts_3-1114	48 ± 6 (15)*	43 ± 8 (16)*	9 ± 6 (13)	6 ± 5 (11)	15 ± 10 (12)
(B6 × C3H)F_1-Ts_3-1127	45 ± 3 (15)*	−14 ± 10 (8)	13 ± 7 (8)	10 ± 4 (13)	88 ± 5 (16)*
(B6 × C3H)F_1-Ts_3-1131	47 ± 3 (15)*	−5 ± 7 (12)	8 ± 5 (12)	68 ± 5 (16)*	6 ± 11 (16)

‡ Groups of mice were immunized with NP-O-Su and were given TsF_3 on the day before and the day of antigen challenge.

§ The data represent the pooled results from three separate experiments. The data were normalized and the percent suppression ± SE was calculated. The number of mice tested is indicated in parentheses. An asterisk indicates significant levels of suppression, $P<0.01$.

TABLE V

Intra H-2 Mapping of (B6 × C3H)F_1-Ts_3-1032 Derived Factor‡

Source of TsF	Normalized percent suppression ± SE§			
	C57BL/6 (H-2^b; Igh^b)	B.C.-8 (H-2^b; Igh^a)	B10.A(3R) (IJ^b; Igh^b)	B10.A(5R) (IJ^k; Igh^b)
BW5147	0 ± 6	0 ± 5	0 ± 7	0 ± 5
F1-Ts_3-1032	73 ± 7*	−2 ± 7	70 ± 7*	3 ± 10

‡ Groups of 4–8 mice were immunized with NP-O-Su and were given TsF on the day before and the day of antigen challenge.

§ The data represent the normalized percent suppression of ± SE. An asterisk indicates significant suppression, $P<0.01$.

analysis. TsF_3 derived from this hybridoma was tested in C57BL/6 (H-2^b, Igh^b), B.C-8 (H-2^b, Igh^a), B10.A (3R) (K^b, IA^b, IB^b, IJ^b, IE^k, IC^d, S^d, D^d; Igh^b), and B10.A(5R) (K^b, IA^b, IB^b, IJ^k, IE^k, IC^d, S^d, D^d; Igh^b) NP-primed mice. As shown in Table V, (B6 × C3H)F_1-Ts_3-1032 factor suppressed NP responses in C57BL/6 mice and B10.A(3R) mice, but failed to suppress NP responses in B10.A(5R) and Igh congenic B.C-8 recipients. Thus, the suppressive activity mediated by (B6 × C3H)F_1-Ts_3-1032–derived factor is restricted by I-J and Igh-linked genes.

Immunochemical Characterization of (B6 × C3H)F_1-Ts_3–derived Factors

The results of previous experiments demonstrated that TsF_3 factors derived from C57BL/6 (H-2^b, Igh^b) donors reacted with anti-I-J^b and anti-NP^b antisera (7). Furthermore, additional indirect evidence predicted that C3H-derived TsF_3 would react with anti-I-J^k and anti-NP^j antisera (7, 12). Thus, it is important to determine which I-J and Igh allelic products are expressed on the (B6 × C3H)F_1 derived TsF_3 and to determine whether these determinants correlate with the genetic restrictions of the factors. Therefore, three of the factors were passed over a series of immunoadsorbent columns. As shown in Table VI, the suppressive activity of (B6 × C3H)F_1-Ts_3-1032 factor which was restricted to C57BL/6 (Igh^b) could be depleted by passage over anti-NP^b but not anti-NP^j columns. Furthermore, the suppressive activity could be specifically recovered following acid elution. In contrast, the suppressive activity of (B6 × C3H)F_1-Ts_3-1131 and (B6 × C3H)F_1-Ts_3-1127–derived factors which suppressed NP-responses in C3H mice (Igh^j) or C3H.SW (Igh^j) mice, respectively, were adsorbed by anti-NP^j columns but not by anti-NP^b immunoadsorbent columns.

In another series of experiments these factors were passed over several anti-I-J columns. The suppressive activity of the control C57BL/6-derived B6-Ts_3-2 factor could be depleted by passage over a monoclonal anti-I-J^b (WF9-40.5) column or columns made by coupling conventional anti-I-J^b alloantiserum (anti-I-J^b) and the activity could be recovered by acid elution (Table VII). In contrast, the suppressive activity of a control CKB-derived CKB-Ts_3-9 factor was adsorbed by two different batches of monoclonal anti-I-J^k (WF8.C2.4 and WF8.D2.4) or

TABLE VI

Fractionation of Hybridoma-derived Suppressor Factors on Antiidiotype Columns‡

Antiidiotype column	Fraction	F_1-Ts_3-1032§	F_1-Ts_3-1127	F_1-Ts_3-1131
None	Unfractionated	54 ± 11*	64 ± 9*	55 ± 6*
Anti-NP^b	Eluate	58 ± 6*	9 ± 8	−17 ± 11
	Filtrate	0 ± 3	60 ± 9*	63 ± 6*
Anti-NP^j	Eluate	−4 ± 6	51 ± 7*	52 ± 8*
	Filtrate	35 ± 9*	4 ± 13	−8 ± 8

‡ C57BL/6, C3H.SW or C3H mice were used as recipients of (B6 × C3H)F_1-Ts_3-1032, (B6 × C3H)F_1-Ts_3-1127 or (B6 × C3H)F_1-Ts_3-1131 factor, respectively. The mice were primed with NP-O-Su. On the day before and the day of antigen challenge, 0.5 ml of the designated column fractions were injected i.v.

§ The data represent the pooled and normalized percent suppression from two or three separate experiments for each factor. An asterisk indicates significant suppression, $P<0.01$.

TABLE VII

Fractionation of Hybridoma-derived Factors on Anti-I-J Immunoadsorbent Columns‡

Anti-I-J column	Fraction	B6-Ts_3-2	CKB-Ts_3-9	F_1-Ts_3-1032	F_1-Ts_3-1127	F_1-Ts_3-1131
None	Unfractionated	44 ± 9*	41 ± 8*	81 ± 3*	74 ± 4*	42 ± 6*
WF8.C2.4	Eluate	5 ± 12	53 ± 10*	−2 ± 9	5 ± 9	56 ± 2*
	Filtrate	32 ± 6*	8 ± 6	43 ± 9*	76 ± 5*	2 ± 2
WF8.D2.3	Eluate	3 ± 7	48 ± 9*	2 ± 5	0 ± 5	35 ± 3*
	Filtrate	55 ± 8*	5 ± 10	65 ± 8*	73 ± 6*	−5 ± 11
WF9.40.5	Eluate	49 ± 2*	10 ± 3	58 ± 3*	85 ± 3*	1 ± 10
	Filtrate	−7 ± 8	46 ± 7*	0 ± 4	1 ± 4	45 ± 3*
Anti-I-J^b	Eluate	33 ± 5*	5 ± 13	65 ± 4*	74 ± 5*	−1 ± 2
	Filtrate	−12 ± 3	49 ± 5*	1 ± 4	4 ± 6	62 ± 11*
Anti-I-J^k	Eluate	−3 ± 3	50 ± 6*	4 ± 5	0 ± 5	46 ± 9*
	Filtrate	56 ± 4*	12 ± 9	60 ± 8*	80 ± 3*	3 ± 9

‡ Groups of mice were primed with NP-O-Su. On the day before and the day of antigen challenge, 0.5 ml of the designated column fractions were injected i.v. C57BL/6 mice were recipients of B6-Ts_3-2 and (B6 × C3H)F_1-Ts_3-1032 factor, B10.BR mice were recipients of CKB-Ts_3-9 factor, C3H.SW recipients were given (B6 × C3H)F_1-Ts_3-1127 factor and C3H mice were given (B6 × C3H)F_1-Ts_3-1131 factor.

§ The data represent the pooled and normalized percent suppression from one to three separate experiments for each factor. An asterisk indicates significant suppression, $P<0.01$.

conventional anti-I-J^k columns. Three (B6 × C3H)F_1-Ts_3–derived factors, (B6 × C3H)F_1-Ts_3-1032, (B6 × C3H)F_1-Ts_3-1127, and (B6 × C3H)F_1-Ts_3-1131, were passed over the same columns and the activity of the filtrate and eluate fractions were tested using C57BL/6, C3H.SW, and C3H mice, respectively. The suppressive activities of (B6 × C3H)F_1-Ts_3-1032 and (B6 × C3H)F_1-Ts_3-1127 factors were absorbed by monoclonal anti-I-J^b (WF9-40.5) and conventional anti-I-J^b columns. In contrast, the suppressive activity of (B6 × C3H)F_1-Ts_3-1131 factor was adsorbed by monoclonal and conventional anti-I-J^k immunoadsorbents (Table VII). The I-J and Igh-related determinants on (B6 × C3H)F_1-Ts_3-1114 derived factor were not analyzed.

Thus, (B6 × C3H)F_1-Ts_3-1032 factor, which suppressed NP-responses in C57BL/6 (H-2^b,Igh^b) recipients, bears I-J^b and NP^b-related idiotypic determinants. Similarly, (B6 × C3H)F_1-Ts_3-1127 factor, which is specific for C3H.SW (H-2^b, Igh^j) recipients, bears I-J^b and NP^j-related idiotypic determinants. TsF_3 derived from (B6 × C3H)F_1-Ts_3-1131 hybridoma cells carries I-J^k and NP^j-related determinants and its activity is restricted to mice carrying the H-2^k and Igh^j haplotypes.

Discussion

We had previously demonstrated that two distinct populations of Ts_3 cells, each restricted to one parental H-2 haplotype, existed in H-2 heterozygous F_1 mice (12). However, those previous studies never evaluated the Igh restrictions of the Ts_3 cells from Igh heterozygous donors. The present studies focus on this issue. Two independent approaches were used. First, heterogeneous populations of Ts_3 cells were induced in (B6 × C3H)F_1 donors and activated in vitro with monoclonal TsF_2. The activated Ts_3 cells were adoptively transferred to Ts_3-

depleted C57BL/6, C3H.SW, C3H, or CKB recipients. Strict genetic restrictions controlled the activation of the F_1-derived Ts_3 populations. Thus, H-2 and Igh homology was always required between the TsF_2 used for activation and the recipient strain (Table I). Thus, it appeared that four distinct Ts_3 subpopulations, each with a distinct combination of restriction specificities, existed within H-2 and Igh heterozygous F_1 animals. Although these conclusions describe the phenomena, they do not unravel the underlying mechanism responsible for the restrictions. One hypothesis to explain these restrictions is based on the previous demonstration that specialized populations of I-J-bearing antigen (or factor)-presenting cells are required for imposing I-J genetic restrictions on suppressor T cells (8, 9). Thus, to account for the restrictions between TsF_2 and the recipients, we postulate that an I-J-bearing acceptor cell population is also involved in the presentation of TsF_2. Such cells could serve to juxtapose the TsF_2 with the Ts_3 cells, thereby permitting the interactions required for Ts_3 activation. Furthermore, since suppressor cell interactions can occur in the absence of an external source of antigen (13, 25), the Igh restriction may reflect an antiidiotypic internal image (which substitutes for antigen). This hypothesis further suggests that such a system of internal images is in some fashion controlled by genes linked to the Igh complex. This speculative hypothesis, which attempts to account for the I-J and Igh restrictions, will require additional experimental support.

The second approach to analyzing Ts_3 specificity involved the hybridization of (B6 × C3H)F_1-derived Ts_3 cells with the BW5147 thymoma. This approach led to the production of four monoclonal hybridoma cell lines. The hybridomas constitutively secreted soluble factors (TsF_3) that mediated NP-specific effector phase suppressor activity (Tables II and III). The evidence that these hybridomas represented Ts_3 cells included these facts: (*a*) the methods of induction and enrichment were based on those used to identify the Ts_3 population, (*b*) the hybridomas had I-J and NP^b phenotypes characteristic of Ts_3 cells, (*c*) the hybridoma cells produced NP-specific soluble suppressor factors that only functioned in the effector phase of the immune response in an H-2 (I-J), and Igh-restricted fashion, (*d*) the factors were active in cyclosphosphamide treated recipients (data not shown), and (*e*) the factors specifically bound to columns containing allele-specific anti-I-J and antiidiotypic antibodies. All the above features are characteristic of Ts_3 cells and their factors and permit one to distinguish Ts_3 cells from the other suppressor cells and factors of the NP suppressor cell cascade.

The production of these (B6 × C3H)F_1-derived Ts_3 hybridomas permitted analysis of the genetic restrictions at the clonal level. In conformation of the adoptive transfer data (Table I), we noted that each hybridoma was restricted to one of the parental H-2 and Igh haplotypes. Of the four hybridomas characterized, we noted three distinct restriction specificities, including one clone, (B6 × C3H)F_1-Ts_3-1131, which was restricted by H-2^k- and Igh^j-linked genes (Table IV). The corresponding subpopulation in primed lymph node cells could not be identified using the adoptive transfer protocol, since C3H-derived TsF_2 was not available. Factors derived from (B6 × C3H)F_1-Ts_3-1127 cells specifically suppressed NP responses in C3H.SW (H-2^b; Igh^j) recipients (Table IV). Thus, the

latter F_1 hybridoma clone demonstrated a "scrambled" restriction specificity, ie., the H-2^b restriction specificity was derived from the C57BL/6 parent and the Igh^j restriction specificity was derived from the C3H parent. Additional data supporting the notion of "scrambled" restriction specificities was obtained from the adoptive transfer experiments in which in vitro–activated (B6 × C3H)F_1-derived Ts_3 subpopulations specific for C3H.SW (H-2^b; Igh^j) and CKB (H-2^k; Igh^b) recipients were identified. The above results imply that the scrambled genetic restrictions may be a consequence of a receptor or an acceptor on the Ts_3 population that recognizes a combined Igh and MHC specificity present in the F_1 recipients, and/or that the Igh and H-2 receptors responsible for these scrambled genetic restrictions are separately encoded in the genome and demonstrate independent assortment in F_1 cells.

Another important purpose of these studies was to evaluate any correlations between the I-J and idiotype-related determinants present on the F_1-derived Ts_3 hybridoma cells and their factors. The level of complement-mediated lysis of these and other hybridoma cells using specific anti-I-J alloantisera, was variable (27, 28). Although there have been claims that this variability was dependent on the cell cycle (29), little is known about such parameters. Our initial screening suggested that all four F_1-derived hybridoma cell lines reacted with both anti-I-J^b and anti-I-J^k alloantisera. Previous studies have also indicated that both parental I-J alleles are expressed on H-2 heterozygous F_1-derived T cells (12, 30). In contrast, the monoclonal suppressor factors derived from these F_1 hybridoma cells reacted with only one set of allele-specific anti-I-J reagents. Both conventional alloantisera and monoclonal anti-I-J reagents, specific for I-J^b and I-J^k allelic products, were used to examine the F_1-derived factors (Table VII). Either one of the TsF_3 products secreted from each of the hybridomas was nonfunctional or the molecules derived from F_1 hybridomas only carry one set of I-J determinants even though both I-J allelic products may be expressed on the cell surface. Data in another system also suggest that H-2 heterozygous F_1 cells may produce TsF with only one parental I-J determinant (21), however, the phenotype of the Ts cells in those experiments was not determined.

The correlation between the phenotypic expression of Igh-controlled idiotype-related determinants of the F_1-derived cells and factors was more consistent. Thus, the (B6 × C3H)F_1-Ts_3-1032 cells were lysed with anti-NP^b, but not anti-NP^j reagents, while the (B6 × C3H)F_1-Ts_3-1132 cells demonstrated the reciprocal pattern. Furthermore, the TsF_3 derived from these hybridomas specifically bound to immunoadsorbent columns containing the appropriate allele-specific antiidiotypic reagents. Cells from one hybridoma, (B6 × C3H)F_1-Ts_3-1127 were not lysed by either antiidiotypic reagent, but TsF_3 derived from these cells was specifically bound by an anti-NP^j immunoadsorbent column. This finding suggested that the sensitivity of the column adsorptions was greater than that of the complement-mediated cytotoxicity tests. Nonetheless, the fact that the antiidiotypic reagents could be used to distinguish among the hybridoma cells and factors suggests that the idiotype-like determinants detected by these reagents are allelically excluded on F_1-derived Ts_3 cells and factors. In contrast, there is codominant expression of I-J determinants on the F_1-derived hybridoma cells, although there is functional allelic exclusion of I-J determinants on F_1-derived

TsF_3. It is interesting that F_1-derived TsF_3 displays many of the same features of allelic exclusion as exhibited by immunoglobulins. Thus, in F_1-derived B cells only one heavy chain and one light chain gene product is expressed (31). There have, however, been several examples of aborted heavy or light chain rearrangements that affect the other parental chromosome (32, 33). Although the latter gene products are generally not secreted, in selected instances, they are produced and can be detected in nonsecreted form (34).

A complete correlation was found between the determinants on F_1-derived TsF_3 and the restriction specificity of these factors. Thus, among the three F_1 hybridoma-derived factors examined, those that were restricted to H-2^b, reacted with anti-I-J^b immunoadsorbent columns, whereas those restricted to H-2^k were bound by anti-I-J^k columns. Furthermore, when the activity of F_1-derived TsF_3 was restricted to strains bearing Igh^b-linked genes, the factors were bound by anti-NP^b columns but not immunoadsorbent columns specific for anti-NP antibodies derived from mice carrying the Igh^j haplotype. The latter antiidiotypic reagents were capable of specifically binding (B6 × C3H)F_1-derived TsF_3, the activity of which was restricted to strains bearing the Igh^j haplotype. These findings appear to conflict with the observations of Yamauchi et al. (35), who reported that the Igh restriction specificity of spleen cell–derived TsF was controlled by the I-J–bearing chain. Although the factor described Yamauchi et al. is also a two-chain heterodimer, it was derived from Lyt-1+ inducer Ts. In contrast, the products described in the present report are derived from Lyt-2^+ effector Ts; consequently direct comparisons of the present data with those of Yamauchi and colleagues may not be valid.

Some recent reports have indicated that the I-J region may not code for a gene product (36). These findings raise many questions as to the nature of the I-J products detected on T cells by this and many other laboratories (1–4, 16–18). In fact, what has been shown in all these reports is that antibodies produced in 3R–5R combinations can bind to suppressor cells and factors. We have recently examined this issue and concluded that the I-J determinants found on antigen-presenting cells (perhaps a modified I-E_B gene product) may represent the restricting elements (8, 9). We postulated that the determinants detected on suppressor T cells and factors are anti-self I-J receptors that are recognized by antiidiotypic antibodies contaminating the conventional anti-I-J alloantisera (9). The monoclonal reagents used in this and other reports may also represent anti-receptor antibodies. In the current experiments, the restriction of TsF_3 to the I-J subregion matching the "I-J phenotype" of the factor may be attributed to recognition of I-J products present in the recipient by clonally restricted anti-self I-J receptors on TsF_3. Since the NP-specific TsF_3 molecule consists of a disulfide-linked heterodimer, containing an antigen-binding chain and a second chain that reacts with anti-I-J antisera (11, 17), it is tempting to create a model of TsF_3 in which each chain controls a distinct restriction specificity, i.e. a two-receptor molecule.

Finally, it is important to evaluate the specificity of suppression in F_1 vs. parental recipients. In the various experiments performed using either the Ts_3 cell transfer protocol or the analysis of F_1-derived TsF_3, we noted that the magnitude of suppression was generally comparable in either parental or F_1

recipients. If TsF_3 is specific for the receptors on CS effector cells we would expect a lower degree of suppression on the CS responses in H-2 heterozygous F_1 animals. Therefore, we must either entertain the possibility that the targets of Ts_3 cells or TsF_3 are not the CS effector population (2, 37, 38) and/or that the final suppressor mechanism may demonstrate bystander suppression (39, 40).

Summary

In the 4-hydroxy-3-nitrophenyl acetyl (NP) contact sensitivity system, the activity of third-order suppressor cells and their factors is restricted by H-2(I-J) and Igh linked genes. The present report analyzes the specificity of NP-specific Ts_3 cells and factors derived from H-2 and Igh heterozygous (B6 × C3H)F_1 mice. Two approaches were used. First, heterogeneous populations of F_1 Ts_3 cells were activated in vitro and then assayed in Ts_3-depleted recipients which carried different combinations of H-2 and Igh alleles. The second approach was to hybridize the Ts_3 cells and analyze the specificity of the F_1-derived TsF_3. The combined data demonstrated four functionally distinct populations of Ts_3 cells. The activity of each population was restricted by a particular combination of H-2 and Igh haplotypes. Thus, Ts_3 cells derived from F_1 donors can demonstrate an apparent scrambling of H-2 and Igh restriction specificities.

There was functional allelic exclusion of the H-2(I-J) and Igh determinants expressed on (B6 × C3H)F_1 hybridoma–derived TsF_3. Thus, TsF_3 from each cloned hybridoma line expressed only one set of I-J and Igh determinants. Furthermore, there was a complete correlation between the I-J and Igh linked determinants expressed on TsF_3 and the restriction specificity.

In view of the recent findings on the molecular biology of the I-J region, an alternative interpretation of the role of I-J determinants on suppressor cells and factors is offered.

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