I-J RESTRICTIONS ON THE ACTIVATION AND INTERACTION OF PARENTAL AND F_1-DERIVED Ts_3 SUPPRESSOR CELLS*

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Data from a variety of systems indicate that several distinct populations of T lymphocytes are involved in the process of immune suppression (1-3). These suppressor T cells (Ts)[1] function in a defined sequence. The nature of these cells and the Ts-derived factors (TsF) involved in the suppressor pathway have not been fully resolved, but in at least two independent systems three separate Ts populations have been identified (4-6). These Ts populations have been termed Ts_1, Ts_2, and Ts_3. Many of the Ts described in the literature have properties similar to one of these three populations. Although it is difficult to classify all Ts reported in this simplified suppressor cell cascade, many of the discrepancies might reflect differences in the various assay conditions used rather than implying the existence of several totally distinct suppressor cell pathways.

One of the most frequently defined Ts cell types appears to correspond to the Ts_3 population identified in the 4-hydroxy-3-nitrophenyl acetyl (NP) suppressor system. This suppressor cell population is derived from antigen-primed mice, may represent the final or effector cell in the Ts pathway, has the Lyt 1^-, Lyt 2^+, I-J^+ phenotype, and produces a soluble TsF that may under selected conditions be nonspecific (4, 7). Ts cells that fit most of these criteria have also been identified in the azobenezenearsonate (6, 8), dinitrophenyl (9), trinitrophenyl (10), keyhole limpet hemocyanin (11), and sheep erythrocyte (12) systems.

This report focuses on the mechanism of Ts_3 cell activation and the specificity of Ts_3 cells, especially those obtained from F_1 hybrid mice. The NP suppressor system was chosen to study these parameters because the methods for assaying Ts_3 activity independent of Ts_1 or Ts_2 activity had been established (4, 5). Furthermore, we previously characterized (13) suppressor factors (TsF_2) derived from a series of monoclonal Ts_2 hybridomas that could be used to activate Ts_3 cells. The present data demonstrate that the suppressive activity of the Ts_3 population is not manifest unless these cells are specifically activated by TsF_2. Furthermore, the data suggest that

* Supported in part by grant SO 7 RR 05381-20 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and by grant CA-14723 from the National Cancer Institute, and a grant from the Cancer Research Institute.

[1] *Abbreviations used in this paper:* CS, cutaneous sensitivity; CY, cyclophosphamide; DMSO, dimethyl sulfoxide; DNFB, 2,4-dinitrofluorobenzene; HBSS, Hanks' balanced salt solution; 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; Ts_1, Ts_2, Ts_3, first, second, or third order suppressor T cells, respectively; Ts, suppressor T cells; TsF_1, TsF_2, TsF_3, suppressor factor derived from Ts_1, Ts_2, or Ts_3, respectively.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/08/0465/15 $1.00 465
Volume 156 August 1982 465-479

distinct clones of F_1-derived suppressor cells are restricted to each parental H-2 haplotype. Thus, Ts cells, like helper T cells, appear to be restricted in their ability to recognize antigen in the context of major histocompatibility complex gene products, but in the Ts pathway, antigen may be associated with I-J products instead of products of the I-A or I-E loci.

Materials and Methods

Mice. All mice were either purchased from The Jackson Laboratory, Bar Harbor, ME, or were bred in the animal facilities at Harvard Medical School, Boston, MA. Mice were used at 3–12 mo of age and were maintained on laboratory chow and acidified, chlorinated water *ad lib.*

Animals used in this 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 Councel (DHEW publication (NIH) 78-23, revised 1978).

Antigens. NP-O-Succinimide (NP-O-Su) was purchased from 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-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 (14).

Treatment of Lymph Node Cells with Anti-I-J Antisera and Complement. 7.5×10^7 NP-immune lymph node cells were pelleted and incubated in 1.0 ml of a 1:5 dilution of B10.A(3R) anti-B10.A(5R) (anti-I-J^k) or B10.A(5R) anti-B10.A(3R) (anti-I-J^b) antisera. After 30 min at room temperature, cells were spun and resuspended in 1.0 ml of rabbit complement diluted 1:5 or 1:8 in Hanks' balanced salt solution. After an additional 30-min incubation at 37°C, the cells were washed three times and then activated with TsF_2, as detailed below.

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. B6-Ts_2-28 and CKB-Ts_2-59-derived TsF_2, which have been characterized and described (13), were used for activation of lymph node Ts_3 cells in vitro. 5×10^7 NP-primed lymph node cells were cultured for 2 or 48 h in 10 ml RPMI 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, or BW5147 cells that were grown in (AKR × B6)F_1, (AKR × CKB)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 (15). 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 in the left footpad with 0.025 ml 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 later. Swelling was determined as the difference, in units of 10^{-3} cm, between the left and right footpad thickness. It should be noted that 1×10^7 immune lymph node cells are not sufficient to transfer immunity under these experimental conditions.

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) (16). 5 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 DNFB + 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 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 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 group receiving Ts_3 cells activated with BW5147 tumor ascites − swelling of group receiving Ts_3 cells activated with TsF_2)/(swelling of group receiving Ts_3 cells activated with BW5147 tumor ascites − 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

In Vitro Activation of Ts_3 Cells. To demonstrate that Ts_2-derived factor could activate Ts_3 cells, we took advantage of past observations on the biological properties of the Ts_3 cell population. Thus, it was previously shown that the Ts_3 population was sensitive to (CY) treatment and, furthermore, that lymph node cells from antigen-primed mice could be used in adoptive transfer experiments to restore Ts_3 activity to the CY-treated recipients (4). To directly activate Ts_3 cells, we incubated 0.05 ml of BW5147 control or Ts_2 hybridoma-derived ascites with 5 × 10^7 NP-O-Su-primed lymph node cells in 10 ml of RPMI 1640 media containing 10% fetal calf serum. The cells were cultured for 48 h at 37° in 10% CO_2. After 48 h of in vitro culture, the cells were washed extensively, and 1 × 10^7 viable lymph node cells were injected intravenously into syngeneic recipients that had been previously primed with NP-O-Su and treated 24 h later with 20 mg/kg CY. In confirmation of previous findings (5), CY-treated recipients were not sensitive to suppression by monoclonal B6-Ts_2-28 or CKB-Ts_2-59 suppressor factor (Table I). However, significant suppression of the cutaneous sensitivity (CS) response was observed when CY-treated recipients were given lymph node cells derived from NP-O-Su-primed C57BL/6 mice that were activated in vitro with B6-Ts_2-28-derived TsF_2. As specificity controls, factors from the BW5147 tumor line or from the CKB-Ts_2-59 line failed to activate suppressive activity in these cells. The failure of CKB-Ts_2-59-derived TsF_2 to activate C57BL/6 antigen-primed lymph node cells is presumably due to the H-2-linked (I-J) genetic restriction of TsF_2 (13). Thus, the B6-Ts_2-28 factor that is derived from C57BL/6 (H-2^b, Igh^b) cells is only active in recipients that are matched at the I-J and Igh regions (13). The CKB-Ts_2-59 factor is of CKB (H-2^k, Igh^b) origin and is also genetically restricted by I-J and Igh genes. To verify that the CKB-Ts_2-59 factor was capable of activating antigen-primed lymph node cells of the appropriate strain, a reciprocal experiment was performed. As shown in Table I, the CKB-Ts_2-59 factor activated Ts_3 suppressive activity when incubated with H-2 and Igh-matched B10.BR lymph node cells, whereas the C57BL/6-derived Ts_2 factor failed to induce suppression under the same experimental conditions.

Kinetics of Ts_3 Activation. Lymph node cells from C57BL/6 mice were cultured with B6-Ts_2-28 or control BW5147-derived factors for various intervals ranging from 5 min to 48 h. The cells were then washed and assayed for suppressive activity in NP-O-Su-primed CY-treated C57BL/6 recipients. As shown in Fig. 1, maximum suppressive activity was noted after 1–2 h of in vitro activation with TsF_2. Activation of Ts_3 cells with TsF_2 for up to 48 h did not result in an increased level of immune suppression.

Specificity of In Vitro Activated Ts_3 Cells. The specificity of in vitro activated Ts_3 cells

TABLE I

*In Vitro Activation of Suppressor Cells with TsF_2**

TsF_2 source	Donor of NP-primed lymph node cells	NP-primed, CY-treated recipients	Footpad swelling ± SE (10^{-3} cm)
BW5147	None	C57BL/6	38.8 ± 1.4
B6-Ts_2-28	None	C57BL/6	38.0 ± 2.0
BW5147	C57BL/6	C57BL/6	37.5 ± 1.0
B6-Ts_2-28	C57BL/6	C57BL/6	15.8 ± 1.8‡
CKB-Ts_2-59	C57BL/6	C57BL/6	35.0 ± 3.8
BW5147	None	B10.BR	27.0 ± 1.8
CKB-Ts_2-59	None	B10.BR	28.0 ± 2.6
BW5147	B10.BR	B10.BR	26.3 ± 1.3
CKB-Ts_2-59	B10.BR	B10.BR	14.3 ± 1.2‡
B6-Ts_2-28	B10.BR	B10.BR	27.3 ± 1.7

* Regional lymph node cells from mice that had been immunized subcutaneously with 2 mg NP-O-Su were cultured for 48 h with TsF_2 or control BW5147 ascites for activation, then washed and transferred to designated recipients. Groups of recipient mice were immunized with 2 mg NP-O-Su. 24 h later, they were treated with intraperitoneal injections of 20 mg/kg CY. On day 6, each mouse received 1×10^7 activated NP-primed lymph node Ts_3, and the recipients were challenged after cell transfer. The data were expressed as the increment of footpad swelling ± SE in units of 10^{-3} cm. The background response of nonimmunized C57BL/6 mice was 12.5 ± 1.3 and that of B10.BR was 7.3 ± 1.1.

‡ Significant suppression, $P < 0.001$.

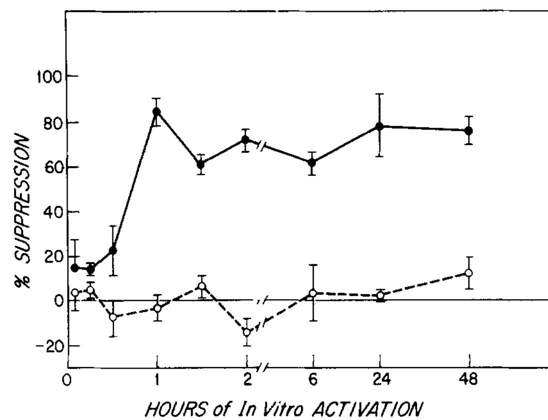

FIG. 1. Kinetics of in vitro activation of lymph node cells from NP-O-Su-primed mice with TsF_2. C57BL/6 mice were immunized with 2 mg NP-O-Su. After 6 d, the regional lymph nodes were removed, teased, and the cells were cultured for 5 min to 48 h with TsF_2 or control BW5147 ascites for activation. The Ts_3 cells were then washed and used for transfer. Groups of recipient mice were immunized with 2 mg NP-O-Su. 24 h later, the recipients were treated with an intraperitoneal injection of 20 mg/kg CY. On day 6, each mouse received 1×10^7 activated NP-primed lymph node Ts_3 cells intravenously. The mice were then challenged. The data represent pooled results from two separate experiments. The data were normalized and the percent suppression ± SE was calculated. ●, TsF_2; ○, BW.

was evaluated in two ways. First, NP-O-Su or DNFB antigen-primed C57BL/6 lymph node cells were used as the source of Ts_3 cells for activation with TsF_2. Second, these activated cells were tested for suppressive activity in syngeneic C57BL/6 recipients primed with either DNFB or DNFB + NP-O-Su. In these experiments the mice were challenged by injection of NP-O-Su into the left ear pinna or by painting the left ear with DNFB or both. The control right ear was untreated. As shown in Table II, the only condition in which significant levels of suppression were observed was when hybridoma-derived TsF_2 was used to activate Ts_3 cells from NP-O-Su-primed mice and when these activated Ts_3 cells were tested in animals primed and challenged with NP-O-Su. The suppression was not due to the carry over of B6-Ts_2-28 factor because intravenous injection of TsF_2 did not suppress CY-treated recipients (Table II). NP-specific TsF_2 would not activate lymph node cells from DNFB-primed mice, even when these cells were tested in DNFB-primed and challenged recipients. Furthermore, there is no apparent suppression of a bystander DNFB response when activated Ts_3 cells are transferred to recipients that had been either doubly primed or challenged with DNFB + NP-O-Su (Table II).

Genetic Restrictions on Ts_3 Cell Activation and Function. One of the advantages of activating Ts_3 cells in vitro is that it permits independent analysis of the genetic restrictions for Ts_3 activation and Ts_3-target cell interactions. Control BW5147, C57BL/6 (H-2^b, Igh^b), and CKB (H-2^k, Igh^b)-derived TsF_2 were incubated with C57BL/6, B10.BR (H-2^k, Igh^b), CKB, or C3H (H-2^k, Igh^j) NP-O-Su-primed lymph node cells. Ts_3 activation was assessed by adoptively transferring the in vitro activated

TABLE II

*Specificity of in Vitro Ts_3 Cell Activation**

48-h Ts_3 activation		Priming of CY-treated recipient	Antigen for ear challenge		
TsF_2 source	Antigen for Ts_3 priming		NP-O-Su	DNFB	NP-O-Su + DNFB
BW5147 (i.v.)	—	NP + DNFB	16.3 ± 0.3	10.8 ± 0.3	
B6-Ts_2-28 (i.v.)	—	NP + DNFB	17.3 ± 0.5	NT‡	
BW5147	NP-O-Su	NP + DNFB	16.5 ± 0.6	10.5 ± 0.3	
B6-Ts_2-28	NP-O-Su	NP + DNFB	8.3 ± 1.0§	10.3 ± 0.5	
BW5147	DNFB	NP + DNFB	14.3 ± 3.4	10.0 ± 1.1	
B6-Ts_2-28	DNFB	NP + DNFB	18.0 ± 0.4	11.8 ± 1.8	
BW5147	—	None	4.0 ± 0.4	1.0 ± 0.6	
BW5147 (i.v.)	—	DNFB			18.0 ± 0.8
B6-Ts_2-28 (i.v.)	—	DNFB			18.8 ± 0.5
BW5147	NP-O-Su	DNFB			17.3 ± 0.5
BW5147	DNFB	DNFB			16.8 ± 0.5
B6-Ts_2-28	NP-O-Su	DNFB			18.8 ± 1.2
B6-Ts_2-28	DNFB	DNFB			17.0 ± 1.2
BW5147	—	None			5.0 ± 0.6

* Regional lymph node cells from mice that had been immunized with DNFB or NP-O-Su were cultured with B6-Ts_2-28 or control BW5147 ascites for activation. Groups of recipient mice were primed with DNFB alone or DNFB and NP-O-Su. 24 h later, all groups were given 20 mg/kg CY. 6 d later, mice were given the in vitro activated Ts_3 cells and challenged with DNFB or NP-O-Su alone or with DNFB and NP-O-Su.

‡ Not tested.

§ Significant suppression, $P < 0.001$.

Ts_3 cells to antigen-primed CY-treated C57BL/6, B10.BR, CKB, or C3H recipients. Activated Ts_3 cells were transferred during the effector phase of the CS response, i.e., on the day of antigen challenge. Such effector phase transfers minimize potential allogeneic effects because the Ts_3 cells are only present in the allogeneic environment for 24 h before termination of the assay. Furthermore, the BW5147-activated Ts_3 lymph node population serves as a control for nonspecific suppression. The data shown in Table III were derived from seven independent experiments that were normalized and pooled. Activation of the Ts_3 population was generally assayed after 2 h of incubation with TsF_2. After activation of the Ts_3-containing lymph node population, suppressive activity was only noted in combinations of TsF_2, Ts_3, and recipients that were matched at the H-2 and Igh gene complexes. Thus, after a 2 h in vitro activation, C57BL/6 (H-2^b)-derived TsF_2 activated C57BL/6 but not B10.BR

TABLE III

Genetic Restrictions on Ts_3 Cell Activation and Function‡*

TsF_2 source	Ts_3 donor	CY-treated recipients	Normalized percent suppression ± SE
BW5147	C57BL/6	C57BL/6	0 ± 3 (7)
B6-Ts_2-28	C57BL/6	C57BL/6	51 ± 5 (8)‡
CKB-Ts_2-59	C57BL/6	C57BL/6	5 ± 7 (4)
BW5147	B10.BR	C57BL/6	0 ± 4 (4)
B6-Ts_2-28	B10.BR	C57BL/6	−5 ± 4 (4)
CKB-Ts_2-59	B10.BR	C57BL/6	2 ± 6 (4)
BW5147	C57BL/6	B10.BR	0 ± 3 (4)
B6-Ts_2-28	C57BL/6	B10.BR	7 ± 8 (4)
CKB-Ts_2-59	C57BL/6	B10.BR	6 ± 19 (4)
BW5147	B10.BR	B10.BR	0 ± 7 (4)
B6-Ts_2-28	B10.BR	B10.BR	3 ± 9 (4)
CKB-Ts_2-59	B10.BR	B10.BR	55 ± 10 (4)‡
BW5147	CKB	B10.BR	0 ± 9 (4)
B6-Ts_2-28	CKB	B10.BR	−1 ± 11 (4)
CKB-Ts_2-59	CKB	B10.BR	80 ± 8 (4)‡
BW5147	C3H	B10.BR	0 ± 6 (8)
B6-Ts_2-28	C3H	B10.BR	−4 ± 7 (7)
CKB-Ts_2-59	C3H	B10.BR	2 ± 7 (8)
BW5147	CKB	CKB	0 ± 6 (9)
CKB-Ts_2-59	CKB	CKB	59 ± 4 (9)‡
BW5147	CKB	C3H	0 ± 7 (8)
CKB-Ts_2-59	CKB	C3H	3 ± 5 (8)
BW5147	None	C57BL/6	0 ± 7 (5)
B6-Ts_2-28	None	C57BL/6	−3 ± 5 (4)
BW5147	None	B10.BR	0 ± 8 (5)
CKB-Ts_2-59	None	B10.BR	−3 ± 19 (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 except for one experiment in which a 48-h activation was used. 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 were normalized and the percent suppression ± SE was calculated. The number of mice is indicated in parentheses.

‡ Significant suppression, $P < 0.01$.

(H-2^k) Ts_3 cells that only functioned when adoptively transferred to syngeneic C57BL/6 recipients. Similarly, after 2 h of activation with CKB (H-2^k)-derived TsF_2, only B10.BR or CKB Ts_3-containing lymph node cells were activated. Furthermore, the activated B10.BR or CKB Ts_3 population would suppress CS responses in H-2 and Igh-matched B10.BR and CKB recipients but not in Igh-disparate C3H mice (Table III). To prove that the suppression was mediated by activated Ts_3 cells instead of TsF_2 that might have been passively transferred along with the Ts_3 cells, we injected TsF_2 intravenously into NP-O-Su-primed CY-treated recipients. As shown in Table III, administration of TsF_2 without added Ts_3 cells was unable to suppress CS responses in antigen-primed CY-treated recipients.

I-J Restriction of Activated Ts_3. Because after a 2-h activation period the TsF_2-Ts_3-target cell interactions are H-2 restricted, we next asked which subregion within the H-2 complex was responsible for this genetic restriction. Based on several previous studies that indicated that suppressor cell restrictions were generally mediated through the I-J subregion (5, 13, 17), we tested two congenic strains of mice, 3R(I-J^b) and 5R(I-J^k), that only differ with respect to their I-J subregions. The data in Table IV demonstrate that using 2-h activation conditions, suppression is only observed when the TsF_2-Ts_3 and the recipient strain are matched at the I-J subregion. The controls for these experiments were similar to those used in the previous experiments and demonstrate that the results are not due to carry over of TsF_2 (Table IV).

Activation and Function of Ts_3 Cells Derived from F_1 Mice. To further analyze the

TABLE IV

*I-J Restrictions of In Vitro Activated Ts_3 Cells**

TsF_2 source	Ts_3 donor	NP-primed, CY-treated recipients	Normalized percent suppression ± SE
BW5147	5R	5R	0 ± 6 (8)
B6-Ts_2-28	5R	5R	7 ± 5 (7)
CKB-Ts_2-59	5R	5R	50 ± 6 (8)‡
BW5147	3R	5R	0 ± 4 (8)
B6-Ts_2-28	3R	5R	3 ± 5 (8)
CKB-Ts_2-59	3R	5R	15 ± 3 (8)
BW5147	None	5R	0 ± 4 (8)
CKB-Ts_2-59	None	5R	8 ± 13 (8)
BW5147	5R	3R	0 ± 5 (8)
B6-Ts_2-28	5R	3R	6 ± 7 (7)
CKB-Ts_2-59	5R	3R	−1 ± 10 (8)
BW5147	3R	3R	0 ± 11 (8)
B6-Ts_2-28	3R	3R	59 ± 8 (8)‡
CKB-Ts_2-59	3R	3R	−11 ± 11 (8)
BW5147	None	3R	0 ± 6 (10)
B6-Ts_2-28	None	3R	7 ± 5 (8)

* Refer to legend for Table III for protocol. Regional lymph node cells from NP-primed mice were cultured with TsF_2 for 2 h. The data represent the pooled results from three separate experiments. The data were normalized and the percent suppression ± SE was calculated.

‡ Significant suppression, $P < 0.001$.

restrictions on Ts_3 cell interactions and to evaluate whether allogeneic effects could influence the results, we activated NP-O-Su-primed (B10 × B10.BR)F_1 lymph node cells with C57BL/6, CKB, or control BW5147-derived TsF_2 for 2 h in vitro. These activated F_1 cells were transferred to NP-O-Su-primed CY-treated C57BL/6 (H-2^b) or B10.BR (H-2^k) recipients. The data in Table V again clearly demonstrate an absolute requirement for H-2 homology between the TsF_2 and the recipient strain to obtain immune suppression. Thus, C57BL/6 (H-2^b)-derived TsF_2 activates (B10 × B10.BR)F_1 Ts_3 cells, but these cells only function in C57BL/6 (H-2^b) not B10.BR (H-2^k) mice. In a reciprocal experiment, CKB (H-2^k)-derived TsF_2 activated (B10 × B10.BR)F_1 Ts_3 cells, but again these F_1 cells only produce suppression when transferred into H-2^k-bearing B10.BR recipients.

The simplest hypothesis that would account for the above observation is that two distinct Ts_3 populations exist in lymph node cells derived from F_1 animals; one population is restricted by I-J^b gene products and the other by I-J^k gene products. This hypothesis parallels the situation observed with helper T cells derived from F_1 mice in which two functionally distinct populations exist and each is restricted by different I region genes (18, 19). Another possibility to account for these observations is that the I-J gene products are allelically expressed on the F_1 cells. To test the latter possibility, B10.BR, C57BL/6, or (B10 × B10.BR)F_1 NP-O-Su-primed lymph node cells were treated with anti-I-J^k or anti-I-J^b alloantisera plus complement before a 2-h activation with TsF_2. As shown in Table VI, treatment of B10.BR Ts_3 cells with anti-I-J^k specifically depleted the ability to generate suppressive activity. In reciprocal groups, treatment of C57BL/6 Ts_3 cells with anti-I-J^b but not anti-I-J^k alloantisera completely eliminated Ts_3 cell activity. When the same anti-I-J alloantisera were used to lyse (B10 × B10.BR)F_1 Ts_3 cells, both anti-I-J^b and anti-I-J^k alloantisera eliminated the ability to generate functional Ts_3 cells. Thus, it appears that Ts_3 cells derived from (B10 × B10.BR)F_1 donors carry both the I-J^k and I-J^b antigenic determinants in a codominant fashion.

Suppression of H-2 Heterozygous F_1 Recipients by Activated Ts_3 Cells. Finally, to evaluate

TABLE V
*Activation of Ts_3 Cells from F_1 Hybrid Mice**

TsF_2 source	Ts_3 donor	CY-treated recipients	Normalized percent CS suppression ± SE
BW5147	(B10 × B10.BR)F_1	C57BL/6	0 ± 4 (8)
B6-Ts_2-28	(B10 × B10.BR)F_1	C57BL/6	43 ± 3 (8)‡
CKB-Ts_2-59	(B10 × B10.BR)F_1	C57BL/6	1 ± 5 (8)
BW5147	(B10 × B10.BR)F_1	B10.BR	0 ± 6 (8)
B6-Ts_2-28	(B10 × B10.BR)F_1	B10.BR	−2 ± 6 (8)
CKB-Ts_2-59	(B10 × B10.BR)F_1	B10.BR	54 ± 6 (7)‡
BW5147	None	C57BL/6	0 ± 5 (10)
B6-Ts_2-28	None	C57BL/6	−2 ± 3 (8)
BW5147	None	B10.BR	0 ± 6 (10)
CKB-Ts_2-59	None	B10.BR	−4 ± 9 (8)

* Refer to legend for Table III for protocol. The 2 h-activation data represent the pooled results from two separate experiments. The data were normalized and the percent suppression ± SE was calculated.

‡ Significant suppression, $P < 0.01$.

TABLE VI

*F_1-derived Ts_3 Cells Bear Both Parental IJ Determinants**

TsF_2 source	Ts_3 donor	Ts_3 treatment	NP-primed CY-treated recipient	Normalized percent CS suppression ± SE
BW5147	B10.BR	—	B10.BR	0 ± 4 (12)
B6-Ts_2-28	B10.BR	—	B10.BR	−1 ± 11 (4)
CKB-Ts_2-59	B10.BR	—	B10.BR	78 ± 4 (12)‡
CKB-Ts_2-59	B10.BR	Anti-IJ^k + C	B10.BR	7 ± 8 (12)
CKB-Ts_2-59	B10.BR	Anti-IJ^b + C	B10.BR	80 ± 6 (8)‡
BW5147	C57BL/6	—	C57BL/6	0 ± 4 (8)
B6-Ts_2-28	C57BL/6	—	C57BL/6	64 ± 5 (6)‡
B6-Ts_2-28	C57BL/6	Anti-IJ^k + C	C57BL/6	43 ± 9 (7)‡
B6-Ts_2-28	C57BL/6	Anti-IJ^b + C	C57BL/6	−1 ± 4 (8)
BW5147	(B10 × B10.BR)F_1	—	B10.BR	0 ± 3 (12)
B6-Ts_2-28	(B10 × B10.BR)F_1	—	B10.BR	2 ± 5 (12)
CKB-Ts_2-59	(B10 × B10.BR)F_1	—	B10.BR	61 ± 5 (11)‡
CKB-Ts_2-59	(B10 × B10.BR)F_1	Anti-IJ^k + C	B10.BR	3 ± 6 (12)
CKB-Ts_2-59	(B10 × B10.BR)F_1	Anti-IJ^b + C	B10.BR	11 ± 7 (8)
BW5147	(B10 × B10.BR)F_1	—	C57BL/6	0 ± 3 (12)
CKB-Ts_2-59	(B10 × B10.BR)F_1	—	C57BL/6	4 ± 4 (12)
B6-Ts_2-28	(B10 × B10.BR)F_1	—	C57BL/6	55 ± 4 (12)‡
B6-Ts_2-28	(B10 × B10.BR)F_1	Anti-IJ^k + C	C57BL/6	5 ± 5 (11)
B6-Ts_2-28	(B10 × B10.BR)F_1	Anti-IJ^b + C	C57BL/6	4 ± 5 (8)

* Before activation of regional lymph node cells from NP-O-Su-primed mice, the lymph node cells were treated with anti I-J antisera and C, as described in Materials and Methods. Activation of the lymph node cells was done as in Table III. The data represent the pooled results from three separate experiments. The data were normalized and the percent suppression ± SE was calculated.

‡ Significant suppression, $P < 0.001$.

the potential role of the recipient strains in directing the genetic restrictions, H-2 heterozygous F_1 recipients were given in vitro activated Ts_3 cells. In the first experiment, C57BL/6 (H-2^b) or B10.BR (H-2^k) NP-O-Su-primed lymph node cells were used as the source of the Ts_3 population. The Ts_3 cells were activated for 2 h in vitro with monoclonal B6-Ts_2.28 (H-2^b origin) or CKB-Ts_2-59 (H-2^k origin) TsF_2 and then adoptively transferred to (B10 × B10.BR)F_1 (H-2^b × H-2^k) NP-O-Su-primed CY-treated recipients. As shown in Table VII, significant suppression was only noted when the TsF_2 and Ts_3 cells were derived from strains that shared H-2 haplotypes. It should be noted that after a 2-h activation, CKB (H-2^k)-derived TsF_2 failed to activate C57BL/6 (H-2^b) Ts_3 cells, even when the potential suppressive activity of these cells was assayed in H-2^b × H-2^k recipients. These data again indicate that under these experimental conditions a definite requirement for H-2 homolgy exists among the TsF_2, Ts_3 cells and the recipient strain.

In a second experiment, the role of genes linked to the Igh complex was also evaluated. Thus, C57BL/6 (H-2^b, Igh^b), CKB (H-2^k, Igh^b), and C3H (H-2^k, Igh^j) Ts_3 cells were activated for 2 h with either C57BL/6- or CKB-derived TsF_2. The activated cells were then adoptively transferred to (C57BL/6 × CBA)F_1 (H-2^b/H-2^k; Igh^b/Igh^j) recipients during the effector phase of the CS response. Only in those combinations

Table VII
*Suppression of F_1 Recipients by Activated Parental Ts_3 Cells**

TsF_2 source	Ts_3 donor	CY-treated recipients	Footpad swelling ± SE
BW5147	C57BL/6	(B10 × B10.BR)F_1	48.5 ± 2.4
B6-Ts_2-28	C57BL/6	(B10 × B10.BR)F_1	30.5 ± 1.9‡
CKB-Ts_2-59	C57BL/6	(B10 × B10.BR)F_1	47.3 ± 2.8
BW5147	B10.BR	(B10 × B10.BR)F_1	50.0 ± 1.9
B6-Ts_2-28	B10.BR	(B10 × B10.BR)F_1	47.7 ± 2.2
CKB-Ts_2-59	B10.BR	(B10 × B10.BR)F_1	35.4 ± 2.9‡
BW5147	C57BL/6	(B6 × CBA)F_1	56.6 ± 2.3
B6-Ts_2-28	C57BL/6	(B6 × CBA)F_1	31.3 ± 2.3‡
CKB-Ts_2-59	C57BL/6	(B6 × CBA)F_1	54.0 ± 3.5
BW5147	CKB	(B6 × CBA)F_1	56.8 ± 2.7
B6-Ts_2-28	CKB	(B6 × CBA)F_1	56.0 ± 2.8
CKB-Ts_2-59	CKB	(B6 × CBA)F_1	35.5 ± 2.2‡
BW5147	C3H	(B6 × CBA)F_1	58.0 ± 2.2
B6-Ts_2-28	C3H	(B6 × CBA)F_1	56.8 ± 2.2
CKB-Ts_2-59	C3H	(B6 × CBA)F_1	59.5 ± 2.5

* Refer to legend for Table III for protocol. The data were expressed as the increment of footpad swelling ± SE in units of 10^{-3} cm. The background response of nonimmunized (B10 × B10.BR)F_1 mice was 12.0 ± 1.2 and that of (B6 × CBA)F_1 mice was 10.0 ± 1.1.

‡ Significant suppression, $P < 0.01$.

in which the donor of the TsF_2, the Ts_3, and the recipients shared genes in both the H-2 and Igh complexes were significant levels of suppression noted (Table VII).

Discussion

The past several years have witnessed numerous advances in our knowledge of the mechanisms of immunoregulation. In some systems, three distinct T lymphocyte subpopulations act in a defined sequence to mediate immune suppression (20, 21). For example, suppression of both cellular (5) and humoral (7) immune responses to the NP require a similar cellular cascade involving Ts_1, Ts_2, and Ts_3 cells as well as factors derived from each of these cell types. Previous reports (5, 13, 16) from our laboratory characterized a series of hybridoma T cell lines representing each of these functional populations. Furthermore, we compared the suppressor factors (TsF) released by each of these Ts cells. The TsF_2 and TsF_3 factors, which both function during the effector phase of the immune response, have similar genetic restrictions. Thus, TsF_2 and TsF_3 only suppress strains of mice that are homologous with the factor-producing strain at both the H-2 complex (I-J subregion) and the Igh complex (5, 13). Because the basis for these dual restrictions had not been clarified, it was postulated that at least some of the restrictions might represent "pseudogenetic restrictions," as were initially described for TsF_1 factors and cells (16, 22, 23). These pseudogenetic restrictions reflect requirements for homology between H-2 or Igh determinants that are present at different ends of the suppressor cell cascade (16). The hypothesis that the dual genetic restrictions of TsF_2 reflected a psuedo-restriction was based on the observation that TsF_2 activity could be absorbed by Ts_3 cells derived

from mice of different H-2 haplotypes (13). The present protocol was designed to determine whether the allogeneic cells that could absorb TsF_2 could become activated. Thus, we developed an experimental system in which the genetics of activation of Ts_3 cells by TsF_2 could be analyzed in vitro, independent of the ability of activated Ts_3 cells to interact with their targets. The transfer of Ts_3 cells was performed during the effector phase, i.e., along with the NP-O-Su challenge and within 24–26 h of the termination of the CS response, to minimize potential allogeneic effects. Additional controls to exclude potential allogeneic affects included the transfer of nonactivated Ts_3 cells that were cultured with control BW5147-derived factor. Furthermore, F_1-derived Ts_3 cells and F_1 recipients were used in combinations in which the direction of the allogeneic effect could be controlled (Tables V and VII).

The data demonstrated that NP-specific Ts_3 cells are generated in NP-O-Su-immune animals concomitant with the CS effector cell population. In contrast to CS effector cells, Ts_3 are very sensitive to low dose CY treatment. The Ts_3 cells must be specifically activated by TsF_2 to manifest suppression (Table I). Normally, in a primary NP-O-Su immune response, the Ts_3 cells are not activated. However, later in the response the Ts_3 cells may play an important immunoregulatory role in modulating both the cellular and humoral immune response (4, 7). The present data directly demonstrate the role of TsF_2 in suppressor cell activation. The triggering of Ts_3 cells with TsF_2 is rapid. Thus, after 1–2 h of in vitro exposure to TsF_2, the activation of Ts_3 cells appears irreversible and results in optimum levels of suppression (Fig. 1). This rapid activation process presumably reflects the fact that the antigen-primed Ts_3 cells have already expanded and differentiated. These cells apparently await a terminal signal for activation and/or release of biologically active mediators, such as TsF_3.

The specificity of Ts_3 cell-mediated suppression was demonstrated in two ways. First, NP-specific Ts_3 cells are generated after NP-O-Su priming, whereas immunization with another antigen (e.g., DNFB) does not generate NP-reactive Ts_3 cells. Furthermore, once NP-O-Su-induced Ts_3 cells are activated with TsF_2, they suppress only NP-O-Su-induced CS responses even in animals that have been doubly primed or challenged with NP-O-Su plus DNFB (Table II). Although under the experimental conditions described in this report immune suppression is antigen specific, nonspecific suppression of immune response has been noted in other systems in which different experimental conditions are used (10–12). This disparity might reflect the requirement for the suppressor cell and the potential targets to be in very close proximity to mediate suppression.

Genetic analyses of the TsF_2-Ts_3-target cell interaction indicated the requirement for Igh homology was absolute. Thus, CKB (Igh^b)-derived TsF_2 would only activate an Igh-compatible Ts_3 population, which in turn only suppressed Igh homologous recipients (Table III). These results, along with previous data (24) demonstrating anti-idiotypic receptors on Ts_2 cells and factors as well as previous data demonstrating the presence of NP^b-related idiotypic determinants on Ts_1 and Ts_3 cells, suggests that suppressor T cell interactions proceed via a series of idiotypic-anti-idiotypic interactions in accord with Jerne's network hypothesis. In addition to the absolute requirement for Igh homology with respect to the cells involved in suppression of the effector phase of the contact sensitivity response, there also is an H-2 restriction that controls the interaction of these cells. Thus, after activation, the series of interactions between TsF_2, Ts_3, and the recipient strain appears to be completely H-2 restricted. These

H-2 restrictions can be more precisely mapped to the I-J subregion of the H-2 complex (Table IV), which has also been shown to regulate suppressor cell interactions in other systems (17, 25, 26). The physiological meaning of this I-J restriction is unknown. We have not yet determined the directionality of the restriction; i.e., do Ts_3 cells have a receptor for I-J determinants on a target population or are the I-J determinants present on Ts_3 cells and factors recognized by the target population?

To further evaluate the genetic restrictions on activated Ts_3 cells, (B10 × B10.BR)F_1 hybrid-derived Ts_3 were cultured with either H-2^b- or H-2^k-derived TsF_2, and the activated Ts_3 were tested for suppressive activity in either C57BL/6 (H-2^b) or B10.BR (H-2^k) recipients. The data again demonstrate that the critical requirements for H-2 homology were between the H-2 type of the TsF_2 donor and the H-2 type of the recipients of activated Ts_3 cells. Thus, a C57BL/6-derived TsF_2 activated (B10 × B10.BR)F_1-derived Ts_3 cells, as evidenced by their ability to suppress NP-induced CS responses in C57BL/6 mice. It should be noted that the same population of activated Ts_3 cells failed to suppress NP-O-Su CS responses in B10.BR recipients (Table V). Reciprocal data were obtained when CKB-derived TsF_2 was used to activate F_1-derived Ts_3 cells (Table V). The simplest explanation for these observations is that two distinct populations of Ts_3 cells exist in heterozygous F_1 donors, each restricted to a parental I-J determinant. This hypothesis is analogous to the findings noted with F_1-derived helper T cells (18, 19). By extending this analogy with helper T cells further, one can postulate that the induction of I-J restrictions might reflect the requirement for the initial presentation of antigen in the context of I-J determinants. Preliminary experiments support the latter postulate.

The next series of experiments was aimed at determining whether I-J determinants were allelically excluded in the H-2 heterozygous Ts_3 population. If only one I-J determinant was expressed on each subset of F_1-derived Ts_3 cells, it could help to explain the directionality of the genetic restriction. The data in Table VI clearly demonstrate that these I-J determinants are not allelically excluded in confirmation of the results reported by Okuda et al. (27), who arrived at similar conclusions in a different type of experimental system. However, because both I-J determinants are expressed on Ts_3 cells of F_1 origin, it will be important to analyze TsF_3 of F_1 origin to determine whether both I-J determinants are also present on these factors. Separate experiments are planned to address these questions.

Finally, we evaluated the role of the recipient strain in these genetic restrictions. By using F_1 recipients, we again confirmed the requirements for homology at both H-2 and Igh complexes. The recipient strain must contain the cells that are the target of the activated Ts_3 population. However, the present data do not permit us to determine the nature of these target cells. The target cells could be the CS effector cells, a Ts_4 population, or even an antigen-presenting cell. Whatever the nature of the target, we expect that it will either bear I-J determinants or receptors for I-J, and it may also bear anti-idiotypic receptors. Furthermore, the data obtained after a 2-h activation argue against the notion that the dual genetic restrictions of TsF_2 and TsF_3 are pseudogenetic restrictions, as were defined for Ts_1-derived factors (16, 22, 23). In addition, some experimental data indicate that TsF_3 may have a two-chain structure, one polypeptide containing I-J determinants and the other idiotypic determinants (28) (Furusawa, et al., unpublished data). The dual genetic restriction of Ts_3 cells and factors might therefore reflect the requirement of target cells to interact with both

portions of the TsF_3 molecule. The significance of these dual restrictions (I-J and Igh) might lie in the fact that two recognition signals are required for the activation of effector-suppressor cells. Such a two-signal model could account for the specificity of suppression as well as the molecular structure of the factor.

Summary

An experimental system was developed to independently analyze the H-2 and Igh genetic restrictions at two steps of the 4-hydroxy-3-nitrophenylacetyl hapten (NP) suppressor cell pathway. This experimental system allowed genetic analysis of the activation of Ts_3 cells by hybridoma-derived TsF_2 and independent analysis of the genetic restrictions that controlled the interaction of the Ts_3 cells with their target population. Thus, Ts_3 cells were activated in vitro with monoclonal H-2^b or H-2^k-derived TsF_2. The activated Ts_3 cells were then adoptively transferred to Ts_3-depleted (cyclophosphamide-treated) recipients of various genotypes. When the Ts_3-containing lymph node population was activated in vitro for 2 h, suppressive activity was only noted in combinations of TsF_2, Ts_3, and recipients that were matched at both the I-J and Igh gene complexes. The data indicate that TsF_2 can activate Ts_3 cells and that both the activation and the interaction of Ts_3 cells are I-J and Igh restricted. Using (B10 × B10.BR)F_1 mice as Ts_3 donors, we noted that H-2^b-derived TsF_2 activated these F_1 Ts_3 cells to suppress NP-specific cutaneous sensitivity responses in H-2^b but not in H-2^k recipients. Reciprocal experiments using H-2^k-derived TsF_2 demonstrated that only an H-2^k-restricted population was activated in the F_1-derived Ts_3 cells. The simplest explanation to account for these observations is that two distinct populations, each of which is restricted to a parental I-J determinants, exists in the heterozygous F_1 Ts_3 population. Furthermore, we demonstrated that both I-J^b and I-J^k determinants are expressed on F_1-derived Ts_3 cells. These observations are discussed in terms of the mechanisms involved in immunoregulation.

We would like to express our appreciation to Mrs. Nancy Axelrod and Mrs. Mary Jane Tawa for their excellent assistance in the preparation of this manuscript. In addition, we thank Ms. Ann Marie Fay for her outstanding technical help.

Received for publication 19 April, 1982.

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