ANALYSIS OF T CELL HYBRIDOMAS

IV. Characterization of Inducible Suppressor Cell Hybridomas*

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During the past several years, our laboratory has studied the mechanism of immune suppression in the 4-hydroxy-3-nitrophenyl acetyl (NP)[1] system. In this model system, both cell-mediated and humoral immunity (1–3) have been analyzed. The findings have permitted integration of the data into a single linear scheme of cellular interactions (3–5). Based on the results obtained in our and other laboratories that have investigated regulation by several types of suppressor T cells, we proposed that at least three distinct T cell subsets were involved in the phenomenon of immune suppression (3–5). These T cell subpopulations were termed Ts_1, Ts_2, and Ts_3. The major criteria that are used to distinguish and identify the various cells in the suppressor cell pathway include: (*a*) the binding specificity of the relevant cells, i.e., do the cells bind antigen or idiotype; (*b*) the surface phenotype of the cells; (*c*) the genetic restrictions at either the H-2 or Igh gene complexes on the interactions of either the cells or their soluble factors (TsF); and (*d*) the kinetics of suppression, i.e., do the cells function in the induction or effector phase of the immune response?

Ts_1 cells express the Ly-1^+2^- phenotype and possess NP^b-related idiotypic receptors that bind antigen (1, 4). Ts_1 cells only function during the afferent or induction phase of the immune response (1), in contrast to the other forms of suppressor cells that can function during the effector or efferent phase of the immune response (4–6). Ts_1 cells and their factors (TsF_1) function by stimulating a population of nonimmune T cells to become Ts_2 cells that are idiotype- rather than antigen-specific (6, 7). It should be noted that these conclusions were mainly derived from experiments that used hybridoma-derived monoclonal TsF_1 to generate Ts_2 cells in the absence of antigen (7). Another important property of TsF_1 is that it can induce Ts_2 cells in any strain of mouse without genetic restrictions (7). Similar populations of Ts_1-like inducer cells have been described in several other suppressor cell systems (8–13).

Ts_2 cells differ radically from Ts_1 in their properties and function. In the NP system, Ts_2 cells are antiidiotypic and can be shown to bind idiotype-coated plates (6), although they do not bind the major NP^b idiotypic determinants (14). Ts_2 cells bear

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[1] *Abbreviations used in this paper*: CS, contact or cutaneous sensitivity; CY, cyclophosphamide; DMSO, dimethylsulfoxide; DNFB, 2,4-dinitro-1-fluorobenzene; HBSS, Hanks' balanced salt solution; KLH, key-hole limpet hemocyanin; MHC, major histocompatibility complex; NP, 4-hydroxy-3-nitrophenyl acetyl hapten; NP-*O*-Su, NP-*O*-succinimide; PBS, phosphate-buffered saline; pTs_3, inducible TsF_3-secreting hybridoma; Ts_1, Ts_2, Ts_3, first, second, or third-order suppressor cells; TsF_1, TsF_2, TsF_3, first, second, or third-order suppressor factors.

Lyt-2 and I-J determinants (4, 15). These cells function in the effector phase of the immune response, i.e., they are able to suppress contact sensitivity or plaque-forming cell responses in immune animals (6, 16). Another very important difference between Ts_1 and Ts_2 cells concerns the genetic restrictions that govern their interactions with targets. Ts_2 cells are restricted by genes in both the Igh and I-J complexes (4, 7). That is, Ts_2 cells or their factors will not function unless adoptively transferred into recipients that are homologous at both the H-2 (I-J) and Igh gene complexes (15).

The efferent activity of Ts_2 cells led us to consider that these cells might be the final effectors of suppression. However, this proved not to be the case. We observed that injection of Ts_2 cells or TsF_2 would not suppress an immune animal if the recipient mouse had been treated with low doses of cyclophosphamide (CY) shortly after antigen immunization (4). This suggested that a highly CY-sensitive cell was the target of TsF_2. We demonstrated that Ts_3 cells were induced as a consequence of conventional immunization that also induced the T cells mediating contact or cutaneous sensitivity (CS) (4). In addition, Ts_3 cells were required for the modulation of NP-specific in vitro B cell responses (5). Antigen-activated Ts_3 cells appeared to be inactive until appropriately triggered by Ts_2 cells or TsF_2 (4, 5, 17).

The Ts_3 cells that modulate CS responses are antigen specific and H-2 and Igh restricted (4, 18). They express the Lyt-2^+ phenotype and also carry I-J determinants (4). Ts_3 cells produce an antigen-specific TsF_3 that mediates suppression in the effector phase of the immune response (18). The suppressive activity of Ts_3 cells and TsF_3 can be demonstrated in CY-treated recipients and, as is the case for TsF_2, is restricted by I-J and Igh genes (18). Ts_3 hybridomas that constitutively secrete TsF_3 have been obtained in the NP system (18).

Because the Ts_3 hybridomas constitutively secreted TsF, analysis of the triggering process required for release of these biologically active mediators was not possible. An understanding of how mature Ts cells are triggered is particularly important for the Ts_3 population since these cells may secrete the effector suppressor molecules. Ts_3 cells are generated by conventional antigen immunization and consequently are present concomitant with helper or delayed type hypersensitivity effector T cells (4, 5, 17). The Ts_3 population apparently expands and matures after immunization, but remains functionally inactive unless triggered by Ts_2 cells or TsF_2 (17, 19). This situation permits the primary immune response to proceed normally in the absence of suppression. Once Ts_2 cells or factors are generated, they will induce the terminal maturation of the Ts_3 population to secrete TsF_3, which in turn rapidly modulates the immune response. Thus, both TsF_2 and TsF_3 function in previously primed animals and can mediate suppression of either T or B cell responses within 24 h (4–6, 16).

To characterize the process of Ts_3 activation, we generated two hybridoma cell lines that fail to release TsF_3 unless they are specifically activated by TsF_2. These inducible TsF_3-secreting hybridomas, termed pTs_3, are the subject of this 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. Mice used were 3–12 mo old and were maintained on laboratory chow and acidified, chlorinated water ad lib. They 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 (20).

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) 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 (21). Guinea pig anti-NP^b antiidiotype antisera were prepared as detailed elsewhere (22). The characterization of these reagents was described previously (21).

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 (18). 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 100 μg NP-KLH i.p. in alum-pertussis (4), were added to purified anti-mouse immunoglobulin-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 were used as the source of Ts_3 cells.

Hybridization and Screening of Ts_3 Hybridoma Lines. Ts_3-enriched CKB or C57BL/6 (B6) lymphocytes were hybridized with BW5147 T lymphoma cells. The polyethylene glycol-mediated hybridizing method was exactly the same as previously reported (21). The hybridized Ts_3 candidates were screened using a cytotoxicity test (18) with allele-specific anti-I-J and anti-NP^b antisera. Two fusions were performed: the CKB-pTs_3-80 line was derived from a fusion of drug-marked BW5147 thymoma cells with NP-*O*-Su-primed lymph node cells, whereas the B6-pTs_3-9 line was obtained from a fusion using antigen-adherent splenic T cells from a NP-KLH-primed B6 mouse. The B6-pTs_3-9 and CKB-pTs_3-80 hybridoma lines were cloned by limiting dilution: 10 of the 11 clones tested had the same phenotype and suppressive activity as the parental line. All of the hybridomas were cultured in RPMI 1640 containing 8% fetal calf serum and 0.01 M Hepes buffer. The two pTs_3 hybridomas described in this report (and two others not discussed) were unstable. Even though frozen aliquots were used to regenerate the lines, activity was lost in a few months. The hybridomas characterized in this report are no longer available.

In Vitro Activation of pTs_3 Hybridoma with TsF_2. B6-Ts_2-28-, CKB-Ts_2-59-, and B6-Ts_2-14-derived TsF_2, which have been characterized and described elsewhere, were used for activation of pTs_3 hybridomas in vitro. 5×10^6 pTs_3 hybridoma cells were cultured for 2 d 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 or CKB Ts_2 hybridomas or BW5147 tumor cells that were grown in (AKR × B6)F_1 or (AKR × CKB)F_1 mice. After culture these activated pTs_3 hybridomas were washed three times with Hanks' balanced salt solution (HBSS), resuspended in 10 ml RPMI 1640 with 10% fetal calf serum and 0.1 mM Hepes, and cultured for 1 d. The culture supernatant was then collected and tested for suppressive activity.

In Vitro Activation of NP-primed Lymph Node Ts_3 Cells with TsF_2. Methods for the preparation and activation of NP-primed lymph node Ts_3 cells were described in detail elsewhere (17). In brief, 5×10^8 regional lymph node cells from mice that were immunized subcutaneously with 2 mg NP-*O*-Su were cultured with C3H.SW-Ts_2-7 hybridoma-derived TsF_2 or BW5147 tumor cell-derived ascites fluid for 2 h, washed three times, resuspended in HBSS, and injected intravenously as activated Ts_3 cells.

Absorption of TsF_2 with pTs_3 Hybridomas. 0.5 ml of 1:20 diluted Ts_2-derived ascitic factor was incubated with 5×10^6 pTs_3 hybridoma or BW5147 tumor cells for 1 h on ice with intermittent mixing. The supernatants were then centrifuged and tested for suppressor activity at a 1:200 dilution.

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

Assay for Suppressive Activity of TsF on NP-mediated CS Responses. The assay for NP-specific CS responses is also described elsewhere (23). Briefly, each animal was primed subcutaneously with 2 mg of NP-*O*-Su in DMSO. 24 h later, the mice were treated with an intraperitoneal injection of either saline or 20 mg/kg CY in saline to eliminate Ts_3 cell generation. 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 intravenously 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 thicknesses.

Assay for Suppressive Activity of Activated NP-primed Lymph Node Cells. Recipient mice were primed subcutaneously with 2 mg NP-*O*-Su. 24 h later, they were treated with an intraperitoneal injection of 20 mg/kg CY in saline. On day 6, they were given 1×10^7 NP-primed lymph node cells activated with C3H.SW-Ts_2-7-derived TsF_2 or BW5147 tumor cell-derived control factor. After transfer each mouse was challenged in the footpad with NP-*O*-Su as described above. Footpad swelling was measured 24 h after challenge.

DNFB CS Responses. CS was induced by two daily paintings on the shaved abdomen with 25 μl of 0.5% DNFB solution 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.

Percent Suppression. The percent suppression 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

pTs_3 hybridoma cells were prepared by the fusion of antigen-primed Ts_3 cells enriched from an antigen plate with the AKR-derived BW5147 thymoma. 2–3 wk after fusion, 230 of 2,000 wells developed hybridoma colonies. Each of the colonies was screened for the presence of NP^b-related idiotypic determinants and I-J determinants by microcytotoxicity testing. 60 lines were selected that bore either of these determinants but failed to constitutively secrete TsF_3. The B6-derived cell lines were cultured in the presence of B6-Ts_2-28 factor while the CKB lines were cultured for 2 d in the presence of CKB-Ts_2-59 factor. The cells were then washed three times with HBSS, and resuspended into fresh hypoxanthine-aminopterin-thymidine media and cultured for 1 d. The culture supernatants were tested for suppressive activity during the effector phase of the CS response. The B6-pTs_3-9 and CKB-pTs_3-80 lines were shown to have specific suppressor activity (Table I). Each of these lines was cloned and subsequent experiments were performed with the cloned hybridoma cell lines. It should be noted that both of these pTs_3 cell lines initially reacted with both anti-I-J and anti-NP^b antisera; however, after prolonged culture it became increasingly difficult to demonstrate the presence of these determinants on the cell lines. To ensure that we were not assaying the suppressive activity of contaminating TsF_2 that may have been carried over into the second culture, suppressive activity was assayed in CY-treated mice that are refractory to TsF_2 (18) (Table I). The data in Table I emphasize that pTs_3 cells must be activated to release a specific suppressor factor. The specificity of these pTs_3-derived factors is demonstrated by their inability to suppress DNFB CS responses in animals that were primed and challenged with DNFB (Table I).

Kinetics of pTs_3 Hybridoma Activation. The cloned B6-pTs_3-9 and CKB-pTs_3-80 hybridoma cells were cultured with suppressor factor derived from B6-Ts_2-28 or CKB-Ts_2-59 cells, respectively, or with BW5147-derived control 'factor' for various intervals

TABLE I

*Antigen Specificity of Activated pTs$_3$ Hybridoma-Derived Factors**

Recipient strain	Antigen priming	CY treatment	TsF Source		CS response‡	
			Hybridoma cells	TsF_2 used for activation	NP-*O*-Su	DNFB
					10^{-3} *cm* ± *SE*	
C57BL/6	+	+	BW5147	B6-Ts$_2$-28	25.8 ± 2.3	9.5 ± 1.6
C57BL/6	+	+	B6Ts$_2$-28	—	27.0 ± 3.4	ND§
C57BL/6	+	−	B6-Ts$_2$-28	—	16.3 ± 2.8‖	ND
C57BL/6	+	+	B6-pTs$_3$-9	—	23.6 ± 1.2	ND
C57BL/6	+	+	B6-pTs$_3$-9	B6-Ts$_2$-28	9.8 ± 1.0‖	9.5 ± 1.2
C57BL/6	−	+	—	—	4.8 ± 1.6	1.3 ± 0.6
B10.A	+	+	BW5147	—	42.6 ± 2.1	9.3 ± 0.8
B10.A	+	+	CKB-Ts$_2$-59	CKB-Ts$_2$-59	41.8 ± 4.2	ND
B10.A	+	−	CKB-Ts$_2$-59	—	17.6 ± 1.8‖	ND
B10.A	+	+	CKB-pTs$_3$-80	—	39.5 ± 3.1	ND
B10.A	+	+	CKB-pTs$_3$-80	CKB-Ts$_2$-59	16.5 ± 2.4‖	11.2 ± 0.6‖
B10.A	−	+	—	—	11.8 ± 0.8	0.5 ± 0.3

* Groups of four or five mice were immunized with either NP-*O*-Su or DNFB. 24 h later some groups were given 20 mg/kg CY i.p. On the day before and the day of antigen challenge, mice were given an injection of 0.4 ml i.v. of either control BW5147 or suppressor factors. Activated pTs$_3$ suppressor factors were obtained by culturing pTs$_3$ hybridomas with TsF_2 for 2 d and then in fresh media for 1 d.

‡ The data are expressed as the increment of footpad or ear swelling.

§ Not done.

‖ Indicates significant suppression, $P < 0.01$.

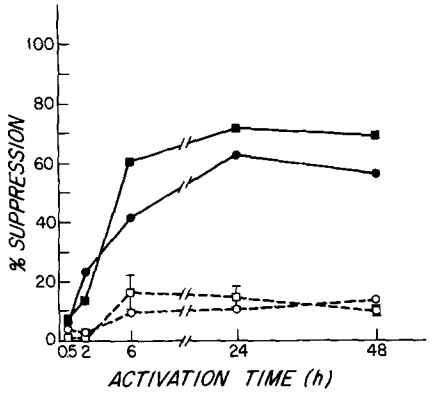

FIG. 1. Kinetics of pTs$_3$ activation. Groups of B6 mice or B10.BR mice were immunized subcutaneously with NP-*O*-Su. 24 h later all mice were given 20 mg/kg CY i.p. During the effector phase, mice were given an injection of 0.5 ml i.v. of either BW5147 or activated suppressor hybridoma factors. Activated B6-pTs$_3$-9 (●) and CKB-pTs$_3$-80 (■) factors were obtained by culturing suppressor hybridomas with B6-Ts$_2$-28 ascites or CKB-Ts$_2$-59 ascites, respectively, for 30 min to 48 h, and then in fresh media for 1 d. The data represent the pooled results from two separate experiments in the case of CKB-pTs$_3$-80 and one experiment with B6-pTs$_3$-9. The data are expressed as the percent suppression of the NP-*O*-Su CS response compared with BW5147 supernatant controls (- - -).

ranging from 5 min to 48 h. The hybridoma cells were washed and cultured in fresh media for 24 h, and the latter culture supernatants were assayed for suppressive activity in NP-*O*-Su-primed CY-treated B6 or B10.BR recipients. As shown in Fig. 1, maximal suppressive activity was noted after 6–24 h of in vitro activation with TsF_2.

Activation of pTs_3 hybridomas with TsF_2 for up to 48 h did not result in increased levels of immune suppression. Over a large series of experiments we noted that the level of suppression obtained with pTs_3-derived factors ranged from 30 to 90%; however most batches of pTs_3-derived factor caused about 50–60% suppression.

Effector Phase Suppression. To compare the kinetics of activity of the pTs_3-derived suppressor factors with other NP-specific suppressor factors, we determined when in the phase of the immune response the pTs_3 hybridoma-derived factors were active. 0.5 ml of pTs_3- or Ts_1-derived factor was injected intravenously (*a*) on the day of and the day after antigen priming (induction phase) or (*b*) the day before and the day of antigen challenge (effector phase). The groups of mice that received control BW5147 or pTs_3-derived factors were injected intraperitoneally with 20 mg/kg CY on the day after antigen priming. In confirmation of previous reports (7, 18), the B6-Ts_1-29 and CKB-Ts_1-17-derived TsF_1 only demonstrated suppressive activity when administered during the induction phase of the immune response (Table II). In contrast, activated B6-pTs_3-9- and CKB-pTs_3-80-derived factors were only active when administered during the effector phase of the NP response (Table II).

Genetic Restrictions on pTs_3-derived Factors. Since the cell surface phenotype of pTs_3 cells and the kinetics of activity of pTs_3-derived factors correlated with those of conventional and hybridoma Ts_3 cells and factors, we wished to further explore the similarities between these cells by evaluating the genetic restrictions on the activity of pTs_3-derived factors. B6-pTs_3-9 and CKB-pTs_3-80 cells were activated with B6- and CKB-derived TsF_2, respectively. Control BW5147 cells were also cultured in TsF_2 for 2 d, after which the cells were washed and recultured in fresh medium. The

TABLE II

*pTs_3-derived Factor Suppresses the Effector Phase of the CS Response**

Recipient strain	CY treatment	Factor source	Footpad swelling‡	
			Time of TsF administration	
			Induction phase	Effector phase
			10^{-3} *cm* ± *SE*	
C57BL/6	+	BW5147	36.4 ± 2.0	36.4 ± 2.0
	−	B6-Ts_1-29	16.0 ± 2.1§	N.D.‖
	+	B6-pTs_3-9.3 activated with B6-Ts_2-28 factor	39.3 ± 0.9	13.5 ± 2.4§
B10.A	+	BW5147	45.6 ± 1.6	45.6 ± 1.6
	−	CKB-Ts_1-17	13.3 ± 2.7§	43.8 ± 2.6
	+	CKB-pTs_3-80.2 activated with CKB-Ts_2-59 factor	45.0 ± 2.2	21.3 ± 1.9§

* 0.5 ml of TsF_1, pTs_3 or control BW5147 supernatants were given intravenously on the day of and the day after NP-*O*-Su priming (induction phase) or the day before and the day of NP-*O*-Su challenge (effector phase). Appropriate groups were treated with CY to prevent Ts_3 generation (groups which received TsF_1 could not be CY treated since these factors fail to suppress CY-treated recipients). Four or five mice were included in each group.

‡ The negative control values, representing the swelling observed in unprimed C57BL/6 and B10.A mice, were 7.3 ± 0.5 and 9.2 ± 1.0, respectively.

§ Indicates significant suppression, $P < 0.01$.

‖ Not done.

supernatants from these activated cells were tested for their ability to suppress NP-induced CS responses in various strains of mice. The data from several experiments are summarized in Table III. In brief, suppressor factors derived from activated B6-pTs$_3$-9 cells only suppress NP responses in H-2- and Igh-compatible B6 recipients. Similarily, suppressor factors from activated CKB-pTs$_3$-80 cells only function in CKB recipients. Thus, matching at both the H-2 and Igh complexes was required for expression of pTs$_3$-derived TsF activity. Mismatching the pTs$_3$ donor and the recipient strain for either H-2 or Igh was sufficient to prevent expression of suppressor activity. Thus, CKB-pTs$_3$-80-derived factors suppress NP-induced CS responses in CKB (H-2^k, Igh^b) mice but not in Igh-congeneic C3H recipients (H-2^k, Igh^j). Furthermore, suppressor factors derived from activated B6-pTs$_3$-9 cells functioned in B6 (H-2^b) mice but not in H-2-incompatible B10.G (H-2^q) recipients (Table III).

To further establish the role of H-2 in restricting pTs$_3$-derived TsF activity, a series of H-2-congeneic strains were used as recipients (Table IV). All the strains carried H-2 recombinant haplotypes in which various regions were of H-2^b origin, thereby enabling precise intra-H-2 mapping of the genes responsible for genetic restriction. The data demonstrate the role of the I-J region in controlling the restrictions on the activity of pTs$_3$-derived factors. Thus, when B6 (I-J^b)-derived pTs$_3$ factor was assayed in I-J-compatible 4R and 3R recipients, significant levels of suppression were observed. However, the same batch of pTs$_3$-derived factor failed to suppress NP responses in I-J^k-bearing 5R or B10.MBR recipients (Table IV). In reciprocal experiments CKB (I-J^k)-derived pTs$_3$ factor suppressed NP responses in B10.MBR and 5R recipients, but not in 3R or 4R mice (Table IV). The critical role of the I-J region is clearly established by the reciprocal patterns of pTs$_3$ activity noted in I-J-congeneic 3R and 5R animals (Table IV).

Specificity of Ts$_3$ Activation. The next series of experiments was designed to evaluate the nature of the signals required for activation of pTs$_3$ cells. pTs$_3$ cells were incubated with hybridoma-derived TsF$_2$ or control BW5147 ascitic fluid (Table V). After 2 d the cells were washed and recultured in fresh media for an additional 24 h. These

TABLE III

*Genetic Restrictions of Activated pTs$_3$-derived Factors**

Factor source	Footpad swelling in recipient strains				
	C57BL/6 (H-2^b, Igh^b)	B10.G (H-2^q, Igh^b)	C57L (H-2^b, Igh^a)	C3H (H-2^k, Igh^j)	CKB (H-2^k, Igh^b)
	10^{-3} cm ± SE				
BW5147	30.3 ± 2.1	35.6 ± 2.0	32.8 ± 7.0	36.6 ± 3.1	41.6 ± 4.1
B6-pTs$_3$-9	16.0 ± 1.6‡	39.4 ± 2.8	32.0 ± 4.4	35.8 ± 5.3	43.5 ± 4.1
CKB-pTs$_3$-80	32.4 ± 4.0	40.6 ± 3.7	ND§	33.3 ± 3.1	16.0 ± 1.5‡
Negative control	8.7 ± 0.8	8.3 ± 1.4	7.2 ± 2.0	7.1 ± 1.7	8.0 ± 1.3

* The data from two to four independent experiments were pooled. Each group contains 7–13 mice, which were immunized with NP-*O*-Su. 24 h later all groups were given 20 mg/kg cyclophosphamide i.p. On the day before and the day of antigen challenge (effector phase), mice were given an injection of 0.4 ml i.v. of either control BW5147 or activated pTs$_3$ hybridoma factors. Activated pTs$_3$-derived factors were obtained by culturing B6-pTs$_3$-9 or CKB-pTs$_2$-80 hybridoma with B6-Ts$_2$-28 or CKB-Ts$_2$-59-derived factor respectively for 2 d and then in fresh media for 1 d.

‡ Indicates significant suppression, $P < 0.001$.

§ Not done.

TABLE IV

*Intra-H-2 Mapping of the Restrictions on Activated pTs$_3$ Hybridoma-derived Factors**

TsF source	TsF$_2$ for activation	NP-*O*-Su priming	Footpad swelling in recipient strains			
			B10.A (3R)	B10.A (4R)	B10.A (5R)	B10.MBR
			10^{-3} *cm* ± *SE*			
BW5147	—	+	48.3 ± 1.8	28.2 ± 2.2	30.2 ± 1.8	40.2 ± 0.8
B6-pTs$_3$-9	B6-Ts$_2$-28	+	29.0 ± 2.0‡	15.3 ± 1.0‡	29.0 ± 0.7	41.0 ± 1.3
CKB-pTs$_3$-80.1	CKB-Ts$_2$-59	+	48.0 ± 3.2	27.8 ± 2.1	19.0 ± 1.0‡	27.5 ± 1.8‡
CKB-pTs$_3$-80.2	CKB-Ts$_2$-59	+	46.7 ± 2.0	28.5 ± 1.0	21.5 ± 1.2‡	31.3 ± 2.3‡
BW5147	—	−	10.3 ± 1.7	6.3 ± 0.9	8.3 ± 1.3	12.3 ± 0.9

* Groups of four or five mice were immunized with NP-*O*-Su. 24 h later all groups were given 20 mg/kg CY i.p. On the day before and the day of NP-*O*-Su challenge, mice were given an injection of 0.5 ml i.v. of either control BW5147 or activated pTs$_3$ hybridoma factors. pTs$_3$ factors were obtained by culturing pTs$_3$ hybridomas with TsF$_2$ for 2 d and then in fresh media for 1 d. Two different subclones of CKB-pTs$_3$-80 were tested in this experiment.

‡ Indicates significant suppression, $P < 0.01$.

activated pTs$_3$ culture supernatants were tested for suppressive activity in NP-primed B6, B10.BR, or C3H.SW CY-treated recipients. Significant levels of suppressive activity were only noted in those combinations in which the H-2 and Igh determinants of the TsF$_2$, pTs$_3$ cells and the recipient strains were matched. Thus, B6 (H-2^b, Igh^b)-derived B6-pTs$_3$-9 cells were activated after incubation with TsF$_2$ factors from either B6-derived B6-Ts$_2$-28 or B6-Ts$_2$-14 cells, but incubation with TsF$_2$ derived from an H-2-mismatched, Igh-matched CKB (H-2^k, Igh^b) source or an H-2-matched, Igh-mismatched C3H.SW source failed to trigger the release of suppressive materials from pTs$_3$ cells (Table V). In accord with the data presented in the previous section, factors from activated B6-pTs$_3$-9 cells failed to suppress H-2- or Igh-incompatible B10.BR (H-2^k, Igh^b) or C3H.SW (H-2^b, Igh^j) recipients. Furthermore, supernatants from B6-pTs$_3$-9 cells incubated with CKB- or C3H.SW-derived TsF$_2$ also failed to function in B10.BR or C3H.SW recipients. Thus, homology between the TsF$_2$ and the recipient strain is not sufficient for expression of suppressive activity. The combined data imply that H-2 and Igh homology is required at two stages: (*a*) for the activation of pTs$_3$ cells and (*b*) for the expression of the suppressive activity mediated by pTs$_3$-derived factors. To verify these conclusions, the genetic restrictions on the activation of CKB-derived pTs$_3$ cells were analyzed in reciprocal fashion with similar results (Table V). As an additional control the biological activity of the C3H.SW-Ts$_2$-7-derived TsF$_2$ was established by incubation with a population of NP-primed C3H.SW lymph node cells. As expected from our previous reports (17, 19), the C3H.SW-derived TsF$_2$ was capable of activating the Ts$_3$ cells in this lymphocyte population. Finally, we attempted to activate B6-pTs$_3$-9 cells with a mixture of Igh^b-compatible CKB-derived TsF$_2$ and H-2-compatible C3H.SW-derived TsF$_2$. The mixture of these two functional TsF$_2$ factors failed to activate B6-derived pTs$_3$ cells (Table V).

Ability of pTs$_3$ Cells to Adsorb I-J-mismatched TsF$_2$. One possible explanation for the failure of Igh- or H-2-mismatched TsF$_2$ to activate pTs$_3$ cells is that these products will not bind the pTs$_3$ cells. In fact, we have previously demonstrated that monoclonal TsF$_2$ has antiidiotypic specificity and will only bind to cells that carry the appropriate idiotypic determinants (15). In the present studies, we evaluated the ability of TsF$_2$ to

TABLE V

*Specificity of pTs_3 Hybridoma Activation**

Strain of CY-treated recipients	pTs_3 or Ts_3 source	TsF source	Normalized percent suppression ± SE‡
C57BL/6	BW5147	None	0 ± 4
	B6-pTs_3-9	BW5147	5 ± 7
		B6-Ts_2-28	49 ± 5§
		B6-Ts_2-14	58 ± 1§
		CKB-Ts_2-59	8 ± 3
		C3H.SW-Ts_2-7	8 ± 4
		CKB-Ts_2-59 + C3H.SW-Ts_2-7	3 ± 4
	CKB-pTs_3-80	BW5147	−6 ± 3
		B6-Ts_2-28	−3 ± 5
		CKB-Ts_2-59	−3 ± 6
B10.BR	BW5147	None	0 ± 6
	B6-pTs_3-9	BW5147	−6 ± 13
		B6-Ts_2-28	0 ± 6
		B6-Ts_2-14	3 ± 9
		CKB-Ts_2-59	−6 ± 13
		C3H.SW-Ts_2-7	−3 ± 14
	CKB-pTs_3-80	BW5147	7 ± 4
		B6-Ts_2-28	−5 ± 8
		B6-Ts_2-14	−1 ± 7
		CKB-Ts_2-59	60 ±5§
		C3H.SW-Ts_2-7	−5 ± 2
C3H.SW	BW5147	None	0 ± 3
	B6-pTs_3-9	B6-Ts_2-28	−4 ± 3
		C3H.SW-Ts_2-7	4 ± 9
	CKB-pTs_3-80	CKB-Ts_2-59	−1 ± 8
		C3H.SW-Ts_2-7	0 ± 2
	C3H.SW NP-primed Ts_3‖	BW5147	0 ± 9
		C3H.SW-Ts_2-7	68 ± 9§

* Recipient mice were immunized with NP-*O*-Su. 24 h later all mice were given 20 mg/kg CY i.p. On the day before and the day of antigen challenge, mice were given 0.4 ml i.v. of either BW5147 or activated suppressor hybridoma factors. Activated pTs_3-derived factors were obtained by culturing B6-pTs_3-9 or CKB-pTs_3-80 hybridoma cells with various kinds of TsF_2 for 2 d and then in fresh media for 1 d.

‡ The data represent the pooled results from six separate experiments (not all groups were included in each experiment). The data were normalized and the percent suppression ± standard error was calculated. Each group represents the pooled normalized data of at least two experiments involving a total of at least seven mice.

§ Indicates significant suppression, $P < 0.01$.

‖ Regional lymph node cells from C3H.SW mice primed subcutaneously with 2 mg NP-*O*-Su 6 d earlier were cultured in vitro with C3H.SW-Ts_2-7 ascitic factor or control BW5147 factor for 2 h. After washing, 1×10^7 activated C3H.SW lymph node cells were transferred into CY-treated C3H.SW mice primed with NP-*O*-Su.

bind to I-J-mismatched pTs_3 cells. Thus aliquots of B6- and CKB-derived TsF_2 were adsorbed with 3×10^7 B6-pTs_3-9, CKB-pTs_3-80, and BW5147 cells/ml. The activity of the absorbed TsF_2 was assayed on NP-*O*-Su-primed recipients. As indicated in Table VI, TsF_2 activity was completely absorbed by both H-2-compatible and H-2-

TABLE VI

*Absorption of TsF_2 with I-J-mismatched pTs_3 Cells**

Recipient strain	NP-*O*-Su priming	TsF source	Cells for TsF_2 absorption	Footpad swelling
				10^{-3} *cm ± SE*
C57BL/6	+	BW5147	—	43.5 ± 2.7
	+	B6-Ts_2-28	BW5147	18.3 ± 0.3‡
	+	B6-Ts_2-28	B6-pTs_3-9	46.3 ± 1.3
	+	B6-Ts_2-28	CKB-pTs_3-80	45.5 ± 1.9
	−	None	—	3.3 ± 0.7
B10.BR	+	BW5147	—	40.0 ± 2.2
	+	CKB-Ts_2-59	BW5147	23.3 ± 1.2‡
	+	CKB-Ts_2-59	B6-pTs_3-9	38.0 ± 3.3
	+	CKB-Ts_2-59	CKB-pTs_3-80	37.8 ± 3.2
	−	None	—	9.0 ± 1.2

* Aliquots of TsF_2 were absorbed with 2.5×10^6 B6-pTs_3-9 (I-J^b), CKB-pTs_3-80 (I-J^k), or control BW5147 cells/ml. The absorbed TsF_2 was then tested for effector phase suppressive activity in previously NP-*O*-Su-primed recipients.

‡ Indicates significant suppression, $P < 0.001$.

TABLE VII

*Fractionation of pTs_3-derived Factors on Immunoadsorbent Columns**

Factor source	Immunoadsorbant column	Column fraction	Footpad swelling		
			Source of suppressor factor‡		
			B6-pTs_3-9	CKB-pTs_3-80	B6-Ts_3-8
			10^{-3} *cm ± SE*		
Activated BW5147	None	—	34.8 ± 2.0	50.2 ± 2.6	29.5 ± 2.0
Nonactivated pTs_3	None	—	35.2 ± 3.4	50.4 ± 3.4	16.8 ± 1.3‡
Activated pTs_3	None	—	13.6 ± 1.4‡	16.2 ± 2.3‡	—
	Anti-I-J^b	Filtrate	40.0 ± 1.1	31.3 ± 3.8‡	28.3 ± 1.7
		Eluate	17.5 ± 4.3‡	43.0 ± 4.0	17.0 ± 1.2‡
	Anti-I-J^k	Filtrate	14.4 ± 1.3‡	47.8 ± 4.4	18.3 ± 1.6‡
		Eluate	34.0 ± 4.0	21.3 ± 1.5‡	30.3 ± 3.2
	NP-KLH	Filtrate	44.3 ± 4.2	47.0 ± 2.0	30.5 ± 2.2
		Eluate	18.5 ± 5.5‡	19.3 ± 3.3‡	15.8 ± 3.0‡
	Anti-NP^b	Filtrate	43.0 ± 3.5	43.3 ± 6.9	25.5 ± 3.7
		Eluate	21.8 ± 1.8‡	19.3 ± 3.3‡	17.3 ± 1.3‡
	NP^b	Filtrate	9.8 ± 2.0‡	31.3 ± 3.8‡	16.3 ± 1.9‡
		Eluate	30.4 ± 2.4	43.0 ± 4.0	27.8 ± 1.7
Negative (background) controls	—	—	8.3 ± 3.4	9.2 ± 2.5	10.0 ± 1.6

* Activated pTs_3 or conventional nonactivated Ts_3 hybridoma-derived TsF containing supernatant fluids were adsorbed onto various columns and the filtrate and acid-eluted fractions were tested for suppressive activity in either C57BL/6 or B10.BR recipients. The TsF_3 from the constitutatively secreting Ts_3 hybridoma (B6-Ts_3-8) is included for comparison. Refer to the legend to Table I for protacol.

‡ Indicates significant suppression, $P < 0.01$.

incompatible pTs_3 cells. Thus it appears that TsF_2 can bind to I-J-mismatched pTs_3 cells but that this binding event is insufficient to cause activation of the pTs_3 cell. The specificity of absorption was demonstrated by the inability of BW5147 cells to absorb TsF_2 activity (Table VI).

TABLE VIII

*Presence of TsF in Extracts of Nonactivated pTs$_3$ Hybridoma Cells**

pTs$_3$ source	TsF$_2$ used for activation	Fraction	Normalized percent CS suppression ± SE
BW5147	None	Cell extract	0 ± 8
CKB-pTs$_3$-80	None	Cell extract	56 ± 6‡
CKB-pTs$_3$-80	None	Supernatant	8 ± 9
CKB-pTs$_3$-80	CKB-Ts$_2$-59	Cell extract	2 ± 9
CKB-pTs$_3$-80	CKB-pTs$_2$-59	Supernatant	40 ± 5‡
BW5147	None	Cell extract	0 ± 6
B6-pTs$_3$-9	None	Cell extract	61 ± 7‡
B6-pTs$_3$-9	None	Supernatant	−12 ± 9
B6-pTs$_3$-9	B6-Ts$_2$-28	Cell extract	27 ± 7
B6-pTs$_3$-9	B6-Ts$_2$-28	Supernatant	40 ± 10‡

* TsF$_2$-activated (see Table I for protocol) or nonactivated pTs$_3$ cells were disrupted by three to four cycles of freeze-thawing; the cell debris was removed by centrifugation and the extract fraction was tested for suppressive activity. Supernatant fractions represent aliquots of the culture supernatants from the activated or nonactivated cells before disruption. The data from two experiments were normalized and expressed as percent suppression. Groups consist of seven to ten mice.

‡ Indicates significant suppression, $P < 0.05$.

Characterization of pTs$_3$-derived Factors. To further document the relationship between pTs$_3$- and Ts$_3$-derived factors, the antigeneic determinants present on the factors were analyzed using a series of affinity columns. As shown in Table VII, the factors derived from activated pTs$_3$ cells carried allele-specific I-J determinants. Thus passage of B6-pTs$_3$-9 factor over anti-I-J^b immunoadsorbants depleted suppressive activity. The suppressive activity could be recovered from the anti-I-J^b column by acid elution (Table VII). Furthermore, the specificity of adsorption was illustrated by the inability of anti-I-J^k immunoadsorbant columns to deplete the B6-pTs$_3$-9-derived suppressor activity. In reciprocal experiments the CKB (H-2^k)-derived pTs$_3$ factors were removed on anti-I-J^k columns and the activity was recovered in the acid eluates. In addition to I-J determinants the pTs$_3$-derived factors have NP^b idiotype-related, antigen-binding determinants. Thus both the B6- and CKB-derived pTs$_3$ factors could be adsorbed onto and eluted from antiidiotype (anti-NP^b) or antigen (NP-KLH) immunoadsorbants (Table VII). To demonstrate that the pTs$_3$-derived factors did not require any residual TsF$_2$ for expression of activity, we passed the factors over columns that contained NP^b idiotype; these columns had been shown to completely deplete TsF$_2$ activity (15). pTs$_3$ factors failed to bind to NP^b idiotype-coupled columns. For further comparison, the results of the pTs$_3$ fractionation are compared with the fractionation of conventional TsF$_3$ from an NP-specific hybridoma (B6-Ts$_3$-8) that constitutatively secretes TsF$_3$ (Table VII).

Presence of Preformed TsF$_3$ in pTs$_3$ Cells. The final series of experiments was directed at determining whether activation of pTs$_3$ cells caused the *de novo* synthesis of TsF$_3$ or if TsF$_3$ was already stored in these cells and activation resulted in the export of this factor into the surrounding medium. Nonactivated CKB-pTs$_3$-80 and B6-pTs$_3$-9 hybridoma cells were disrupted by freeze-thawing and the cell extract was injected into NP-primed CY-treated CKB and B6 recipients, respectively. These extracts of nonactivated pTs$_3$ cells contained suppressive activity whereas extracts from BW5147

control cells failed to suppress NP-induced CS responses (Table VIII). The antigen specificity of the extracted factor was established by determining that this material did not suppress DNFB-induced CS responses (data not shown). As additional controls, the culture media in which the nonactivated pTs_3 cells were grown was also tested for suppressive activity. As expected, supernatants from nonactivated pTs_3 cells failed to suppress NP CS responses.

In addition, pTs_3 cells that had been activated by TsF_2 and then allowed to release factor for 24 h in fresh medium were disrupted and the cellular extracts tested for suppressive activity. Little or no suppressive activity was demonstrable in the extracts of activated pTs_3 cells (Table VIII). As expected, the supernatants of activated pTs_3 cells contained suppressive activity.

Discussion

The pTs_3 hybridomas described in this report display numerous similarities to the Ts_3 cells previously described in the NP system. The origin, phenotype, and activation requirements of the pTs_3 cells as well as the genetic restrictions and antigenic determinants found on pTs_3 hybridoma-derived factors are identical to those previously noted on conventional Ts_3 cells and factors (4, 5, 17–19). Thus, the pTs_3 and Ts_3 hybridomas were derived from the fusion of antigen-adherent T cells obtained from immune mice. Ts_3 and pTs_3 cells and their factors bear I-J- and NP^b-related idiotypic determinants. The factors only function during the effector phase of the immune response and can suppress CS responses in CY-treated recipients. The activity of the suppressor factor is restricted by at least two sets of genes, one associated with the I-J region and the other with the Igh complex. In view of all of these similarities, we have termed these hybridoma cells, pTs_3. The prefix denotes that the cells must be specifically activated to release their biologically active product. This feature clearly distinguishes this series of hybridomas from those which constitutively release TsF_3.

These inducible hydridomas presumably represent a stable stage in the differentiation cycle of Ts_3 cells, i.e., a resting, nonsecretory stage of the mature Ts_3 cell. We have previously noted that after conventional antigen-priming, Ts_3 cells are generated concomitantly with helper and CS effector T cells. However, the mature Ts_3 cells will not mediate suppressive activity unless specifically activated by TsF_2. Thus, mature Ts_3 populations normally remain in an inactive resting state, thereby allowing expression of helper or CS activity. However, after release of TsF_2, activation of Ts_3 cells and suppression occur. This process allows for a rapid regulation of immune responses. The establishment of an inducible hybridoma line confirms these conclusions, which were originally hypothesized after analysis of the activation of heterogeneous populations of splenic or lymph node Ts_3 cells. The latter lymphocyte populations contained Ts_3 cells as well as other NP-specific T cells including helper and/or CS effector cells. The present report describes hybridoma clones that have retained the properties of a mature, non-TsF_3-secreting Ts_3 cell. Furthermore, this report directly documents the interaction of TsF_2 with Ts_3 cells.

Other investigators (24–26) have described an analogous series of major histocompatibility complex (MHC)-restricted hybridoma or cloned T cells that represent inducible helper or proliferating cells. Activation of these hybridomas or clones also requires corecognition of two components, i.e., antigen and I-A- or I-E-encoded MHC products. Similarly, activation of the inducible pTs_3 hybridomas also required rec-

ognition of two elements: MHC components encoded by the I-J subregion and a specific T cell-derived idiotype (which may also be regarded as an antigenic determinant or internal image). The genetic restrictions and the mixing experiment used to analyze the activation of pTs_3 hybridomas indicated that both I-J and idiotypic determinants must be present on the same molecular complex. Similar activation requirements were noted for cloned or hybridoma helper T cells, which required presentation of MHC products and antigenic determinants on the same cell. Thus, similar principles apply for the activation of both helper and suppressor T cell populations. However, it appears that cells of the suppressor cascade are programmed to recognize or interact with I-J molecules whereas helper cells interact with I-A or I-E molecules.

Taniguchi and associates (27) have also identified an I-J-positive inducible suppressor cell hybridoma that was obtained after fusion of antigen-adherent cells from KLH-primed mice. These hybridoma cells bore a receptor (acceptor) for a KLH-specific suppressor factor (27). After activation with suppressor factor, the inducible hybridoma described in the KLH system produced its own distinct, genetically restricted factor that displayed carrier specificity. However, the factors produced by the latter inducible suppressor cell hybridoma had different binding properties than those described in the present report. Thus, Taniguchi et al. (27) demonstrated that the suppressor factor that was required to activate hybridoma pTs cells bound to KLH whereas the factor released from pTs cells failed to bind to KLH. Furthermore, the authors concluded that the pTs-derived factor was antiidiotypic, since it bound to other T cell hybridomas that presumably bore idiotypic determinants specific for KLH (27). The data described in this report represent a reciprocal pattern. Thus, in the NP system, the TsF_2 factor required for pTs_3 activation bound to idiotype while the pTs_3-derived factor bound to antigen. Several hypotheses can reconcile the latter two series of observations. (*a*) These pTs hybridomas represent distinct elements of a common suppressor cell cascade. Presumably, the TsF_3 released by pTs_3 cells could activate a complimentary antiidiotypic suppressor T cell (Ts_4) similar to that described by Taniguchi et al. (27). (*b*) Both idiotypic and antiidiotypic Ts_2 and Ts_3 cells and factors exist. Kapp and Araneo (13) have interpreted data in the L-glutamic acid^{50}-L-tyrosine^{50} system in support of this contention. The method of Ts induction and the possibility of antigen bridging would presumably determine the idiotypic or antiidiotypic nature of the different suppressor elements. (*c*) Perhaps two distinct Ts pathways are involved. This would imply that the suppressor cells described in the NP and KLH systems are members of independent suppressor cell pathways.

The identification of inducible T cell hybridomas permits analysis of the activation processes required for suppressor cell function. Although the activation of pTs_3 cells requires triggering with ascites containing TsF_2, additional requirements for activation may also exist. Thus, it is possible that the ascites contains additional substances that are involved in pTs_3 activation. Nonetheless, the data demonstrate that pTs_3 cells require specific activation with TsF_2. Furthermore, analysis of the genetic restrictions on pTs_3 cell activation indicated that the H-2 and Igh complexes of the donor had to match the strain from which the pTs_3 cells were derived. Homology at only one gene complex (H-2 or Igh) was not sufficient to trigger activation of the pTs_3 cells. The failure of Igh-matched H-2-mismatched TsF_2 to trigger pTs_3 cells was not due to the inability of these materials to bind to the pTs_3 cells. Thus, I-J-incompatible pTs_3 cells

could completely absorb activity. These findings suggest that two distinct steps are required for pTs_3 triggering, i.e., binding of TsF_2 to the idiotypic receptor and interaction of the cell with the I-J (or anti-I-J) determinant on TsF_2. These two triggering signals must be delivered in a very specialized spacial or temporal configuration, presumably by a single molecule or molecular complex. Thus, incubation of pTs_3 cells with two distinct TsF_2 factors, one having H-2 homology and the other Igh homology, was insufficient for pTs_3 activation. The simplest interpretation of this observation is that the triggering of the receptor (acceptor) site on pTs_3 cells requires activation by two spacially related determinants. Several laboratories (28–33) have demonstrated that TsF from a variety of sources is a dimer that contains one chain with MHC (I-J) determinants and another with antigen-binding or Igh-linked determinants. The physiological significance for this type of molecular complex may lie in the mechanism required for suppressor cell triggering.

Another important conclusion that can be derived from the present series of observations is that antigen is not required for the triggering of pTs_3 cells. In previous systems it was not possible to evaluate the requirements for antigen in this phase of the suppressor cell cascade, since the Ts_3 cells are obtained from antigen-primed mice and the assay for CS immunity requires the presence of antigen in the effector phase. However, the present observations clearly demonstrate that antigen is not required for activation of pTs_3 hybridomas and presumably is also not required for activation of conventional Ts_3 lymphocytes.

It is also known that antigen is not required for Ts_2 cell induction (11, 21). Thus, once the cellular elements of the suppressor cell cascade are generated, immune suppression can proceed in the absence of additional antigen. Antigen is apparently only required for the initial induction of Ts_1 and Ts_3 cells (4, 19). Subsequent cellular activation presumably proceeds via idiotype antiidiotype and I-J anti-I-J interactions. Thus, the cascade of regulatory interactions observed in NP systems conforms with the basic predictions of the Jerne network theory (34).

In a series of preliminary experiments, we tried to activate pTs_3 hybridoma cells with either antigen, antiidiotype, or anti-I-J. Neither NP-bovine serum albumin, antigen-modified syngeneic cells, guinea pig antiidiotype, auto-antiidiotype, or anti-I-J alloantiserum was able to activate the pTs_3 cells under the experimental conditions used (data not shown). Although these attempts to activate pTs_3 cells were unsuccessful, the therapeutic value of identifying alternative mechanisms for Ts_3 activation is potentially great for selected diseases.

In addition it is important to distinguish among the mechanisms of TsF activation. As shown in this report, TsF_2 acts on a previously expanded and at least partially differentiated population of antigen-specific Ts_3 cells, causing the release of soluble mediators. In contrast, the soluble factors obtained from first-order suppressor cells (TsF_1) induce the generation and differentiation of another suppressor cell population from normal splenic T cells (7, 8, 11). Thus, whereas TsF_2 activates, TsF_1 induces suppressor cells.

Finally, it is important to note that the activation of pTs_3 cells by TsF_2 does not appear to involve the *de novo* synthesis of TsF_3. Thus, lysis of nonactivated pTs_3 hybridoma cells released an antigen-specific suppressor factor that functioned in the effector phase of the CS response. TsF_3 had already been synthesized by pTs_3 cells before activation. After TsF_2 activation, the pTs_3 cells cleared the TsF_3 from the

intracellular pool and released it into the surrounding medium. Thus, pTs_3 cells represent a mature differentiated stage in the life cycle of the Ts_3 suppressor cell in which a specific activation step is required for the rapid (<6 h) release of antigen-specific suppressor factors into the local environment. The ability of Ts_3 cells to store TsF_3 permits the immune system to rapidly modulate immune responses.

Summary

The Ts_3 subset of suppressor cells is generated after antigen priming, but, in order to express suppressor activity these cells require an additional activation step involving triggering with specific suppressor factors (TsF_2). This report characterizes two cloned hybridoma cell lines (pTs_3 hybridomas) that represent this stage of Ts_3 cell differentiation. These hybridoma cells could be specifically activated with TsF_2 to release another antigen-specific suppressor factor (TsF_3) within 6 h. The inducible feature of these cells permitted analysis of the signals necessary for Ts_3 activation. Antigen was not required for activation. Only TsF_2 factors derived from antiidiotypic second-order suppressor cells could activate pTs_3 hybridoma cells. There were stringent genetic restrictions on the ability of Ts_2 to activate pTs_3 cells. Triggering of pTs_3 required corecognition of two determinants on the TsF_2 molecular complex, i.e., the I-J and Igh-related idiotypic determinants. Thus, although pTs_3 cells could absorb TsF_2 from an I-J-mismatched source, these pTs_3 were not activated by the allogeneic TsF_2. For activation to occur, the H-2 (I-J) and Igh complexes of the TsF_2 donor had to match those of the strain from which the pTs_3 cells were derived. Mixing two distinct TsF_2, one derived from an H-2-matched source and the other from an Igh-matched source, failed to activate pTs_3 cells. Once activated, the pTs_3 cells released a suppressive material that was indistinguishable from the TsF_3 factors previously characterized in this system. Finally, the activation of the pTs_3 cells apparently does not induce the *de novo* synthesis of TsF_3 since the suppressive activity could be extracted from nonactivated pTs_3 cells. Thus, the inducible pTs_3 hybridomas represent a mature stage in the differentiation cycle of Ts_3 cells and provide a means for studying the nature of the specific signals required for Ts_3 activation.

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