Proliferative Signals for Suppressor T Cells

Helper Cells Stimulated with Pokeweed Mitogen In Vitro Produce a Suppressor Cell Growth Factor

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Abstract

To define molecular signals elaborated by inducer populations supporting growth or differentiation of $T8^+$ cells, we collected supernatants of pokeweed mitogen (PWM)-stimulated cultures depleted of $T8^+$ cells. When added to purified $T8^+$ cells, these supernatants caused significant proliferation. PWM plus interleukin 2 (IL-2) in amounts equivalent to those in the supernatant could not reconstitute the response caused by the supernatant. $T8^+$ cells activated by supernatants obtained from PWM-pulsed $T4^+$ cells suppressed fresh PWM cultures. Although exhibiting little proliferation, $T8^+$ cells cultured for 6 d in PWM plus IL-2 still suppressed a fresh PWM response. The supernatants therefore contain an additional T suppressor cell growth factor (TsGF). Elaboration of TsGF required radiosensitive $T4^+Leu8^+$ cells. Molecular weight determination by high performance liquid chromatography gave a single peak of TsGF activity at ~8,000. Finally, whereas TsGF in the absence of IL-2 could not support the proliferation of T suppressor cells, it did cause $T8^+$ cells to become strongly IL-2 receptor-positive.

Introduction

Considerable difficulty has been encountered in the in vitro culturing of suppressor T cells (Ts).[1] Although human HLA class I-restricted cytotoxic T cells (CTLs) and Ts both belong to the $T8^+$ subset, different activation steps are required for each (1, 2). If the growth requirements for Ts proliferation are fundamentally different than those needed by other T lymphocytes, identification of the necessary signals would be valuable. Indeed, the lack of understanding of growth and differentiative requirements for Ts constitutes a major impediment to the in vitro analysis of regulatory interactions.

Pokeweed mitogen (PWM) activation of peripheral blood mononuclear cells (PBMC) offers an in vitro model to investigate soluble signals provided to Ts by $T4^+$ cells. Human lymphocyte proliferation stimulated by PWM involves a regulatory interaction between T cell subsets (3). Culturing PBMC in PWM for 6 d or longer yields an excess of $T8^+$ cells, which are capable of suppressing the response of fresh $T4^+$ cells to PWM. However, stringent depletion of $T4^+$ cells before culturing with PWM abrogates the response. Suppressor–inducer cells contained within the $T4^+$ subset are therefore necessary for the proliferation of PWM-activated suppressor cells contained within the $T8^+$ subset.

The interaction between suppressor–inducer and suppressor–effector cells could be mediated by cell–cell contact, by a secreted molecule(s), or by a combination of the two. By activating $T4^+$ cells with PWM for 48 h and collecting the supernatant, we found that the components necessary to promote the proliferation of $T8^+$ suppressor cells were transferred in a cell-free supernatant. In the presence of interleukin 2 (IL-2), the T suppressor cell growth factor (TsGF) contained in the T4 supernatant greatly enhanced proliferation of $T8^+$ cells. The data further suggest that the factor acts by inducing expression of IL-2 receptors on $T8^+$ suppressor cell precursors. TsGF was purified by high performance liquid chromatography (HPLC) and was found to be an ~8,000 M_r protein distinct from interleukin 1 (IL-1), IL-2, or interferon-gamma (IFNγ).

Methods

Leukocytes. PBMC were obtained by leukapheresis of healthy volunteers and isolation on Isolymph gradients (Gallard-Schlesinger Corp., Carle Place, NY). Cells were stored in liquid nitrogen in 7.5% dimethyl sulfoxide until used. Cells were washed in Hanks' balanced salt solution (HBSS) containing 1.0% pooled human AB^+ heat-inactivated serum and, unless stated otherwise, were not exposed to bovine serum or sheep erythrocytes.

Cell proliferation. To measure incorporation of [^{3}H]thymidine, 1.0 μCi (2 Ci/mmol, Amersham International, Amersham, UK) was added to each of three to five replicate microtiter wells on 96-well round-bottomed plates (Costar, Cambridge, MA) for the final 24 h of culture; the nucleic acid was then isolated on filtermats (Skatron, Inc., Sterling, VA) and counts per minute were determined. Viable cell recovery was measured by replicate cell counts and trypan blue exclusion.

Monoclonal antibodies. OKT4 and OKT8 (Ortho Pharmaceutical Corp., Raritan, NJ) and Leu2, Leu3, Leu7, Leu8, antitransferrin receptor, anti-human HLA-DR, and anti-human IL-2 receptor (Becton, Dickinson Monoclonal Center, Inc., Mountain View, CA) monoclonal antibodies were used for phenotyping PBMC.

Cytotoxicity. $T4^+$, $T8^+$, or $Leu8^+$ cells were cytolytically eliminated from PBMC before culture by a method modified from Reinherz et al. (4). PBMC were incubated in a 1/200 dilution of OKT4, OKT8, or Leu8 monoclonal antibody on ice with occasional shaking for 30 min. Pretitered baby rabbit complement (Pel-Freez Biologicals, Rogers, AR) was added for 30 min at 37°C, 5% CO_2. Cultures depleted by this method contained not >2% of the eliminated population, as determined by analysis on a flow cytometer (EPICS V, Coulter Electronics, Inc., Hialeah, FL).

Preparation of TsGF-containing supernatant. After depletion of $T8^+$ cells, the T4-enriched population was cultured at 5×10^6 cells/ml in a 1:80 dilution of PWM (Gibco, Grand Island, NY) in medium which has been described previously (5). The $T4^+$ cells were incubated at 37°C for

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1. *Abbreviations used in this paper:* HPLC, high performance liquid chromatography; IFNγ, interferon-gamma; IL-1, interleukin 1; IL-2R, interleukin 2 receptor; PBMC, peripheral blood mononuclear cell; PWM, pokeweed mitogen; rIL-2, recombinant IL-2; TR, transferrin receptor; TsGF, T suppressor cell growth factor; Ts, T suppressor lymphocyte.

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48 h. Culture supernatants were harvested by centrifugation and passed through a 0.45-μm filter to remove any residual cellular debris. All supernatants used in these studies were negative for *Mycoplasma* contamination by a method adapted from Chen (6).

Testing of supernatants for TsGF activity. Serial twofold dilutions of test supernatants were made in triplicate in 96-well U-bottomed microculture plates and 5×10^4 T4-depleted cells were added to each well. As controls, equal numbers of cells were cultured in medium alone, 1:80 PWM, recombinant IL-2 (rIL-2, Amgen Biologicals, Thousand Oaks, CA), or 1:80 PWM + rIL-2, with six replicates for each of the controls. Bulk culture of T4-depleted cells was performed at 1×10^6 cells/ml in Falcon 2051 tubes (Falcon Labware, Oxnard, CA) at a volume of 2 ml per tube.

Suppressor cell activity. To test cells for the ability to suppress a primary PWM response, $T8^+$ cells incubated for 7 d were washed, irradiated, (2,000 rads), and added back in varying numbers to 5×10^4 unfractionated PBMC plus 1:80 PWM per well. [^{3}H]Thymidine was added after 5 d, and the cells were harvested 24 h later.

IL-1, IL-2, and IFNγ assays. IL-1 activity was measured by proliferation of C3H/HeJ thymocytes (7). Thymocytes were cultured at 1×10^6/well with serial dilutions of T4-supernatant or an IL-1 standard (cell line-derived IL-1, Genzyme, Boston, MA) in a 72-h assay. The number of units of IL-1 contained in the supernatant was determined by probit analysis of the counts per minute of [^{3}H] after a 24-h [^{3}H]thymidine pulse.

Culture supernatants were assayed for IL-2 content on the basis of their ability to support [^{3}H]thymidine incorporation of the IL-2-dependent HT-2 mouse cell line (8) as described by Gillis et al. (9). Briefly, 5×10^3 washed HT-2 cells were added to each well of triplicate serial twofold dilutions of test supernatants. After 20 h at 37°C in 5% CO_2, [^{3}H]thymidine was added for an additional 4 h before harvesting. Incorporated [^{3}H] counts per minute were subjected to probit analysis, and were compared to the NIH Jurkat-IL2 standard (10) to calculate the dilution for half-maximal proliferation and units of activity.

The titer of IFN was defined as the reciprocal of the last dilution that protected human fetal fibroblasts from lysis by vesicular stomatitis virus. This cytopathic effect assay was modified from the method of Tilles and Finland (11), and utilized 100 μl of supernatant dilutions placed on 4×10^4 fibroblasts per microculture well. On d1, 100 μl of virus (100 tissue culture infective $doses_{50}$) was added, and 0.5% crystal violet in 70% methanol was added to each well on d3. Wells were counted as positive for IFN activity if >50% of the cells remained unlysed.

Removal of IL-2 from T4-supernatant. Concentrated supernatant was passed through an immunoadsorbent column as described by Smith et al. (12). In short, an IL-2-binding monoclonal antibody, DMS-1, was bound to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA). Effluent was shown to be IL-2-free in an IL-2 assay, performed as described above.

Column chromatography. Molecular weight determination of TsGF was performed by HPLC on an Altex 3000 SW Spherogel-TSK column using a Beckman HPLC system (Beckman Instruments, Inc., Palo Alto, CA). The column was run in phosphate-buffered saline at a flow rate of 0.4 ml/min. Molecular weight markers for calibration included bovine serum albumin (68,000), ovalbumin (45,000), lactoglobulin (18,500), myoglobin (17,000), cytochrome C (12,000), insulin (5,700), and insulin β-chain (3,500).

Enzyme treatments. 1-ml aliquots of T4-supernatant were incubated in various enzymes (1 unit of lipase, 25 μg of pronase, and 25 μg of proteinase K [Sigma Chemical Co., St. Louis, MO]) for 1 h at 37°C. To inactivate the enzymes, 200 μl of heat-inactivated human AB^+ serum was added to each aliquot.

Detection of cell surface antigens. Indirect immunofluorescence was performed by incubating 1×10^6 cells with antibody for 15 min at 4°C. After washing twice in HBSS, fluorescein-conjugated goat anti-mouse IgG was added for 15 min at 4°C; the cells were then washed twice and fixed in suspension in 1.0% paraformaldehyde in 0.85% NaCl. Two-color direct immunofluorescence was performed by simultaneously incubating 1×10^6 cells with two monoclonal antibodies, one conjugated with fluorescein and the other conjugated with phycoerythrin. Stained cells were analyzed by flow cytometry. T4-depleted cells were ~50% $T8^+$, T8-depleted cells were ~70% $T4^+$. E-rosette positive T4-depleted cells were >95% $T8^+$.

Results

Supernatants from PWM-stimulated $T4^+Leu8^+$ cells cause $T8^+$ proliferation. To determine whether a cell-free supernatant from a PWM culture would replace the $T4^+$ cell requirement for $T8^+$ cell proliferation, we depleted PBMC of either $T4^+$, $T8^+$, or $Leu8^+$ cells, and then incubated each culture in a 1:80 dilution of PWM for 48 h. The supernatants were then collected and tested for the ability to induce the proliferation of a T4-depleted population (Fig. 1). Whereas PWM plus rIL-2 could not promote the growth of T4-depleted cells, T4-supernatants (from T8-depleted cultures) caused significant proliferation of the $T8^+$ cells. Throughout all experiments, proliferation seen with PWM plus rIL-2, in amounts consistent with those in the T4 supernatant, was much less than that caused by the T4 supernatant. The IL-2 levels used in the control were saturating, inasmuch as no additional rIL-2 caused increased proliferation. Proliferation occurred equally well when the $T8^+$ cells were purified by E rosetting and then T4 depletion (data not shown); thus the proliferating cells were not likely to be contained within the B cell or macrophage populations.

Mohagheghpour et al. (13) demonstrated that only those $T4^+$ cells that also expressed the Leu8 marker were capable of inducing antigen-specific suppression by $T8^+$ cells. We similarly observed that depletion of $Leu8^+$ cells before culturing in PWM yielded a supernatant incapable of inducing a $T8^+$ proliferative response (Fig. 1). Mixing the cells remaining after T4 depletion with cells remaining after Leu8 depletion also could not generate a supernatant able to cause $T8^+$ proliferation, suggesting that the T4 and Leu8 molecules were coexpressed on the factor-producing cells.

T4 supernatant contains a TsGF. Experiments were performed to determine what soluble products were necessary to

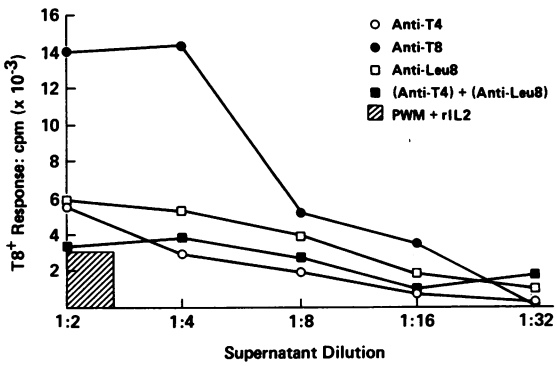

Figure 1. Supernatants from PWM-stimulated $T4^+Leu8^+$ cells cause $T8^+$ proliferation. PBMC were depleted of $T4^+$ cells (○), $T8^+$ cells (●), or $Leu8^+$ cells (□) by antibody and complement treatment. These three populations, plus a mixture of equal numbers of $T4^-$ cells and $Leu8^-$ cells (■) were cultured in PWM for 48 h. Supernatants were collected and added to 5×10^4 T4-depleted cells per well for 6 d. The concentration of IL-2 contained in the supernatant from T8-depleted cells (T4 supernatant) was determined prior to this assay, and the same number of units of rIL-2 were added to a 1:80 PWM dilution as a control supernatant (bar). [^{3}H]Thymidine was added to the cultures for the final 24 h. Mean cpm of triplicate wells is shown.

activate precursor Ts into functional Ts. T4-depleted cells were cultured for 7 d in either T4-supernatant, PWM plus rIL-2, or medium only. As seen in Fig. 2, the suppression caused by cells incubated in either PWM plus IL-2 or T4-supernatant was similar on a cell-to-cell basis. However, because the yield from the culture grown in T4 supernatant (120%) was much larger than that recovered from the PWM plus rIL-2 culture (40%), the T4 supernatant produced substantially more suppressive activity per culture. The viable cell recovery from the PWM plus rIL-2 cultures was only slightly better than that from the medium control (30%), suggesting that T4 supernatants contained a factor supporting suppressor cell growth (TsGF) rather than differentiation. Because the two culture conditions could produce cells with equal suppressive activity, it can be said that either T4 supernatant or PWM plus rIL-2 could fully activate $T8^+$ cells to become functional Ts.

Separation of TsGF from IL-1, IL-2, and IFNγ. The role of IL-1 in T cell growth includes the promotion of IL-2 receptor (IL-2R) synthesis (14). Therefore, we tested whether a combination of IL-1, IL-2, and PWM could induce $T8^+$ proliferation. IL-1 content of the T4 supernatant was determined by a mouse thymocyte proliferative assay, and similar units of purified IL-1 and IL-2, with and without PWM, were added to T4-depleted cells. Although T4 supernatant promoted a significant response, the purified lymphokines and PWM did not (Fig. 3). In order to investigate further the possibility that TsGF may be identical with IL-1 or IL-2 cleavage fragment, we tested TsGF for trypsin sensitivity and IL-1 activity. In confirmation of previous reports (15), IL-1 activity in a mouse thymocyte proliferation assay was trypsin resistant; in contrast, purified TsGF could not induce thymocyte proliferation, and TsGF activity on T4-depleted cells was abolished by trypsin digestion (data not shown).

Preliminary studies indicated TsGF had a molecular weight of <15,000. To physically separate TsGF from IL-2 and IFNγ, an aliquot of T4 supernatant was placed into dialysis tubing with a pore size of 12,000–14,000, and was dialyzed for 24 h at 4°C against culture medium. This dialyzed supernatant was then compared with undialyzed supernatant from the same preparation (Fig. 4). The undialyzed supernatant was able to cause $T8^+$ cell proliferation, whereas the dialyzed supernatant was not. In contrast, both contained the same IL-2 activity, demonstrating that TsGF is distinct from IL-2. TsGF was also much more temperature sensitive than IL-2. Incubation of T4-supernatant for 24 h at 37°C or 7 d at 22°C caused the loss of TsGF activity, even though no effect on IL-2 content was seen (data not shown).

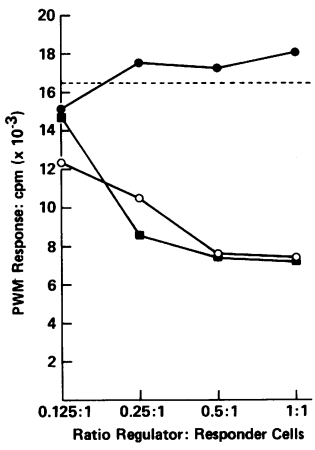

Figure 2. T4 supernatant contains a TsGF. Aliquots of 40×10^6 T4-depleted cells were cultured for 7 d in medium alone (●), 1:80 PWM plus 2.0 U/ml rIL-2 (○), or T4-supernatant (■). Yields of viable cells after culturing were 48×10^6 (T4 supernatant), 16×10^6 (PWM + rIL-2), and 12×10^6 (medium). After harvesting, various dilutions of these cells were cultured for 6 d in the presence of 5×10^4 fresh autologous PBMC plus 1:80 PWM. [^{3}H]Thymidine was added to the cultures for the final 24 h. Mean counts per minute of triplicate wells is shown. Dashed line represents response of PBMC to PWM in the absence of regulator cells.

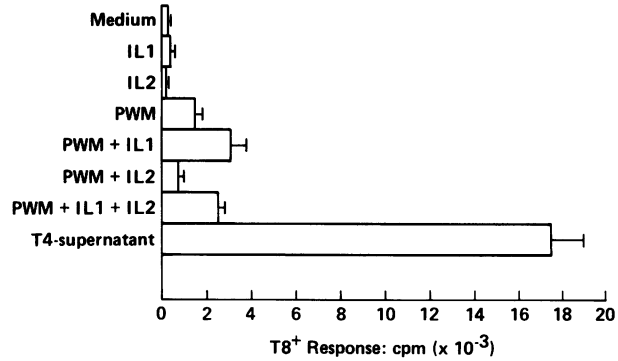

Figure 3. IL-1 cannot replace TsGF. 5×10^4 T4-depleted cells per well were cultured under various conditions for 6 d. Units of IL-1 (20 U/ml) and IL-2 (1.5 U/ml) contained in the T4 supernatant were previously determined, and an equal number of units of column-purified IL-1 and rIL-2 were used in this assay. [^{3}H]Thymidine was added to the triplicate wells for the final 24 h.

The dialyzed and undialyzed supernatants were also analyzed for IFN activity. Both the dialyzed and undialyzed supernatants had antiviral activity at dilutions up to 1:256, as determined in a cytopathic effect assay utilizing vesicular stomatitis virus. Thus TsGF activity of supernatants was abrogated upon removal of molecules < 12,000–14,000, while IL-2 and IFN activities remained.

Both TsGF and IL-2 are necessary for Ts proliferation. IL-2 has been described as a proliferative signal for many T cell populations (16). The function of IL-2 in the growth of suppressor cells was therefore investigated. It was possible that TsGF alone might cause Ts proliferation; alternatively, TsGF and IL-2 could act as cofactors. Fig. 5 shows that when IL-2 was removed from a T4 supernatant by passage over a solid-phase anti-IL-2 immunoadsorbent, the remaining TsGF had a reduced ability to cause proliferation of $T8^+$ cells. The absence of IL-2 in the column effluents was verified by an IL-2 assay on HT-2 cells. When exogenous rIL-2 was added back to the adsorbed super-

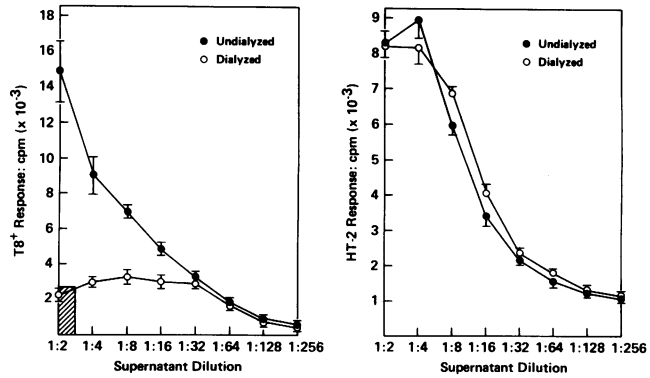

Figure 4. Separation of TsGF from IL-2. T4 supernatant was dialyzed to exclude molecules of molecular weight < 12,000–14,000. Dialyzed (○) and undialyzed (●) supernatants were compared for TsGF (*left*) and IL-2 content (*right*). TsGF assay was on OKT4-depleted cells as in Fig. 1, and IL-2 assay was performed on IL-2-dependent HT-2 cells. Bar on TsGF assay represents the mean counts per minute of T4-depleted cells in 1:80 PWM + rIL-2.

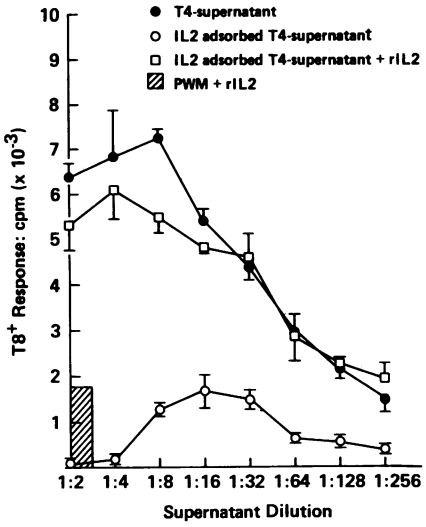

Figure 5. Both TSDF and IL-2 are necessary for Ts proliferation. OKT4-depleted cells were incubated in T4 supernatant (●), IL-2-adsorbed T4 supernatant (○), and IL-2-adsorbed T4 supernatant + rIL-2 (□). Supernatants were assayed as in Fig. 1. IL-2 content of the supernatants was 1.1 U/ml before adsorption and 0.0 U/ml after adsorption. Bar represents the mean cpm of the T4-depleted cells in 1:80 PWM + 1.1 U/ml rIL-2.

natant, most of the activity was reconstituted. Once again, the same amount of IL-2 contained in the supernatant could not induce proliferation, with or without added PWM. Thus, suppressor cells, similar to helper and cytotoxic T lymphocytes, require IL-2 for proliferation.

Molecular weight analysis of TsGF. In order to estimate the molecular weight of TsGF a supernatant was prepared in serum-free conditions. After concentrating the supernatant by lyophilization a sample was analyzed by HPLC on a gel filtration column. Fractions were collected and tested for IL-2 and TsGF activity (Fig. 6). The IL-2 activity appeared at the expected molecular weight of ~15,000. When testing the fractions for TsGF activity, T4-depleted cells were cultured in dilutions of the HPLC fractions, both with and without exogenous IL-2. Proliferation of the $T8^+$ cells was seen only in the presence of the added IL-2, even though the cells did not respond to the rIL-2 alone. TsGF activity eluted as a single peak with a molecular weight of ~8,000. No single fraction contained both TsGF and IL-2 activity.

TsGF is a protein. The molecular nature of TsGF was investigated by incubating aliquots of a serum-free supernatant in various enzymes. After quenching the reaction with the addition of 20% heat-inactivated human AB^+ plasma, the samples were tested for TsGF activity in the presence of rIL-2. As seen in Fig. 7, proteolytic enzymes were capable of destroying the TsGF activity; lipase was not. To verify that the enzymes were not being carried over into the assay, sham supernatant aliquots (serum-free RPMI) were incubated at the same time with the enzymes. After quenching with 20% serum, the sham supernatants were tested for the ability to abrogate a $T8^+$ cell proliferative response. Unlike PWM, PHA elicits a very strong proliferative response by T4-depleted cells. None of the sham supernatants interfered with a PHA response (data not shown), so the proteolytic enzymes did not exert their effect directly on the cells used in the assay.

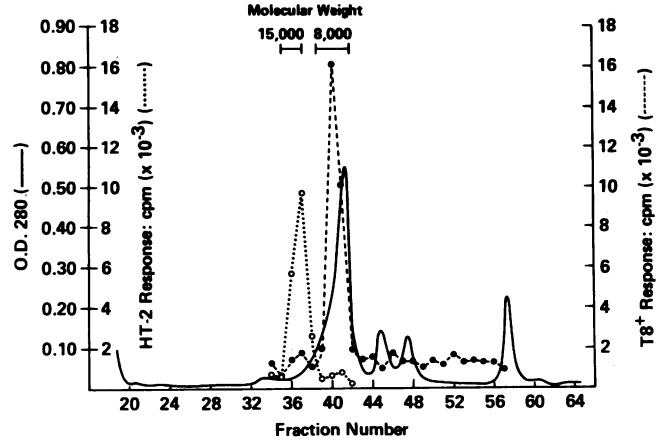

Figure 6. Molecular weight of TsGF. Serum-free T4 supernatant was analyzed by HPLC on a TSK-3000SW column, and fractions were analyzed for protein content at 280 nm (——), IL-2 activity (· · ·), and TsGF activity (– – –). T4-depleted cells were incubated in a 6-d proliferative assay with a 1:2 dilution of each fraction in the presence of 2.0 U/ml rIL-2 to test for the presence of TsGF. The T4-depleted cells had counts per minute of <1,000 when incubated in rIL-2 alone or the fractions in the absence of exogenous IL-2. IL-2 content of the fractions was determined by a 24-h HT-2 assay.

TsGF production is radiosensitive. The induction of suppressor cells has been described as radiosensitive (17), whereas IL-2 synthesis is relatively radioresistant (18). It was thus of interest to investigate whether TsGF production could be altered by irradiation of the suppressor-inducer subset. Various supernatants were prepared from irradiated or nonirradiated T8-depleted cells (Fig. 8). When tested for the ability to cause the

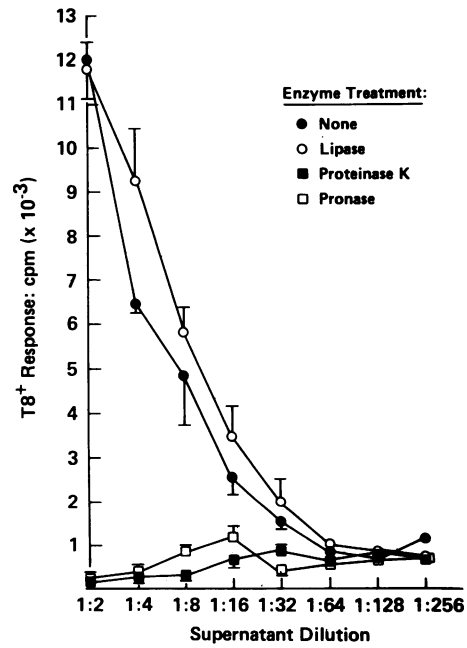

Figure 7. TsGF is a protein. Aliquots of T4 supernatant were incubated in medium (●), lipase (○), proteinase K (■), and pronase (□) for 1 h at 37°C. After IL-2 levels were reconstituted in the pronase and proteinase K samples, the aliquots were tested for TsGF activity as in Fig. 1.

proliferation of $T8^+$ cells, it was seen that supernatants of irradiated cells had very little TsGF activity. IL-2 production, conversely, was not reduced by up to 2,000 rads of γ-irradiation.

Dual-color fluorescence analysis of cultured $T8^+$ cells. $T8^+$ cells grown in various culture conditions were phenotyped to analyze the effects of TsGF on expression of antigens associated with Ts activation and differentiation. T4-depleted cells cultured for 6 d were stained at d0 and d6 with a number of conjugated antibodies and the samples were analyzed for $T8^+$ cells that also displayed other markers. When cells stained by phycoerythrin-conjugated OKT8 were analyzed, the percent staining positive with fluorescein isothiocyanate-conjugated antibodies to IL-2R, HLA-DR, and transferrin receptor (TR) increased after incubation in T4-supernatant (Table I). In contrast, fewer cells were positive for these markers after incubation in either PWM plus IL-2 or medium alone. No change in the percentage of T4 supernatant-stimulated $T8^+$ cells which coexpressed Leu7 or Leu8 on d6 was observed when compared with d0 cultures. The suppressor activity of these same $T8^+$ cultures was described previously in Fig. 2.

Incubation of $OKT8^+$ cells in TsGF induces the expression of IL-2 receptors and HLA-DR. If neither TsGF nor IL-2 alone was sufficient for Ts proliferation, one of the two may have activated $T8^+$ cells to become responsive to the other. To study this possibility, IL-2 was removed from a TsGF-containing supernatant by passing a small volume of concentrated factor through an immunoadsorbent column. The IL-2-free supernatant was then added to the same T4-depleted cells described in Table I to determine whether TsGF incubation of $T8^+$ cells activated IL-2R expression. Whereas before incubation $\sim$ 5% of the $T8^+$ cells were $IL\text{-}2R^+$, after a 6-d TsGF incubation 40% of the $T8^+$ cells were $IL\text{-}2R^+$ (Table I). Dual-bearing $T8^+HLA\text{-}DR^+$ cells also dramatically increased during this same period of time, as did the number of $T8^+$ cells bearing the TR. During this incubation in IL-2-free TsGF, very little proliferation occurred, in accord with the data presented in Fig. 5.

*Table I. Dual-Color Staining of $T8^+$ Cells**

$T8^+$ cells that are also	Day 0	Day 6 cultured in			
		Medium	PWM + rIL-2‡	T4 supernatant	IL-2-free T4 supernatant
	%	%	%	%	%
$Leu7^+$	20	5	4	11	13
$Leu8^+$	50	72	66	70	79
TR^+	3	3	8	22	30
$HLA\text{-}DR^+$	2	3	8	20	21
$IL2\text{-}R^+$	5	3	6	26	40

T4 supernatant was passed over an immunoadsorbent column to remove IL-2. IL-2 content of the supernatant was 1.2 U/ml before column passage and 0.0 U/ml after passage, as determined in an HT-2 proliferative assay.

* 1×10^4 cells per sample were analyzed by flow cytometry after staining with phycoerythrin-conjugated anti-T8 and a fluorescein isothiocyanate-conjugated second monoclonal antibody.

‡ 1:80 dilution of PWM plus 2.0 U/ml rIL-2 were added to cells at initiation of the 6-d culture period.

Discussion

Upon activation, $T4^+$ cells elaborate hormonal products including IL-2, IFNγ, and a variety of other B cell and T cell growth and differentiation factors (16, 19, 20). Secretion of these mediators can be stimulated in a number of ways, including incubation of the $T4^+$ cells with a T cell mitogen. As hormones, these molecules lack antigen specificity but distinguish between target subsets based upon differential receptor display (21). Thus, a responding cell must either have the receptor on its surface constitutively or be stimulated in some manner to express it.

The plant lectin PWM was utilized to activate cellular populations that might have an inducer influence on Ts. Experiments were performed which showed that supernatants from activated $T4^+Leu8^+$ cells (termed T4 supernatants) contain all the components required for the proliferation of T4-depleted cultures. PWM plus rIL-2 in amounts similar to those contained in these supernatants lacked the necessary proliferative signals. No amount of rIL-2 plus PWM ever caused more than slight proliferation of a T4-depleted culture; residual responses were likely due to incomplete removal of $T4^+$ cells by complement-mediated lysis.

$T8^+$ cells grown in T4 supernatant were tested for the ability to suppress a primary PWM response. The suppressive capability of a culture grown in T4 supernatant was greatly increased over a culture of $T8^+$ cells grown in PWM plus rIL-2, reflecting proliferation of the Ts subset of $T8^+$ cells. However, the cells in both cultures were equally suppressive on a cell-to-cell basis. It is for this reason that we designated this factor a T suppressor cell growth factor, or TsGF. Possible effects on proliferation of cells other than Ts remain to be investigated.

The role of monocytes in the activation of Ts has not yet been completely defined. The activation of $T4^+$ cells by PWM is a macrophage-dependent process (22). Because macrophages were present at comparable levels in the culture depleted of $T4^+$ cells, and were incapable of producing the growth factor, it seems clear that helper T cells were required. The possibility that $T4^+Leu8^+$ cells activate macrophages to produce TsGF is presently being addressed. We have recently discovered that certain antigen-specific $T4^+$ T cell lines stimulated by Sepharose-bound OKT3 in the absence of macrophages may also produce supernatants capable of supporting the proliferation of T4-depleted

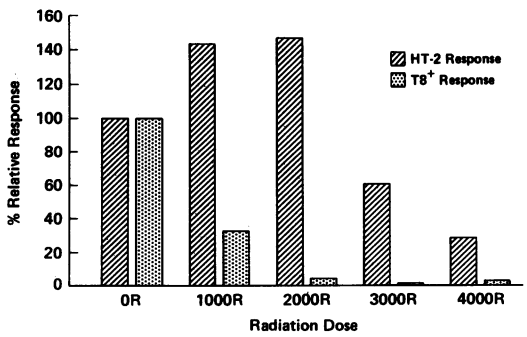

Figure 8. Radiosensitivity of TsGF production. OKT8 + C treated cells were incubated for 48 h at 5×10^6/ml in 1:80 PWM with increasing doses of γ-irradiation (R, rads). Supernatants were collected from these cultures and were assayed for TsGF activity on T4-depleted cells and on HT-2 cells for IL-2 activity. Bars represent the relative response of a 1:2 dilution of the supernatants. % Relative response = (cpm [test supernatant])/(cpm [unirradiated cell supernatant]) $\times$ 100%.

cells, suggesting that macrophages may be unnecessary for TsGF production and Ts proliferation. In contrast, in studies of requirements for induction of Ts functional differentiation rather than proliferation, we have observed a requirement for monocytes in the inductive culture.[2]

While the molecular weight of TsGF (8 kD) indicated that it was smaller than the intact IL-1 molecule (15 kD), Dinarello et al. (23) have reported that a breakdown product of IL-1 with a molecular weight of ~4 kD retains IL-1 activity. For this reason, we investigated whether TsGF was an IL-1 breakdown product capable of inducing IL-2R production in Ts precursors. We found that this was not the case because TsGF and IL-1 have molecular differences (as determined by trypsin sensitivity) and functional differences (as determined by activity on mouse thymocytes and T4-depleted cells).

Reduced levels of TsGF from irradiated $T4^+Leu8^+$ cells may help explain the difficulty in maintaining a long-term culture of suppressor cells. Limited success has been achieved by restimulation of Ts clones with antigen or mitogen in the presence of fresh irradiated feeder cells (24). This method is sufficient for CTL or helper clones, inasmuch as no radiation-sensitive product is required from the feeder cells. Exogenous IL-2, plus the presentation of antigen or mitogen by the fresh adherent cell layer is adequate for the restimulation of these cell types. Ts, on the other hand, are best stimulated by either fresh nonirradiated $T4^+Leu8^+$ cells, which is not practical for clonal expansion, or else a nutritive medium containing a source of TsGF and IL-2. Definition of possibly unique nutritive requirements for Ts is clearly important to efforts to clone such cells consistently.

Our studies show that cell–cell contact between $T4^+$ and $T8^+$ cells is unnecessary for Ts proliferation, as no recognition structures other than those provided in soluble form were provided. Damle et al. (25) have suggested that activation of Ts by alloantigens requires dual recognition of HLA class I molecules and T3-associated structures. We have shown that PWM can replace the need for recognition of cell-surface determinants between $T4^+$ and $T8^+$ cells by causing the production of a soluble suppressor cell growth factor. Utilizing PWM plus rIL-2, $T4^+$ cells were also shown to be unnecessary for the induction of suppressor function within the $T8^+$ subset.

IL-2 was necessary but not sufficient for Ts proliferation. Removal of IL-2 by passage over an immunoadsorbent column or by molecular weight separation by HPLC abrogated the ability of the T4 supernatant to promote $T8^+$ proliferation. IL-2 has been shown to be both necessary and sufficient for the proliferation of murine CTL which were stimulated with lectin (2). In contrast, rIL-2, with or without PWM, was insufficient as a growth hormone for Ts. The difference in the requirements of these populations for proliferation may be based on a differential receptor display, or by special requirements by Ts for the production of the IL-2 receptor.

IL-2 has been shown to up-regulate the production of its own receptor (26), although IL-2 was found to be insufficient to maintain optimal levels of IL-2R on the surface of the cells for a prolonged period of time. In our studies, PWM plus IL-2 were unable to cause more than 6% of $T8^+$ cells to become $IL\text{-}2R^+$, so an additional signal was necessary for the production of IL-2 receptors. IL-2 receptor expression after incubation of $T8^+$ cells in IL-2-free supernatant indicates a possible mechanism of action for TsGF. The production of $T8^+IL\text{-}2R^+$ cells after TsGF incubation may have generated cells capable of proliferating in response to IL-2. The expansion of the $T8^+IL\text{-}2R^+$ subset could be explained by an expansion of the cells already expressing IL-2R, or the induction of receptors on previously $IL\text{-}2R^-$ cells. Because of the large (eightfold) increase in the percentage of $T8^+$ cells expressing IL-2R, a net increase in the number of $IL\text{-}2R^+$ cells occurred over the period of incubation. Furthermore, Fig. 5 indicates that T4-depleted cells do not proliferate in response to IL-2-adsorbed T4 supernatant, suggesting that proliferation of preexistent $IL\text{-}2R^+$ cells does not account for the observed increase.

The induction of IL-2R is likely to be an important regulatory step in Ts proliferation. Whereas PBMC respond to PWM by the generation of IL-2-responsive T cells, Ts in the absence of TsGF could not express IL-2R. The hormonal signal TsGF may then explain the perceived difference in proliferative responses of the various T cell subsets. We have defined a pathway whereby a peptide hormone generated from an inducer population of T cells cause Ts to become responsive to a second signal (IL-2). These factors, not replaced by signals sufficient for IL-2R induction in other T cell subsets, lead to greatly enhanced proliferation of Ts. Further characterization of the TsGF molecule, the gene(s) regulating its production, and its cell-surface receptor will provide insights into the specific roles of Ts in immune regulation.

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