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CHARACTERIZATION OF A NOVEL MURINE T CELL-ACTIVATING FACTOR[1]

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Purified resting peripheral lymph node T cells can be activated to produce interleukin 2 (IL 2) and to proliferate in the presence of Concanavalin A (Con A) and an apparently novel lymphokine that we call T cell activating factor (TAF). TAF is biochemically distinct from IL 1, IL 2, IL 3, and other colony stimulating factors, IL 4 (BSF-1) and interferons. Furthermore, of the recombinant and natural cytokines tested, only IL 2 and TAF are active in the TAF assay. In the presence of Con A, TAF stimulates an increase in the steady-state level of IL 2 mRNA in T cells, the secretion of active IL 2 into the culture medium, and the proliferation of the T cells. We propose that TAF is a previously undescribed molecule the function of which is to stimulate IL 2 production by T cells that have encountered antigen, and we propose that TAF has an important role in primary T cell immune responses.

Activation and proliferation of most resting thymus-derived (T) lymphocytes can be divided into at least two necessary events, given that T cells generally require the T cell growth factor interleukin 2 (IL 2) for proliferation (1). First, resting T cells must be induced to express high-affinity IL 2 receptors, because they are not expressed before mitogenic or antigenic stimulation (2, 3). Second, T cells must be induced to produce IL 2, because they appear to be the sole cellular source of this molecule, and do not produce it constitutively (4). Both of these events have been extensively studied in murine and human systems by using purified peripheral T cells and T cell lines and clones. Both stimulation of IL 2 receptor expression and production of IL 2 appear to require engagement of the T cell antigen receptor complex by the appropriate ligand, either by antigen in the context of products of the major histocompatibility complex (MHC) genes or experimentally by antibodies directed against the antigen receptor or associated structures such as the T3 complex (5, 6). In the case of mitogenic lectins such as concanavalin A (Con A), it is postulated that their activity depends upon interaction with carbohydrate residues on the receptor glycoproteins or associated glycoproteins (7). In addition, some studies suggest that purified peripheral T cells or T cell clones require additional signals, including interleukin 1 (IL 1) for the production of IL 2 (8, 9), or for induction of responsiveness to IL 2 (10). However, the IL 1 requirement for stimulating these events in T cell clones and in purified T cell populations has not been demonstrated in several studies (11, 12).

We have investigated the factor requirements for stimulating IL 2 production and proliferation by purified normal resting murine lymph node T cells in response to Con A. In no case do we observe a requirement for exogenous IL 1 in this assay system. Instead, a novel lymphokine, which is produced by Con A-stimulated murine spleen cells and T cell hybridomas, restores the ability of macrophage-depleted peripheral T cell populations to produce IL 2. We have provisionally named this lymphokine T cell-activating factor (TAF).[2] This new lymphokine is functionally and biochemically distinct from IL 1, IL 2, interleukin 4 (IL 4; also called B cell stimulatory factor-1 or BSF-1), the interferons including immune interferon (IFN-γ), and several colony stimulating factors (CSF) including interleukin 3 (IL 3). Addition of TAF to Con A-stimulated lymph node T cell populations induces transcription of the IL 2 gene. We propose that TAF serves a crucial role in the early phases of resting T cell activation.

MATERIALS AND METHODS

Mice. BALB/c AnN mice were bred and were maintained in the Center for Cancer Research Animal Facility, M.I.T. C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Culture medium. RPMI 1640 culture medium (GIBCO, Grand Island, NY) supplemented with glutamine, antibiotics, and 5% or 10% fetal calf serum (FCS) was used as a basic medium to maintain all cell lines and for cytokine assays. In some cases, the appropriate growth factors were added to maintain factor-dependent cell lines.

Cytokines. Murine IL 1, purified from P388D1 cell supernatants, was kindly provided by S. Mizel (Bowman Gray Medical School, Winston-Salem, NC). In some experiments, supernatant fluid from UV-irradiated P388D1 cells was used as a crude source of IL 1. Recombinant human IL 1-α and IL 1-β were purchased from Genzyme (Boston, MA). Jurkat cell-derived purified human IL 2 (IL 2J) was kindly provided by R. Robb (DuPont, Glenolden, PA). Recombinant human IL 2 (rIL2) was purchased from Amgen Biologicals (Thousand Oaks, CA). Recombinant mouse IFN-γ (rIFN-γ) was kindly provided by P. Gray (Genentech, South San Francisco, CA). Conditioned medium from the myelomonocytic cell line WEHI-3 and the fibroblast cell line L929 were used as crude sources of IL 3 and CSF-1, respectively.

Cytokine bioassays. The IL 1 murine thymocyte co-mitogenesis assay was performed as described (13) by using thymocytes from 3 wk old C3H/HeJ mice. The IL 2 assay was performed, and units of IL 2 were determined as described by Gillis and Smith (14) by using the CTLL-2-15H indicator cell line and an IL 2 standard provided by R. Robb. One Robb unit of IL 2 activity is approximately 10 half-maximal units or 8.2 ng of IL 2. IL 3 activity was measured by the ability of test samples to support the growth of the indicator cell line 32DC1 as described (15). A general liquid culture assay for CSF was

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[2] Abbreviations used in this paper: TAF, T cell-activating factor.

performed as described by Watson et al. (16). IFN was measured by the ability of test samples to protect monolayers of L929 cells from the cytopathic effect of vesicular stomatitis virus as described (17).

TAF assay. T cells were purified from the peripheral lymph nodes of BALB/c AnN mice by passage of the cells over nylon wool columns followed by treatment of the cells with the anti-Ia monoclonal antibodies plus complement as described (18). Purified T cells were cultured at 2×10^5/flat or round-bottomed microculture well in the presence or absence of 2 μg/ml Con A. Samples to be tested for TAF activity were added to these microcultures at various dilutions. After incubation for 40 hr at 37°C, the microcultures were pulsed for 4 hr with 0.5 μCi [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) and were processed for scintillation counting by using a Skatron Cell Harvester (Skatron, Sterling, VA). In some experiments, both the nylon wool column passage and the anti-Ia plus complement treatments were carried out twice sequentially to obtain highly purified T cells that no longer respond to Con A plus IL 2. These cells were washed with RPMI 1640 containing 0.2% bovine serum albumin, were incubated with *Vibrio cholerae* neuraminidase (1:20, Worthington, Freehold, NJ) at 37°C for 1 hr, were washed, and were resuspended in complete medium for use in the TAF assay. Lyt-2$^-$ L3T4$^+$ T cells were prepared by treatment of purified lymph node T cells with anti-Lyt-2 monoclonal antibodies plus complement as described (19). T-depleted spleen cells ("accessory cells") were prepared by treatment of BALB/c AnN spleen cells with anti-Thy-1.2 monoclonal antibody J_1J (20) plus complement, followed by Ficoll-Isopaque purification of the viable cells. In some experiments, the proliferative response of purified lymph node T cells in the presence of 2 μg Con A/ml was reconstituted by the addition of an equal number of "accessory cells".

Preparation of TAF-containing conditioned medium. Spleen cells obtained from BALB/c AnN mice were cultured at 5×10^6/ml in 75 cm^2 culture flasks (Falcon Plastics, Oxnard, CA) in a vol of 50 ml in the presence of 2 μg Con A/ml for 36 hr. The cell-free conditioned medium was then concentrated 20-fold by ultrafiltration by using an Amicon YM-10 membrane (Amicon, Danvers, MA). FS7.20.7.1 T hybridoma cells (kindly provided by J. Kappler and P. Marrack; National Jewish Hospital, Denver, CO) were cultured at 1×10^6/ml for the production of TAF in the presence of 5 μg Con A/ml during a 24-hr culture period.

Absorption of IL 2 on CTLL-2-15H cells. IL 2 was absorbed from the conditioned medium from Con A-activated FS7.20.7.1 cells by using the IL 2-dependent cell line CTLL-2-15H as described (18).

Gel filtration chromatography. Concentrated conditioned medium was applied to a 3 x 90 cm Sephacryl-200 column (Pharmacia) equilibrated at 1.0 ml/min with phosphate-buffered saline (PBS), pH 7.2, and 12.5 ml fractions were collected for assay. In some experiments, 0.5 ml aliquots of partially purified TAF (pre-incubated in the appropriate buffer) were applied to 1 x 25 cm Sephacryl-200 columns equilibrated at 0.5 ml/min with 6 *M* guanidine-HCl/PBS, pH 7.2, or with 6 *M* guanidine-HCl/1 *M* 2-mercaptoethanol/PBS, pH 7.2, and 0.5 ml fractions were collected and were dialyzed against PBS, pH 7.2. All columns were calibrated in the appropriate buffer with bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), trypsinogen (25,000 daltons), and ribonuclease A (14,000 daltons). The void volumes and the included volumes were determined by using blue dextran and riboflavin, respectively. All samples were filtered (0.45 μm) before chromatography.

Anti-IL 2 immunoaffinity chromatography. The DMS-1 hybridoma was kindly provided by K. Smith (Dartmouth Medical School, Hanover, NH). DMS-1 was derived from mice immunized with purified human IL 2, but cross-reacts with mouse IL 2 (21). The IgG2a antibodies were purified from ascites fluid by protein A-Sepharose chromatography and were coupled to CNBr-activated Sepharose beads at a concentration of 10 mg antibody/ml beads according to the manufacturer's protocol (Pharmacia, Uppsala, Sweden). Material to be depleted of IL 2 was warmed to 37°C and was passed over a 5-ml anti-IL 2 column equilibrated at 0.5 ml/min with PBS, pH 7.2, at 37°C. The column was then eluted with glycine-buffered saline, pH 2.3, to remove the bound IL 2.

Phenyl-Sepharose chromatography. Samples were dialyzed against 0.8 M ammonium sulfate/PBS, pH 7.2, filtered and applied to a 1.75 x 25 cm phenyl-Sepharose column equilibrated at 0.5 ml/min with the same buffer at 4°C. The column was then eluted with an increasing linear gradient of ethylene glycol superimposed on a linear decreasing gradient of ammonium sulfate as indicated in the figures. Fractions were collected and were dialyzed against PBS, pH 7.2.

Polyuridylic acid-Sepharose chromatography. Samples were dialyzed against 10 m*M* Tris, pH 7.5, were filtered, and were applied to a 4-ml polyuridylic acid-Sepharose column equilibrated at 0.5 ml/min with the same buffer at 22°C. The column was then eluted either with a linear gradient of NaCl or with 1 *M* NaCl in 10 m*M* Tris, pH 7.5. Fractions were collected and were dialyzed against PBS, pH 7.2, before assay.

Reversed-phase high pressure liquid chromatography (HPLC). A Beckman HPLC system (Beckman Instruments, Berkeley, CA), consisting of a Model 421A controller, 2 Model 110A pumps, a Model 210A sample injector, and a Model 160 absorbance detector was used for all experiments. Samples were acidified to pH 2.1 with trifluoroacetic acid (TFA), were filtered, and were applied to an Ultrapore RPSC C_3 reversed-phase column (4.6 mm x 7.5 cm) equilibrated at 1 ml/min with 0.1% TFA at 22°C. The column was then eluted with a gradient of acetonitrile as shown in the figure. One milliliter fractions were collected and were dialyzed against PBS, pH 7.2, before assay.

Trypsin treatment. One milliliter of TAF, partially purified from CASN by sequential gel filtration, anti-IL 2, phenyl-Sepharose, and polyuridylic acid-Sepharose chromatography was incubated at 37°C in the presence of trypsin-TPCK (Cooper Biomedical, Malvern, PA). After 1 hr, 0.1 ml FCS was added to inhibit trypsin activity (18). As controls, similar samples of TAF were incubated under the same conditions in the absence of trypsin, or were incubated with trypsin after the addition of FCS.

RNA preparation and ribonuclease protection analysis. Total cellular RNA was prepared from various cell preparations by using the guanidinium/cesium chloride method as described (22). Ribonuclease protection analysis was performed as described (19, 23) by using a ^{32}P-labeled RNA probe derived by in vitro transcription of DNA from the fourth exon and 3′-untranslated regions of a murine genomic IL 2 clone kindly provided by W. Fiers (State University of Ghent, Ghent, Belgium).

RESULTS

Restoration of the proliferative response of accessory cell-depleted T cell populations. The Con A-stimulated proliferative response of lymph node T cells purified by a single passage through a nylon wool column followed by treatment with anti-Ia antibody plus complement is virtually abolished by these treatments, but can be restored by the addition of γ-irradiated, T cell-depleted syngeneic spleen cells (18). In addition, the proliferative response of these cells can be restored by cell-free conditioned medium preparations derived from Con A-activated mouse spleen cells (18) or the T cell hybridoma FS7.20.7.1 activated with Con A (Fig. 1*A*). Both purified JURKAT-derived human IL 2 (Fig. 1*A*) and recombinant human IL 2 (see below) are also capable of restoring this response. Subsequently we will refer to this assay system as the TAF assay.

Removal of IL 2 from TAF-containing preparations. Each source of factors capable of restoring the proliferative response of T cells in the experiments described above contained IL 2. To determine whether IL 2 is the

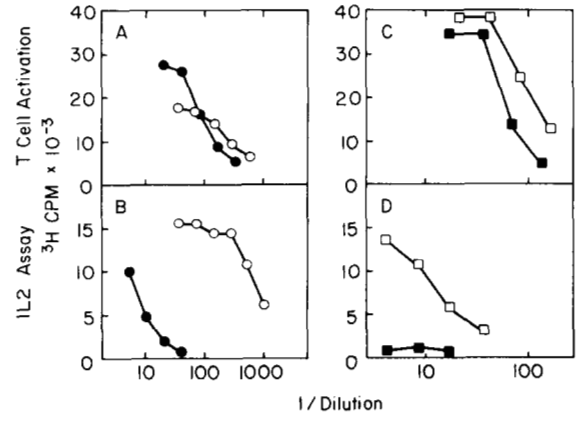

Figure 1. TAF activity in FS7-20.7 culture fluid after removal of IL 2. Conditioned medium from Con A-activated FS7-20.7.1 cells (●, □), conditioned medium from Con A-activated FS7-20.7.1 cells absorbed with CTLL-2-15 cells (■), and purified human IL 2 (10 U/ml, ○) were tested in the assay for T cell activation (*A* and *C*) and in the IL 2 assay (*B* and *D*).

only factor in conditioned medium from activated FS720.7.1 cells that is active in this assay, IL 2 was removed by absorption on the IL 2-dependent cell line CTLL-2-15H. As shown in Figure 1C and *D*, all measurable IL 2 activity was removed by the absorption, leaving behind molecules active in the TAF assay. This result suggests that T cell hybridomas produce a molecule distinct from IL 2 that stimulates T cell proliferation in this assay system. Similar results were obtained previously by using conditioned medium from Con A-stimulated spleen cells (18). As described below, the active molecules obtained from these two sources have similar biochemical properties.

Partial purification of spleen cell-derived TAF. Two liters of conditioned medium from Con A-stimulated mouse spleen cells were concentrated 20-fold by ultrafiltration and were applied to a calibrated gel filtration column in 10 ml aliquots. Fractions from the column were assayed for IL 2 activity and TAF activity. Molecules active in the IL 2 and TAF assays co-eluted in fractions corresponding to an apparent m.w. of approximately 30,000 to 35,000 (Fig. 2*B*), and active fractions from several column runs were pooled (bracket; Fig. 2).

To remove IL 2 from the gel filtration pool, monoclonal anti-IL 2 (DMS-1) antibody bound to Sepharose beads was used as an immunoadsorbent. DMS-1 was produced in mice immunized with human IL 2 but cross-reacts with mouse IL 2 (21). The gel filtration pool (Fig. 2*B*) was passed over the anti-IL 2 column, and the material that bound to the column was eluted with glycine-buffered saline, pH 2.3. The material that passed through the column contained substantial activity in the TAF assay but only 1 to 3% (in different experiments) of the IL 2 activity; most of the IL 2 was recovered in the acid eluent (Table I). Anti-IL 2 columns are thus useful for removal

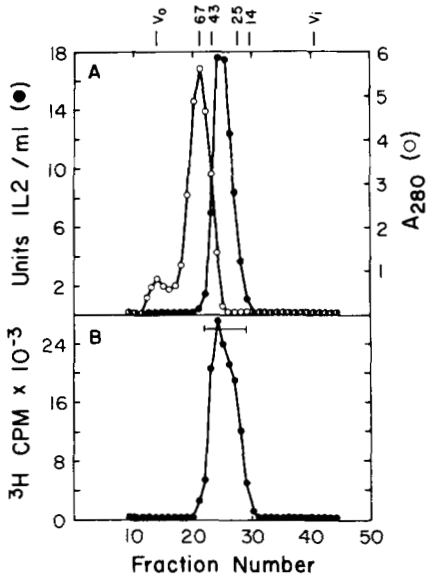

Figure 2. Gel filtration of concentrated conditioned medium from Con A-activated spleen cells. Ten milliliters of 20× CASN were applied to an S-200 column equilibrated with PBS, pH 7.2, and were calibrated with bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), trypsinogen (25,000 daltons) and ribonuclease A (14,000 daltons). The void volume (V_o) and included volume (V_t) were determined by using blue dextran and riboflavin, respectively. In *Panel A*, fractions were tested for absorbance at 280 nm (○) and in the IL 2 assay (●). In *Panel B*, fractions were tested at 1/8 in the TAF assay. *Bracket* indicates pool for additional chromatography.

TABLE I

Removal of IL 2 from the TAF-containing gel filtration pool by using immunoaffinity chromatography

Sample	Dilution	^{3}H cpm	
		TAF assay	IL 2 assay
Gel filtration pool (1.65 U IL 2/ml)	1/2	20,083	NT[a]
	1/6	16,062	27,285
	1/8	7,884	17,385
	1/54	NT	4,842
Material not bound to anti-IL 2 Sepharose (0.01 U IL 2/ml)	1/2	10,208	NT
	1/6	5,065	628
	1/18	2,934	35
	1/54	NT	30
Material eluted from anti-IL 2 Sepharose (24.28 U IL 2/ml)	1/2	14,774	NT
	1/6	10,450	26,932
	1/18	8,840	32,084
	1/54	NT	30,012
	1/162	NT	21,511
Medium control	—	143	25
Con A control	—	196	NT

[a] Not tested.

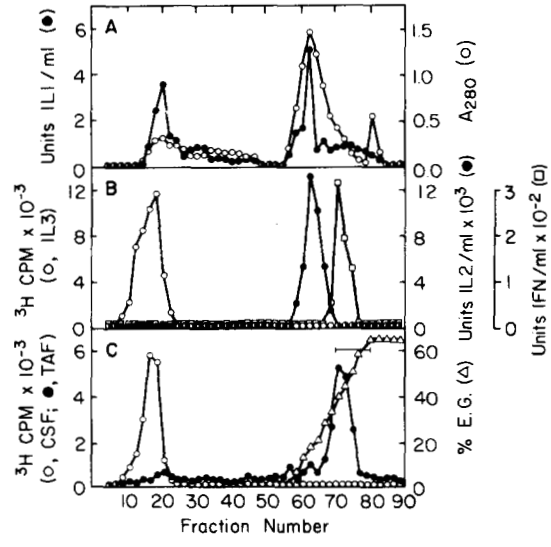

Figure 3. Phenyl-Sepharose chromatography of the anti-IL 2-Sepharose TAF pool. Material that did not bind to the anti-IL 2 column was applied to a phenyl Sepharose column equilibrated with 0.8 *M* ammonium sulfate-PBS, pH 7.2, and the column was eluted with a linear gradient of 0 to 60% ethylene glycol in PBS. In *Panel A*, fractions were monitored for absorbance at 280 nm (○) and were tested in the thymocyte co-mitogenesis assay for IL 1 (●). In *Panel B*, fractions were tested at 1/6 in the IL 3 assay (○), in the IL 2 assay (●), and in the IFN assay (□). In *Panel C*, fractions were tested at 1/6 in a general assay for CSF (○) and at 1/6 in the TAF assay (●). *Brackets* indicate fractions pooled for additional chromatography. *Triangles* indicate percentage ethylene glycol (E.G.) in the fractions.

of most of the IL 2 from these samples, and as in the absorption experiment shown in Figure 1, substantial TAF activity remains.

The material that did not bind to the anti-IL 2 column was additionally fractionated by hydrophobic chromatography. The sample was applied to a phenyl-Sepharose column equilibrated with PBS containing 0.8 *M* ammonium sulfate, and bound material was eluted with a linear gradient of 0 to 60% ethylene glycol superimposed on a gradient of 0.8 to 0.0 *M* ammonium sulfate. In this analysis each fraction was tested in several defined cytokine bioassays. As shown in Figure 3, IL 3 activity and material active in a general assay for CSF passed through the column, whereas IL 2, IFN, and TAF activities bound to the column and were eluted during the gradient. Al-

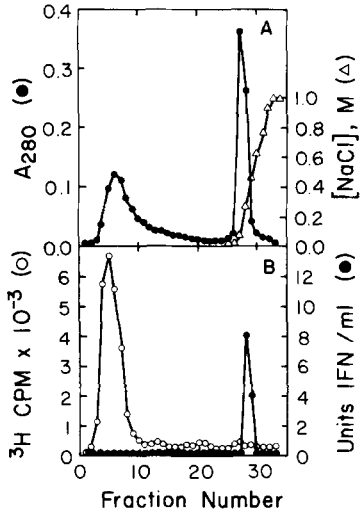

Figure 4. Polyuridylic acid-Sepharose chromatography of the phenyl-Sepharose TAF pool. The TAF pool from phenyl-Sepharose chromatography was applied to a polyuridylic acid-Sepharose column equilibrated with 10 m*M* Tris, pH 7.5, and the column was eluted with a linear gradient of 0.0 to 1.0 *M* NaCl. *Panel A* shows the absorbance of the fractions at 280 nm. In *Panel B*, fractions were tested at ½ in the TAF assay (○) and in the IFN assay (●).

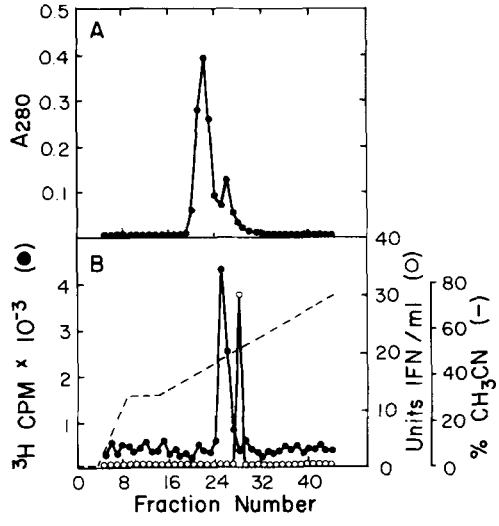

Figure 5. Reversed-phase HPLC of the phenyl-Sepharose TAF pool. Eight milliliters of phenyl-Sepharose purified TAF were applied to a C_3 HPLC column equilibrated with 0.75% CH_3CN in 0.1% TFA, and the column was eluted with a gradient of CH_3CN. *Panel A* shows the absorbance of the fractions at 280 nm. In *Panel B*, fractions were tested at 1/18 in the TAF assay (●) and in the IFN assay (○).

though IL 2 was eluted from the column in approximately 25% ethylene glycol, the TAF and IFN activities were eluted in approximately 42% ethylene glycol. Note that although IL 2 is active in the TAF assay (Fig. 1), the amount of IL 2 in the sample applied to the phenyl-Sepharose column was extremely low (0.5 U; based on a standard provided by R. Robb; 0.5 Robb units corresponds to approximately 5 half maximal units) explaining the low response of the IL 2-containing fractions in the TAF assay. Very little IL 1 activity was recovered in fractions distinct from the IL 2 peak fractions (IL 2 is active in the IL 1 assay), and most of the material active in the IL 1 assay was resolved from the molecules active in the TAF assay. These results show that phenyl-Sepharose chromatography separates molecules active in the TAF assay from CSF, IL 3, IL 2, and IL 1, but not from

TABLE II

TAF activity is destroyed by treatment with trypsin

Sample	Dilution	³H cpm in TAF Assay
TAF[a]	1/2	18,932
	1/6	15,435
	1/18	6,450
(TAF + trypsin) + FCS[b]	1/2	129
	1/6	728
	1/18	307
(TAF + FCS) + trypsin[c]	1/2	7,875
	1/6	14,635
	1/18	7,201
Con A control	—	322
Medium control	—	57

[a] TAF partially purified by using sequential gel filtration, anti-IL2, phenyl-Sepharose and polyuridylic acid-Sepharose chromatography.
[b] TAF incubated with trypsin at 37°C for 1 hr followed by addition of FCS.
[c] TAF incubated with trypsin at 37°C for 1 hr with prior addition of FCS.

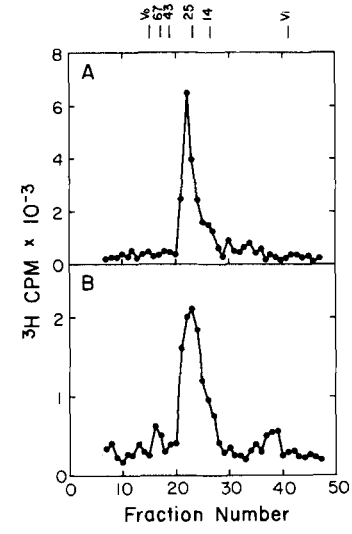

Figure 6. Gel filtration of TAF under denaturing or reducing and denaturing conditions. Two milliliters of polyuridylic acid-Sepharose-purified TAF were applied to an S-200 column equilibrated with 6 *M* guanidine HCl (*Panel A*) or (*Panel B*) 6 *M* guanidine HCl, 1 *M* 2-mercaptoethanol. Fractions were tested at ½ in the TAF assay after dialysis against PBS, pH 7.2. Elution positions of m.w. markers (see Fig. 2 legend) are indicated.

IFN. The major IFN species present in the gel filtration pool is probably IFN-γ, because Con A-activated T cells produce this molecule (24). Furthermore, most of the IFN activity in this pool can be destroyed by prolonged incubation at low pH, suggesting that the major IFN species is IFN-γ (data not shown).

Polyuridylic acid-Sepharose chromatography was used for separating the TAF activity from IFN-γ. When a pool of active fractions from phenyl-Sepharose chromatography was applied to a polyuridylic acid-Sepharose column, TAF activity was found in the material that did not bind to the column, whereas IFN activity bound to the column and was eluted during a gradient of sodium chloride (Fig. 4). This analysis resolves the TAF activity from IFN present in the hydrophobic chromatography pool.

To additionally examine the chromatographic properties of TAF, a pool of active fractions from phenyl-Sepharose chromatography was applied to a C_3 column equilibrated with 0.1% TFA and 0.75% CH_3CN, and was eluted with a gradient of CH_3CN as shown in Figure 5. The

fractions were immediately dialyzed against PBS, pH 7.2, and were tested in the TAF and IFN assays. As shown in Figure 5, TAF activity was eluted from the column with 45% CH_3CN, whereas IFN activity that survived the short exposure to pH 2.1 was eluted with 50% CH_3CN. Recovery of TAF activity from the C_3 column was approximately 45%, and recovery of IFN activity was 30%. No IL 2 activity was detected in any of the HPLC fractions (data not shown). These results indicate that reversed-phase HPLC may be a useful procedure for additional purification of TAF.

Trypsin treatment of TAF. Although the chromatographic behavior of molecules active in the TAF assay suggested that this activity is mediated by a polypeptide, it was important to determine whether or not TAF is a protein. The data in Table II show that TAF activity is sensitive to trypsin treatment, indicating that the molecules active in the TAF assay are protein in nature. Interestingly, it has been reported that tryptic peptides of IL 1 retain activity in the thymocyte assay for IL 1, additional evidence that IL 1 and TAF are distinct molecules (25).

Gel filtration of TAF under denaturing and reducing conditions. The data presented in Figure 3 and in Table II suggest that TAF activity is mediated by a protein molecule with an approximate M_r of 30,000 to 35,000 as judged by gel filtration in a physiologic buffer. To provide data concerning the possibility that TAF activity may be mediated by a molecule with subunits that are noncovalently associated or disulfide linked, poly U-Sepharose-purified TAF was subjected to gel filtration in 6 *M* guanidine HCl with or without 1 *M* 2-mercaptoethanol. As shown in Figure 6, TAF activity was eluted from the column with an approximate M_r of 30,000 under denaturing conditions (Fig. 6*A*) and under denaturing and reducing conditions (Fig. 6*B*). These results suggest that TAF activity is mediated by a single protein molecule without disulfide-linked subunit structure. However, because the recovery of activity from denaturing and reducing conditions was lower than from denaturing conditions, intrachain disulfide bonds may be important in maintaining a conformation that results in TAF activity.

Partial purification of T cell hybridoma derived TAF. In separate experiments TAF activity was partially purified from conditioned medium from Con A-activated FS7.20.7.1 T hybridoma cells. The properties of TAF derived from this T cell hybridoma are similar in all respects to TAF derived from spleen cells. However, the level of TAF produced by these cells is much lower than that found in culture fluid from Con A-stimulated spleen cells (data not shown). FS7.20.7.1-derived TAF exhibits an apparent approximate M_r of 30,000 to 35,000 on gel filtration columns equilibrated with PBS, pH 7.2 (data not shown). TAF derived from FS720.7.1 cells bound to phenyl-Sepharose columns and was eluted with 42% ethylene glycol (Fig. 7) as did spleen cell-derived TAF (Fig. 3). Phenyl-Sepharose chromatography was useful for separating FS7.20.7.1-derived TAF from CSF and IL 2 (Fig. 7). The amount of IFN-γ in the phenyl-Sepharose fractions was below the level of detection in our assay, however, presumably due to nonspecific losses of this molecule during prior handling. FS7.20.7.1 cells produce a small amount of IFN-γ and do not produce detectable IL 3 (data not shown).

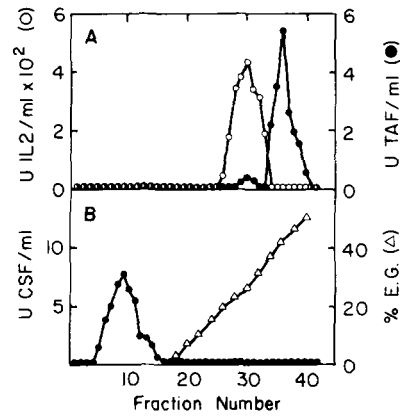

Figure 7. Phenyl-Sepharose chromatography of FS7.20.7.1-derived TAF. Culture fluid from Con A-activated FS7.20.7.1 cells was subjected to DEAE-cellulose chromatography, and fractions active in the TAF assay were applied to a phenyl-Sepharose column equilibrated with 0.8 *M* ammonium sulfate-PBS, pH 7.2. The column was then eluted with a linear 0 to 50% gradient of ethylene glycol in PBS. In *Panel A*, fractions were tested in the IL 2 (O) and TAF assays (●). In *Panel B*, fractions were tested in a general assay for CSF (●). One unit in the TAF and CSF assays gave 50% of the maximal [^{3}H]thymidine incorporation; one unit of IL 2 gave 50% incorporation at a dilution of 1/10.

TABLE III

TAF is not active in the CSF, IL 2, IL 3, or IFN assays

Assay	Sample	^{3}H cpm Dilution 1/2	1/6	1/18	1/54
CSF	L929 supernatant	48,677	50,097	24,537	5,294
	TAF[a]	635	293	213	336
	Medium	122	—	—	—
IL 2	IL 2J[b] (10 U/ml)	NT[c]	35,939	36,896	30,804
	TAF	40	25	23	24
	Medium	39	—	—	—
IL 3	WEHI-3 supernatant	NT	21,893	16,296	11,699
	TAF	458	108	89	68
	Medium	65	—	—	—
		Units/ml			
IFN	rIFN-γ	300			
	TAF	0			
	Medium	0			

[a] TAF partially purified by using sequential gel filtration, anti-IL 2, phenyl-Sepharose and polyuridylic acid-Sepharose chromatography.
[b] Purified human IL 2.
[c] Not tested.

Relationship of TAF activity to other lymphokine activities. Table III summarizes a series of experiments in which partially purified TAF and other lymphokines were tested in bioassays for IL 1, IL 2, IL 3, IFN, CSF, and TAF. As shown in Table III, TAF partially purified by the above procedures had no significant activity in any of these assays; conversely, of six factors assayed, only IL 2 and partially purified TAF were significantly active in the TAF assay (Table IV).

Because under certain conditions both TAF and IL 1 will stimulate the production of IL 2 by T cells or thymocytes in the presence of a lectin, it was particularly important to distinguish between these two activities. To address this issue, partially purified TAF and recombinant human IL 1-α and IL 1-β were tested in both the TAF assay and the standard murine thymocyte co-stimulator IL 1 assay. As shown in Figure 8, levels of human rIL 1-α and rIL 1-β giving plateau activity in the IL 1 assay

TABLE IV

TAF and IL 2 are strongly active in the TAF assay

Dilution	^{3}H cpm in TAF Assay							
	Sample							
	IL 1[a] (100 U/ml)	IL 2[b] (10 U/ml)	L929 sup	WEHI-3 sup	rIFN-γ (300 U/ml)	TAF[c] Expt. 1	TAF[c] Expt. 2	TAF[c] Expt. 3
1/2	808	NT[d]	452	161	431	9,437	7,491	6,668
1/4	1,075	11,155	405	542	709	5,120	4,320	5,898
1/8	645	12,722	477	469	702	1,892	3,242	5,264
1/16	427	8,613	NT	NT	648	NT	NT	NT
Con A	587	587	390	398	587	587	390	398
Medium	80	80	187	45	80	80	187	45

[a] Purified mouse IL 1.
[b] Recombinant human IL 2.
[c] TAF partially purified by using sequential gel filtration, anti-IL 2, phenyl-Sepharose and polyuridylic acid-Sepharose chromatography.
[d] Not tested.

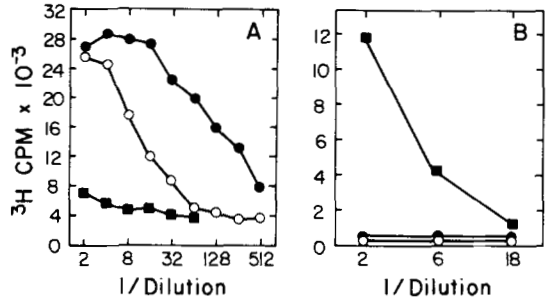

Figure 8. Functional difference between TAF and IL 1. Polyuridylic acid-Sepharose-purified TAF (■), recombinant human IL 1-α (●) and recombinant human IL 1-β (○) were tested at various dilutions in the murine thymocyte co-mitogenesis assay for IL 1 (*A*) and in the TAF assay (*B*).

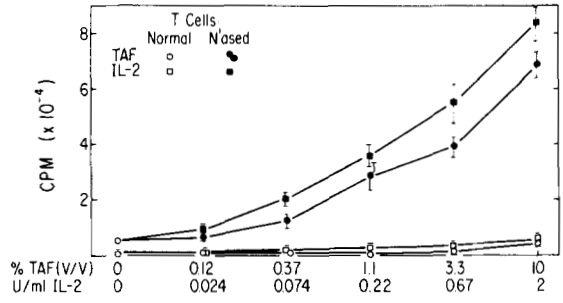

Figure 9. Effect of pretreatment of purified T cells with neuraminidase on TAF activity. Highly purified T cells (two nylon wool passages and two anti-Ia plus complement treatments) were tested for proliferation with Con A plus purified human IL 2 (□) or partially-purified TAF (○) or were tested for proliferation after neuraminidase treatment (IL 2, ■; TAF, ●).

gave background activity in the TAF assay. Conversely, a concentration of TAF very near plateau in the TAF assay resulted in minimal activity in the IL 1 assay. Similar results were found by using purified murine IL 1 (see Table IV). These results indicate that IL 1 and TAF are functionally distinct molecules, although under certain conditions both activities can lead to the production of IL 2.

Functional aspects of TAF activity. An important issue is whether TAF acts directly on T cells or indirectly through contaminating "accessory cells" such as macrophages. Recent studies have suggested that lymph node T cells purified in a manner similar to the procedure used in our standard TAF assays are contaminated with functionally active "accessory cells" (26). Specifically, it was shown that more exhaustive purification of T cells (for example, by repeating both the nylon wool column passage and anti-Ia plus complement treatments) yields a T cell population that will not proliferate in response to Con A plus IL 2. Additional studies showed that the proliferative response of the more purified T cells to Con A plus IL 2 was restored if the cells were pretreated with neuraminidase (27). The interpretation of these and other experiments was that "polyvalent" forms of Con A (for example, cells coated with Con A molecules) are required for optimal T cell triggering, and whereas non-T accessory cells are effective at "presenting" Con A molecules to T cells, resting T cells are ineffective in this role. Resting T cells have high levels of surface sialic acid, creating a high negative charge character that may result in the inability of the cells to present Con A to each other due to charge repulsion.

In light of these studies, it was of interest to determine whether more purified T cell populations would respond to TAF plus Con A. Our experiments confirmed and extended the results of Hunig et al. (26, 27). T cells purified by two consecutive passages on nylon wool columns followed by two consecutive anti-Ia plus complement treatments responded poorly to Con A plus IL 2, but proliferated vigorously to Con A plus IL 2 if they were pretreated with neuraminidase (Fig. 9). Similarly, the more purified T cells responded poorly to Con A plus TAF, but proliferated vigorously to Con A plus TAF if pretreated with neuraminidase (Fig. 9). These results strengthen the case that TAF acts directly on T cells. In addition, these data strongly suggest that TAF activity is not a consequence of a neuraminidase-like activity mediated by the TAF molecule.

It was also important to determine whether the resting T cells in the TAF assay require Con A for proliferation in the presence of TAF. As shown in Table V, TAF alone does not cause proliferation of T cells in the TAF assay, but requires Con A, presumably to cross-link their antigen receptors. Thus TAF is a co-stimulating activity, suggesting it may act in concert with antigen stimulation in the T cell immune response.

TAF induces IL 2 production by resting T cells activated with Con A. The data presented thus far demonstrate that an apparently novel lymphokine, TAF, stimulates the proliferation of Con A-activated purified T cells. An important question is whether TAF is a novel T

TABLE V

The activity of TAF is dependent upon the presence of Con A in the TAF assay

Sample	2 μg/ml Con A	Dilution	^{3}H cpm in TAF Assay
Medium	–	—	195
Medium	+	—	537
TAF[a]	–	1/2	365
		1/6	270
		1/18	270
TAF	+	1/2	13,823
		1/6	8,206
		1/18	3,552

[a] TAF partially purified by using sequential gel filtration, anti-IL 2, phenyl-Sepharose, and polyuridylic acid-Sepharose chromatography.

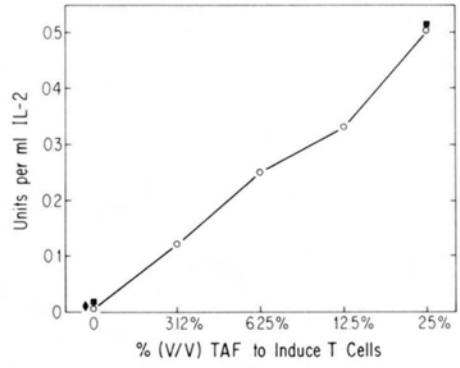

Figure 10. Induction of IL 2 secretion by TAF. Supernatant fluids from purified Lyt-2$^-$ L3T4$^+$ T cells activated with 2 μg Con A/ml plus various concentrations of partially purified TAF (○) were tested for IL 2 activity. Purified murine IL 1 (10 U/ml, ■) was added to the cultures alone or with 25% TAF. IL 2 produced by purified T cells without Con A is indicated (◆).

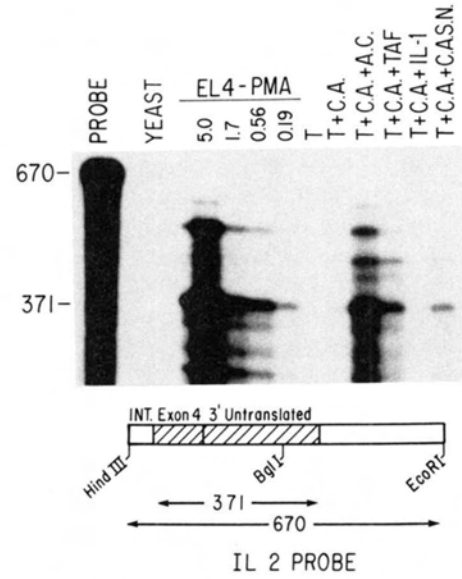

Figure 11. Analysis of IL 2 mRNA levels in T cell populations by ribonuclease protection assay. The indicated amounts of total RNA isolated from PMA-stimulated EL4 cells and 3 μg total RNA isolated from purified neuraminidase-treated Lyt-2$^-$ L3T4$^+$ T cells (T) alone or cultured with 2 μg/ml Con A (CA), T-depleted spleen cells (AC), polyuridylic acid-Sepharose-purified TAF, purified murine IL 1 (10 U/ml), or conditioned medium from Con A-stimulated spleen cells (CASN) were analyzed by ribonuclease protection assay by using a ^{32}P-labeled RNA probe derived by in vitro transcription of 670 base pairs (bp) of genomic IL 2 DNA indicated. The 371 bp protected fragment corresponds to IL 2 exon 4 and the 3′-untranslated region of IL 2 mRNA. The larger protected fragments probably result from IL 2 mRNA that have used alternative polyadenylation sites. Yeast soluble RNA was used as a negative control; EL4 RNA was used as a positive control.

cell growth factor, or alternatively stimulates production of the previously defined T cell growth factor, IL 2. As shown in Figure 10, the latter explanation appears to be correct. For these experiments, we used highly purified Lyt-2$^-$ L3T4$^+$ (helper phenotype) peripheral T cells that had been pretreated with neuraminidase (see the previous section) to ensure maximal activation. The cells were cultured with Con A and graded doses of factor, and the culture supernatants were then titrated in the IL 2 bioassay to determine the amount of IL 2 released. Culture of the T cells with TAF caused a dose-dependent release of IL 2 into the culture medium, whereas culture of the cells with purified murine IL 1 caused no measureable IL 2 release. Furthermore, IL 1 neither augmented nor inhibited the release of IL 2 caused by TAF. These results show that TAF plus Con A stimulates production of IL 2 by highly purified T cells of the helper phenotype, whereas IL 1 plus Con A does not.

Induction of IL 2 mRNA by TAF. The stimulation of IL 2 release by TAF could be the result of transcriptional, translational, and/or post-translational events. To determine whether TAF influences the level of IL 2 mRNA in stimulated cells, we assayed total cellular RNA samples from stimulated or nonstimulated neuraminidase-treated Lyt-2$^-$ L3T4$^+$ T cells for IL 2 mRNA. mRNA levels were determined in a ribonuclease protection assay by using a ^{32}P-labeled RNA probe complementary to the fourth exon of IL 2 mRNA; the probe was derived by in vitro transcription of DNA derived from a murine genomic IL 2 clone that was subcloned in reverse orientation into the SP64 plasmid (19, 23). In a pilot experiment we found that IL 2 mRNA is undetectable in resting T cells, but becomes detectable by 2.6 h after stimulation with Con A plus T-depleted spleen cells (data not shown). The level of IL 2 mRNA increased additionally and appeared to plateau by 12 hr after stimulation. IL 2 mRNA was undetectable in T cells stimulated with Con A alone or with Con A plus IL 2, although the latter stimulation resulted in T cell proliferation (data not shown). Therefore, it appears that IL 2 plus Con A alone is not sufficient to induce accumulation of IL 2 mRNA during the first 12 hr of culture. However, as shown in Figure 11, stimulation of T cells for 12 hr with Con A plus TAF resulted in the accumulation of IL 2 mRNA in the cells, whereas stimulation with Con A plus IL 1 did not. As before (Fig. 10), IL 1 did not cause the release of measurable amounts of IL 2 above control levels, whereas TAF stimulated the release of a significant amount of IL 2 (data not shown). Taken together, the data show that TAF stimulates both T cell proliferation and accumulation of IL 2 mRNA, IL 2 stimulates T cell proliferation but not accumulation of IL 2 mRNA, and IL 1 stimulates neither T cell proliferation nor accumulation of IL 2 mRNA in highly purified T cell populations activated with Con A.

DISCUSSION

Our results describe an apparently novel lymphokine, which we call TAF. A preliminary description of this activity was reported previously by us (12). The relationship of TAF to an activity produced by a human T cell hybridoma (28) is unknown. Here we show that TAF can be chromatographically separated from molecules active in IL 1, IL 2, IL 3, CSF, and IFN bioassays. In addition, all sources of recombinant and/or natural IL 1, IL 3, CSF-

1, and IFN-γ that we have tested thus far have no activity in the TAF assay. Although we have not assayed for IL 4 (also called BSF-1) directly, the reported behavior of IL 4 on phenyl-Sepharose columns suggests that TAF and IL 4 are distinct molecules (IL 4 elutes in 20% ethylene glycol, before IL 2, whereas TAF elutes in 42% ethylene glycol, after IL 2) (29) (Figs. 3 and 7). A recent report has also shown that IL 4 is much less hydrophobic than IL 2 when analyzed by reversed-phase HPLC (30), whereas TAF is more hydrophobic than IL 2 under the same conditions (data not shown). Moreover, IL 4 (16,000 to 20,000 daltons) is smaller than TAF (30,000 to 35,000 daltons) as measured by gel filtration chromatography (31) or by the size predicted from the IL 4 cDNA sequence (32). Finally, we have recently shown that an excess of the anti-IL 4 monoclonal antibody 11B11 does not inhibit TAF, further demonstrating that TAF and IL 4 are distinct molecules (data not shown).

In the presence of Con A, TAF stimulates an increase in the steady-state level of IL 2 mRNA in T cells, the secretion of active IL 2 into the culture medium, and the proliferation of the T cells. Partially purified TAF does not stimulate the proliferation of resting T cells in the absence of Con A as a ligand to engage the T cell antigen receptor complex. Preliminary experiments have also demonstrated that Sepharose bead-bound anti-T cell receptor β chain antibody (F23.1) can be used to stimulate the proliferation of purified T cells in the presence of TAF, and T cell proliferation stimulated by Con A plus TAF can be blocked by the addition of anti-IL 2 receptor monoclonal antibodies to the TAF assay (data not shown). We therefore propose that TAF is a previously undescribed molecule the function of which is to stimulate IL 2 production by T cells that have encountered antigen. It seems likely that TAF plays an important role in primary T cell immune responses.

Thus far, we have found no requirement for exogenous TAF in the proliferative responses of cloned T cell lines, which presumably are representative of T cells in various stages of activation. In our hands, cloned helper T cell lines produce IL 2 in response to Con A alone and do not produce more IL 2 when TAF is added (data not shown). In this respect, we are in agreement with several other investigators, who find that many cloned T cells respond to Con A alone or to Sepharose bead-bound anti-T cell receptor antibodies alone (3, 5, 6). Cloned T cells, which have previously been activated, may differ from primary resting T cells in one or both of two relevant respects; such cells may no longer require TAF for IL 2 production, or alternatively, they may themselves produce sufficient TAF without accessory cells so that exogenous TAF is not required. However, whether there is a subtype of T cell clone that requires exogenous TAF remains to be determined.

TAF can be obtained by fractionation of conditioned medium from cultures of spleen cells activated with Con A, although the identity of the cells in the spleen that produce TAF cannot be determined from our experiments. However, TAF is produced by at least two T cell hybridomas (the FS7.20.7.1 line studied here, and also the AOFS21.10.9 line, data not shown), suggesting that it is a T cell product. Several non-T cell lines we have tested do not produce detectable TAF, including P388D1 (macrophage; data not shown), WEHI-3 (myelomonocytic leukemia), and L929 (fibroblast). Although we cannot yet be certain that TAF is produced only by T cells, it seems worth addressing models that can account for the observations reported here as well as other findings concerning T cell activation.

A prevalent model for the activation of resting T cells involves presentation of the antigen (or mitogen) and production of IL 1 by accessory cells, resulting in IL 2 production, IL 2 receptor expression, and subsequent proliferation of the T cells. This model was based in part on studies showing that crude preparations of IL 1 restored the ability of purified T cells to produce IL 2 in response to Con A (8, 9). More recent studies including those reported here have failed to demonstrate such an effect of IL 1 (11, 12) (T. Hunig, personal communication). Instead, we find that TAF stimulates IL 2 production in such cultures and restores the proliferative response of the T cells. Nevertheless, an additional requirement for IL 1 in such cultures cannot be ruled out, because it may be provided in vivo before cell purification in vitro by small numbers of contaminating macrophages or even by the T cells themselves (33).

Because T cells may be the sole producers of TAF, TAF may act in an autocrine manner to stimulate IL 2 production. Obviously it is of interest to determine in subsequent experiments the signals necessary for TAF production. These signals, which are absent from cultures of purified T cells, are presumably provided by "accessory cells". IL 1 (plus mitogen) cannot be a sufficient signal for TAF production, because if this were the case, T cells should respond to IL 1 plus Con A. As discussed earlier, all sources of IL 1 that we have tested thus far are inactive in the TAF assay.

Partially purified TAF is inactive in the IL 1 co-mitogenesis assay, a finding that is somewhat surprising, because the two assays appear to be so similar. It may be significant that the TAF assay uses a Con A concentration that is optimal for T cell proliferation, whereas the thymocyte co-mitogenesis assay depends upon a suboptimal concentration of PHA to prevent proliferation independent of exogenous factors. We have found that TAF activity in the TAF assay falls off rapidly with decreasing concentrations of Con A (data not shown), which may explain why TAF is inactive or only weakly active in the thymocyte co-mitogenesis assay. We have not ruled out the possibilities that the differences in the cell source (thymocyte or peripheral T cell) or mitogen (Con A or PHA) may contribute to the apparent functional difference between TAF and IL 1. However, it is noteworthy in this regard that lymph node T cells better represent those T cells active in immune responses than do thymocytes.

In contrast to our findings and those of others (10, 11), there are reports that IL 1 restores IL 2 production and proliferation of T cells (29, 34). The reasons for this discrepancy are not clear, but we suggest that IL 1 may augment T cell proliferative responses in the presence of suboptimal but significant accessory cell contamination. As an extension of this idea, IL 1 may play an important role in augmenting T cell immune responses.

Current efforts are being directed at purification of the TAF molecule and at developing strategies for the molecular cloning of the TAF gene. Such studies should provide important reagents for additional characterization of the role of TAF in T cell immune responses and for determin-

ing the relationship of the TAF molecule to the well-characterized cytokines.

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