TWO TYPES OF MOUSE T HELPER CELL

IV. Th2 Clones Secrete a Factor that Inhibits Cytokine Production by Th1 Clones

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Many long-term mouse Th cell clones can be divided into two types, based on the pattern of cytokines secreted in response to antigen or lectin stimulation. Th1 clones secrete IL-2, IFN-γ, and lymphotoxin (LT),[1] whereas Th2 clones express IL-4, IL-5, IL-6, and a gene of unknown function, P600 (1–4). Both types of clone secrete IL-3, granulocyte-macrophage CSF (GM-CSF), TNF-α, preproenkephalin, and several other induction-specific proteins. The differences between Th1 and Th2 cytokine synthesis patterns lead to markedly different functions (5–8). Th2 clones are normally better B cell helpers (8, 9), while Th1 clones, although they can provide B cell help in some circumstances (7, 10, 11), preferentially induce macrophage activation (12) and delayed-type hypersensitivity (DTH) (6). Recent evidence (Street, N. E., J. H. Schumacher, T. A. T. Fong, H. Bass, D. F. Fiorentino, J. A. Leverah, and T. R. Mosmann, manuscript in preparation) (13–15) suggests that other differentiation states of mouse Th cells exist, and that the Th1 and Th2 phenotypes represent activated effector cells.

The existence of these two major types of Th cell may explain the well-documented separation of immune responses into DTH or antibody production, which often appear to be mutually exclusive (16). If B cell help and DTH are predominantly mediated by Th2 and Th1 cells, respectively, this suggests that Th1 and Th2 cells may be mutually inhibitory (17, 18). The Th1 product IFN-γ inhibits proliferation of Th2 clones in vitro (19, 20), while an unknown product of a Th2 clone may inhibit the proliferation of Th1 clones (21). In addition to crossinhibition of proliferation, we considered the possibility that inhibitors of Th1 and Th2 effector function also exist. Here, we report the preliminary characterization of a cytokine produced by Th2 clones that inhibits the synthesis of several cytokines by Th1 clones. This cytokine, which we have called cytokine synthesis inhibitory factor (CSIF), has been distinguished from the other known Th2 cytokines.

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[1] *Abbreviations used in this paper:* CSIF, cytokine synthesis inhibitory factor; DTH, delayed-type hypersensitivity; GM-CSF, granulocyte-macrophage CSF; ppCSIF, partially purified CSIF; TGF-β, transforming growth factor β.

Materials and Methods

Mice. BALB/c, C57BL/6, and CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and the Institute of Medical Research (San Jose, CA). Female mice 8–12 wk old were used in all experiments.

Cell Lines. Th1 clones included: HDK-1, BALB/c anti-KLH (2); MD13-2, BALB/c anti-CRBC (1); MD13-10, BALB/c anti-CRBC (1); GK15-1, CBA/J anti-CRBC (11); LB2-1, C57BL/6 anti-CRBC (1); ML3-A-C1-2-Ba, C57BL/6 anti-BALB/c; and ML3-A-C2-6-Nb, C57BL/6 anti-BALB/c. Th2 clones included: D10.G4.1 (D10), AKR/J anti-conalbumin, obtained from C. Janeway (Yale University, New Haven, CT) (22); CDC25 and CDC35, C3D2 anti-rabbit IgG, obtained from D. Parker (University of Massachusetts, Worcester, MA) (23); D9, C57BL/6, obtained from G. Nabel (Harvard University, Boston, MA) (24); MB2-1, C57BL/6 (1); and M411-2 and M411-6, BALB/c anti-CBA/J. The maintenance and stimulation of antigen-specific T cell lines have been described previously (1, 2). The mouse T cell line HT2 (25) was obtained from S. Strober (Stanford University, Stanford, CA) and the WEHI 164.13 line (26) from M. Palladino (Genentech, South San Francisco, CA).

Media. Assay medium consisted of RPMI 1640 (J. R. Scientific Inc., Woodland, CA) with 10% FCS (J. R. Scientific Inc.), 0.05 mM 2-ME (Sigma Chemical Co., St. Louis, MO), and 20 mM Hepes (Gibco Laboratories, Grand Island, NY). T cell growth medium consisted of assay medium containing 330 U/ml mouse rIL-2.

Antibodies. mAbs against IL-2 (S4B6), IL-5 (TRFK4 and TRFK5), and IFN-γ (XMG1.2) have been described previously (1, 2, 27). The anti-IL-3 mAbs 8F8.11 and 43D.11 (28), as well as the anti-GM-CSF antibodies 22E9.11 and 35E10.11, were obtained from J. S. Abrams (DNAX), the anti-IL-4 antibody 11B11 (29) was from W. E. Paul (National Institutes of Health, Bethesda, MD), and the 6B4 anti-IL-6 antibody (30) from J. van Snick (Ludwig Cancer Institute, Brussels, Belgium). The hamster anti-mouse T3 mAb 145-2C11 (31) was obtained from J. Bluestone (N. I. H.). Polyclonal rabbit IgG antibodies specific for transforming growth factor β (TGF-β) were obtained from R & D Systems (Minneapolis, MN), and control rabbit IgG antibodies were obtained from R. Coffman (DNAX).

T Cell Supernatants. Antigen-induced T cell supernatants were prepared by incubating T cell clones (2×10^5 cells/ml) with irradiated APC (5×10^6 cells/ml) and antigen in assay medium. Serum-free Con A–induced T cell supernatants were prepared by incubating T cell clones (5×10^6 cells/ml) with Con A (5 μg/ml; Sigma Chemical Co.) in RPMI 1640 lacking phenol red (Gibco Laboratories) and containing 0.05 mM 2-ME and 20 mM Hepes for 24 h.

Cytokines. TGF-β was obtained from R & D Systems. Purified mouse P40 (32) was generously provided by J. van Snick (Ludwig Cancer Institute). Purified mouse IL-5 (33) was depleted of known Th2 cytokines and provided by R. Coffman, DNAX. Purified recombinant mouse cytokines were provided by: M. Howard, DNAX (IL-4); R. Kastelein, DNAX (IL-4); F. Lee, DNAX (IL-6 and IL-7); J. Schreurs, DNAX (IL-3 and GM-CSF); G. Zurawski, DNAX (human IL-1α); Schering Research, Bloomfield, NJ (IL-2 and IFN-γ); and M. Palladino, Genentech (TNF-α).

Cytokine Bioassays. The MTT assay (34), as modified (1), was used to measure IL-2, IL-4, and LT/TNF-α using mAbs to establish monospecificity for IL-2 and IL-4 assays (2, 26). LT/TNF-α was assayed on WEHI 164.13 cells essentially as described by Espevik and Nissen-Meyer (26), except that 5×10^3 target cells/well were used, and the assay was carried out in the presence of saturating (>2 ng/ml) amounts of IFN-γ. Units of activity were calculated as described previously (1).

Cytokine ELISAs. Two-site sandwich ELISAs for IL-3, IL-5, and IFN-γ were carried out as described previously (2, 27, 28). The ELISA for GM-CSF was carried out essentially as described for the IL-5 assay, using the mAbs 35E10 and 22E9. Intracellular IFN-γ levels were measured after lysing cells in 1% Triton X-100 (Sigma Chemical Co.) and removing nuclei by centrifugation.

Column Chromatography. 1–2.5-liter batches of serum-free Con A–induced D10 supernatant were concentrated ~10-fold using YM-5 membranes (Amicon Corp., Danvers, MA), passed through a 5-ml mannose-conjugated agarose column (E-Y Laboratories, San Mateo,

CA), then further concentrated another three- to fivefold, for a total concentration of 30–50-fold. This material was fractionated by HPLC on a hydroxylapatite-based support (Bio-Gel HPHT; Bio-Rad Laboratories, Richmond, CA) and on a gel filtration column (TSK-G 3000 SW, 60-cm length; LKB Instruments, Gaithersburg, MD) as previously described (33). After sequential chromatography on these two media, the fraction with CSIF activity is referred to as partially purified CSIF (ppCSIF).

Antigens. Keyhole limpet hemocyanin was obtained from Pacific Bio-Marine Laboratories, Inc. (Venice, CA) and used at a final concentration of 150 μg/ml. TNP-KLH (132 TNP residues per 10^6 daltons protein) was used at 1 μg/ml (35). Conalbumin (Sigma Chemical Co.) was used at 100 μg/ml. Chicken red blood cells were obtained from PMK farms (Vacaville, CA) and used at 0.02% packed volume.

Anticytokine Affinity Columns. Six affinity columns were prepared by coupling the mAbs XMG1.2 anti-IFN-γ, 22E9.11 anti-GM-CSF, S4B6 anti-IL-2, 8F8.11 anti-IL-3, 11B11 anti-IL-4, and TRFK5 anti-IL-5 to Affi-Gel 10 (Bio-Rad Laboratories). Each 1–2-ml column contained ~10–20 mg of coupled antibody.

CSIF Assay. Samples were diluted in 96-well flat-bottomed microtiter trays (Falcon Labware, Oxnard, CA) in a volume of 0.05 ml. Th1 cells (5×10^4 cells/well), irradiated (2,500 rad) syngeneic spleen cells (5×10^5 cells/well), and antigen were added in a volume of 0.15 ml. 11B11 anti-IL-4 mAb (10 μg/ml) was added to the assay if samples were suspected of containing IL-4. Levels of IFN-γ and other cytokines in the 24-h supernatants were assayed. Omission of either antigen or APC resulted in negligible cytokine synthesis. When initially assayed, one batch of ppCSIF inhibited IFN-γ production by ~50% at a dilution of 1:200 in an assay volume of 0.2 ml, and so the standard unit (SU) was defined by assigning a value of 1,000 SU/ml to this CSIF preparation. CSIF activity in unknown samples was quantitated by comparing levels of inhibition of IFN-γ synthesis by the unknown with that of the standard in each assay using a four-parameter curve-fitting program (Assay Zap; Biosoft, Cambridge, UK). Means ± SD of triplicate cultures were determined.

mRNA Measurement. Th1 cells were stimulated with antigen plus APC that had been depleted of erythrocytes by centrifugation over Ficoll (Histopaque; Sigma Chemical Co.). At 8 and 12 h, aliquots of the cells were withdrawn and cytoplasmic RNA was isolated (36). Dilutions of RNA were bound to filters and hybridized with synthetic DNA probes that were ^{32}P-labeled by fill-in synthesis of overlapping DNA oligonucleotides as described previously (2). The DNA probes corresponded to bases 900–989 for IFN-γ (2) and 1262–1363 for γ-actin (37). Filters were exposed to x-ray film and the autoradiographs quantitated by laser densitometry.

Results

Supernatants from Th2 but not Th1 Clones Inhibit Synthesis of IFN-γ by Th1 Clones. Con A–induced supernatants from seven Th2 clones all inhibited IFN-γ production by HDK-1 cells (Fig. 1 *A*). This inhibitory activity was produced by D10 cells in response to Con A, antigen plus APC, or anti-T3 plus APC (Fig. 1 *B*). The inhibitory activity appeared to be produced only by stimulated cells, since Con A–stimulated CDC25 and CDC35 cells produced 866 and 708 U/ml of inhibitory activity, respectively, whereas unstimulated cells produced <40 U/ml. We will call the factor responsible for this activity CSIF.

The decrease in IFN-γ production could be due to a decrease in synthesis and/or secretion. If a block in secretion was solely responsible for the reduced IFN-γ supernatant levels, then the intracellular levels of IFN-γ should be greatly elevated in the presence of CSIF. When HDK-1 cells were stimulated with antigen plus APC, the maximum extracellular IFN-γ levels at 24 h were 250 and 1,100 ng/ml in the presence and absence of CSIF, respectively. The corresponding maximum intracellular levels were 1.0 and 4.5 ng/ml.

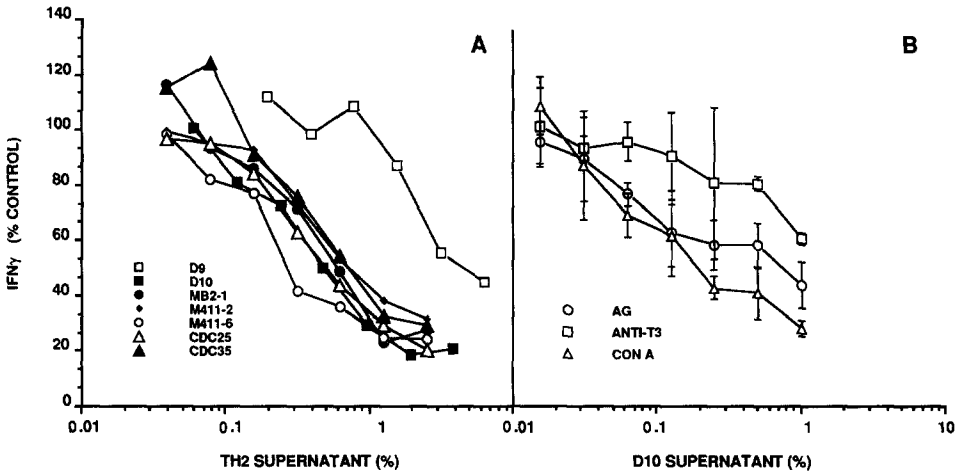

FIGURE 1. Th2 clones produce an inhibitor of IFN-γ synthesis by Th1 cells. HDK-1 cells were stimulated with antigen plus APC in the presence of supernatants from seven Con A-induced Th2 clones (*A*) or from D10 cells induced with Con A, Ag plus APC, or anti-T3 mAb (4.5 μg/ml) plus APC (*B*). IFN-γ secretion is expressed as a percentage of the levels produced in the absence of the Th2-derived inhibitor.

Four Con A-induced Th1 supernatants were depleted of IFN-γ and IL-2 (which would interfere with the CSIF assay) by passage over monoclonal anticytokine affinity columns, and then tested for CSIF activity. These Th1 supernatants, even at a dilution of 1:10, did not inhibit IFN-γ production (Fig. 2 *A*). The slightly increased IFN-γ levels in cultures treated with the highest concentration of M264-37 superna-

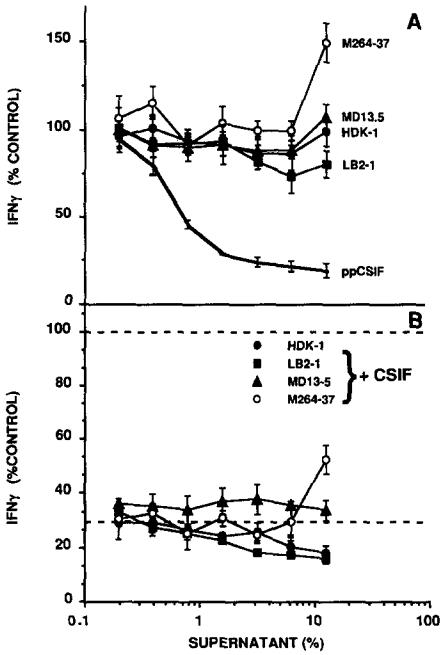

FIGURE 2. Th1 clones do not secrete CSIF. Supernatants from four Con A-induced Th1 clones were depleted of IL-2, IL-3, IFN-γ, and GM-CSF, and tested for CSIF activity using HDK-1 cells (*A*). Inhibition by ppCSIF is shown for comparison. The same samples were also tested in the presence of 14 U/ml ppCSIF (*B*). The upper and lower dashed lines represent the levels of IFN-γ in the absence and presence of CSIF, respectively. All values are expressed as a percentage of the IFN-γ levels produced in the absence of both ppCSIF and Th1 supernatants.

tant could be accounted for by the residual IFN-γ in this sample. Thus, Th1 supernatants either do not contain CSIF, or they contain both CSIF and an inhibitor that masks its activity. This second possibility is ruled out by the demonstration that IL-2- and IFN-γ-depleted Th1 supernatants did not counteract CSIF activity (Fig. 2 *B*). Taken together, these experiments demonstrate that Th2 but not Th1 supernatants contain CSIF.

CSIF Inhibits Production of Several Th1 Cytokines. Since ppCSIF still contained GM-CSF and IL-3, the levels of these cytokines were further reduced by using anticytokine affinity columns before testing for CSIF's ability to inhibit the synthesis of several other Th1 cytokines. Production of IFN-γ was consistently inhibited in all Th1 clones tested (e.g., Table I). The synthesis of IL-2, LT/TNF, IL-3, and GM-CSF was inhibited in some cases but not others; e.g., IL-2 production was inhibited in HDK-1 cells but not in MD13-10 cells. When inhibition was observed, the extent of maximum inhibition varied between different clones and cytokines, ranging from ~60 to >95%. Even for a single clone, different cytokines were maximally inhibited at different concentrations of CSIF. In particular, the synthesis of LT/TNF was inhibited by relatively low concentrations of CSIF, but the extent of maximal inhibition was much less than that of IFN-γ.

CSIF Inhibits Late but not Early Cytokine Synthesis. The kinetics of inhibition of cytokine synthesis were examined for antigen-stimulated HDK-1 cells (Fig. 3). In general, the synthesis of all cytokines was not inhibited by CSIF at early times, e.g., before 8.5 h (before 6.5 h for IL-2). Two cytokines synthesized mainly at early times (GM-CSF and LT/TNF) were inhibited slightly or not at all, whereas the synthesis of cytokines produced mainly at later times (IFN-γ and IL-3) was inhibited substantially. Differences in the exact kinetics of CSIF action and cytokine synthesis could thus account for some of the variability of inhibition seen in Table I.

Inhibition of IFN-γ Synthesis Occurs at the mRNA Level. HDK-1 cells were stimulated with antigen plus APC in the presence or absence of ppCSIF, and total cytoplasmic RNA was prepared from cells at 8 and 12 h after stimulation. RNA samples were tested for IFN-γ and γ-actin mRNA levels by dot-blot hybridization. IFN-γ

TABLE I

Effect of CSIF on the Synthesis of Th1 Cytokines

Cell line	Cytokine	Percent of control synthesis level		
		14 U/ml CSIF	42 U/ml CSIF	125 U/ml CSIF
HDK-1	IFN-γ	47.6 ± 0.8	29.1 ± 1.0	18.6 ± 0.7
	IL-2	71.7 ± 2.3	59.6 ± 3.9	40.4 ± 6.1
	LT/TNF	41.9 ± 1.4	45.1 ± 2.3	42.8 ± 1.1
	IL-3	63.9 ± 11.4	52.6 ± 6.8	38.4 ± 1.9
	GM-CSF	86.9 ± 8.5	79.1 ± 7.8	66.8 ± 9.3
MD13-10	IFN-γ	36.0 ± 4.9	27.5 ± 3.4	23.2 ± 2.6
	IL-2	88.2 ± 5.1	109.3 ± 13.3	96.0 ± 16.4
	IL-3	60.2 ± 5.1	63.0 ± 2.7	51.0 ± 6.5
	GM-CSF	109.9 ± 13.0	119.9 ± 7.4	97.6 ± 19.2

HDK-1 and MD13-10 cells were stimulated with antigen plus APC in the presence of varying dilutions of ppCSIF that had been depleted of GM-CSF and IL-3. Cytokine levels in the 24-h supernatants are expressed as a percentage of the amount produced in the absence of CSIF.

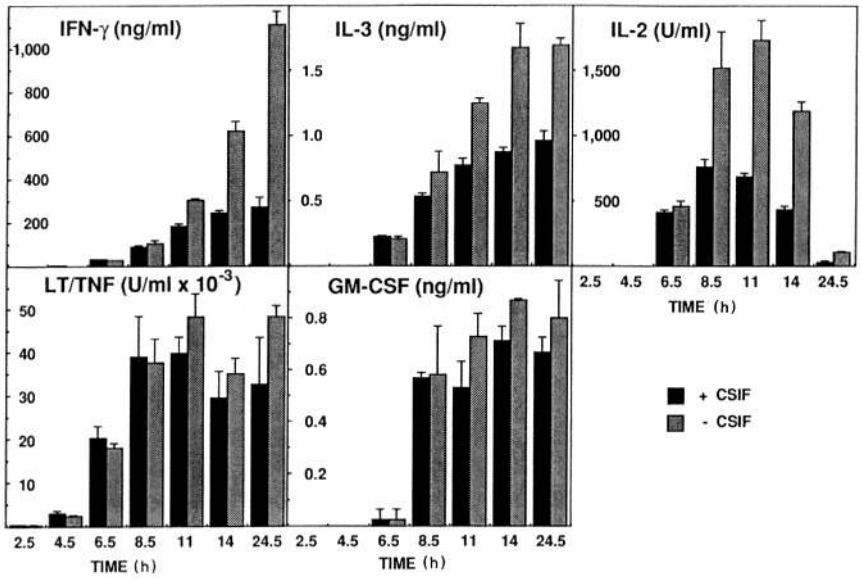

FIGURE 3. Kinetics of inhibition of cytokine synthesis by CSIF. HDK-1 cells were stimulated with antigen plus APC in the presence or absence of 100 U/ml ppCSIF that had been depleted of IL-3, IL-4, IL-5, and GM-CSF. Supernatants were collected at the indicated times and assayed for cytokine levels.

mRNA levels were moderately reduced by CSIF at 8 h, and strongly reduced at 12 h over a range of 5–40 U/ml CSIF (Fig. 4). In contrast, γ-actin mRNA levels were not significantly reduced by CSIF at either 8 or 12 h, and possibly showed a slight enhancement at 12 h. The inhibition of IFN-γ protein levels in the 24-h supernatants by CSIF was slightly less than the inhibition of mRNA levels at 12 h.

Th1 but not Th2 Clones Respond to CSIF. ppCSIF was tested on seven Th1 clones that were derived from three mouse strains and were specific for particulate antigens,

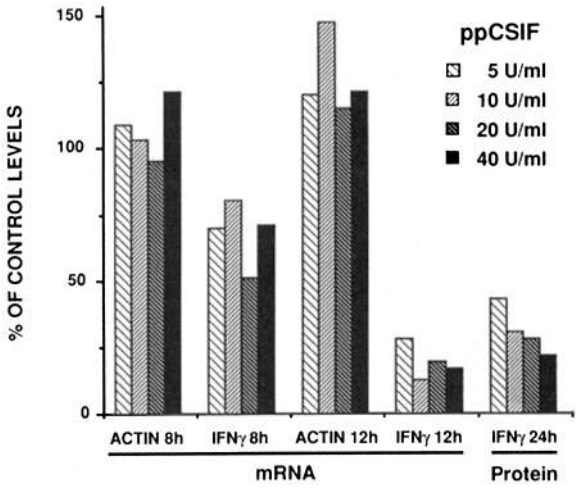

FIGURE 4. CSIF reduces mRNA levels for IFN-γ but not actin. HDK-1 cells were stimulated with antigen plus APC in the presence or absence of ppCSIF. Cells were harvested at 8 and 12 h for cytoplasmic mRNA isolation, and supernatants were taken at 24 h for IFN-γ protein determination. mRNA levels were assessed by hybridization with synthetic DNA probes, and protein was measured by ELISA. All values are expressed as a percentage of levels produced in the absence of CSIF.

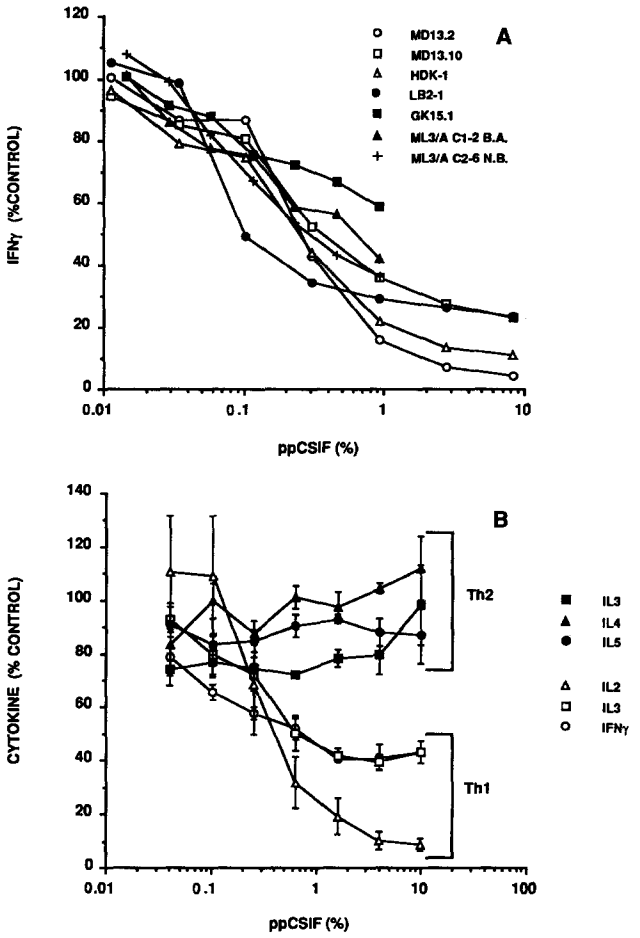

FIGURE 5. Th1 but not Th2 clones respond to CSIF. Seven Th1 clones were stimulated with antigen plus APC in the presence of dilutions of ppCSIF, and IFN-γ secretion was measured (*A*). HDK-1 (Th1) and D10 (Th2) cells were stimulated with antigen plus APC in the presence of dilutions of ppCSIF that had been depleted of IL-3, IL-4, IL-5, and GM-CSF (*B*). Levels of IL-2, IL-3, and IFN-γ (HDK-1) or IL-3, IL-4, and IL-5 (D10) were assayed in the 24-h supernatants. All values are expressed as a percentage of the cytokine levels produced in the absence of ppCSIF. .

soluble proteins, or alloantigens. IFN-γ synthesis by all seven clones was inhibited by ppCSIF (Fig. 5 *A*). Since some of the Th2 cytokines may have been present in the ppCSIF preparation, IL-3, IL-4, and IL-5 were depleted by passage over mAb affinity columns. The resulting CSIF preparation did not suppress the production of IL-3, IL-4, or IL-5 by the Th2 clone D10 (Fig. 5 *B*). Two additional experiments with D10 yielded similar results, and in other experiments (not shown), CSIF did not inhibit the production of IL-5 by the Th2 clones MB2-1, CDC25, or CDC35, nor did it inhibit IL-4 synthesis by MB2-1. Synthesis of other cytokines in response to antigen plus APC in these experiments was too low to reliably determine CSIF effects. In general, we have not seen convincing inhibition of the production of any cytokine by Th2 cells responding to antigen plus APCs.

CSIF Action on Th1 Cells May Be Indirect. CSIF inhibited IFN-γ synthesis in response to antigen plus APC or anti-T3 plus APC, but not IFN-γ synthesis stimulated by anti-T3 bound to a polystyrene surface, or by Con A (Table II). Since only the stimulations involving APC were inhibitable, CSIF may act indirectly on the

TABLE II

CSIF Is Effective in Stimulations Involving APC

Exp.	Stimulation	Percent of control IFN-γ synthesis			
		5 U/ml CSIF	19 U/ml CSIF	74 U/ml CSIF	294 U/ml CSIF
1	Antigen plus spleen	72.6 ± 12.8	29.1 ± 3.5	20.5 ± 6.6	
	Anti-T3 plus spleen	88.7 ± 8.0	78.6 ± 2.8	68.5 ± 2.6	
	Anti-T3 (coated)	95.7 ± 6.7	103.3 ± 6.7	94.5 ± 9.3	
	Con A	102.7 ± 4.2	95.4 ± 5.3	94.7 ± 5.0	
2	Antigen plus spleen		17.5 ± 5.9	12.8 ± 1.6	8.2 ± 1.8
	Anti-T3 plus spleen		54.5 ± 3.1	45.6 ± 6.0	42.0 ± 7.2

HDK-1 cells were stimulated with antigen plus APC, anti-T3 mAb (9 μg/ml) plus APC, anti-T3 bound to 96-well flat-bottomed trays, or Con A (5 μg/ml), each in the presence or absence of CSIF-containing D10 supernatant. Trays were coated with anti-T3 by incubating with 50 μg/ml anti-T3 mAb in 50 μl PBS for 1 h at 37°C, following which, the plates were extensively washed. IFN-γ levels in 24-h supernatants are expressed as a percentage of the amounts produced in the absence of CSIF, using the corresponding stimulation condition.

T cells, via a cell type in the APC population. CSIF acts mainly at later times (Fig. 3), consistent with the hypothesis that CSIF is first modified into, or induces a secondary factor that then inhibits IFN-γ synthesis. Additional evidence for indirect action was obtained by varying the CSIF assay conditions. 10-fold changes in T cell and/or APC numbers did not affect the extent of inhibition of IFN-γ synthesis by CSIF or the sensitivity of detection of CSIF (data not shown). In contrast, saturating amounts of CSIF inhibited IFN-γ synthesis more effectively (to 16% instead of 35% of control IFN-γ amounts) if the assay volume were reduced twofold. If the residual IFN-γ levels represent the cytokine secreted at early times before CSIF action, these results are consistent with the inhibition of IFN-γ synthesis being dependent on a soluble factor produced in the assay. In this case, such a factor would accumulate to effective concentrations more rapidly in a smaller assay volume.

CSIF Does not Directly Inhibit Th1 Proliferation. In contrast to its effect on Th1 cytokine synthesis, CSIF had no measurable effect on the ability of Th1 cells to proliferate in response to IL-2. In the presence of exogenous IL-2 (500 ng/ml), antigen-stimulated HDK-1 cells proliferated 5.2-fold by day 3, and 42.6-fold by day 6, as assessed by cell counting. The corresponding proliferation values in the presence of IL-2 plus 100 U/ml ppCSIF were 5.0-fold and 40.0-fold. Similar results (not shown) were obtained using the MTT assay. However, in the absence of exogenous IL-2, ppCSIF was sometimes able to decrease proliferation of HDK-1 cells when added at the time of antigen stimulation (results not shown); presumably, this inhibition was indirect and was a result of CSIF's ability to reduce IL-2 production by HDK-1 cells (Table I, Fig. 3).

Other Cytokines that Affect IFN-γ Synthesis. In some but not all experiments, exogenous IL-4 and especially IL-2 increased the production of IFN-γ by Th1 clones (data not shown). Since IL-2 was synthesized in variable amounts by stimulated Th1 cells, exogenous IL-2 and IL-4 might only be effective in experiments in which nonsaturating amounts of endogenous IL-2 were synthesized. In the experiment shown in Fig. 6 *A*, anti-IL-2 antibody inhibited the production of IFN-γ, indicating that normal IFN-γ synthesis was enhanced by IL-2 produced by the Th1 cells responding to an-

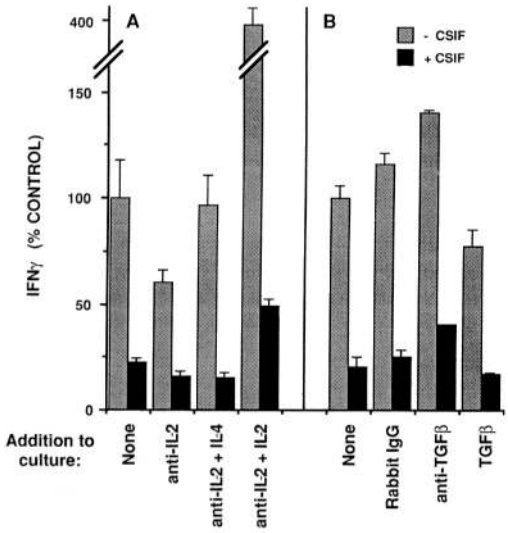

FIGURE 6. Effects of IL-2, IL-4, and TGF-β on IFN-γ synthesis. HDK-1 cells were stimulated with antigen plus APC with various combinations of cytokines and antibodies: IL-2 (500 ng/ml), IL-4 (50 ng/ml), TGF-β (10 ng/ml), ppCSIF (125 U/ml), S4B6 anti-IL-2 (10 μg/ml), rabbit anti-TGF-β (50 μg/ml), and normal rabbit IgG (50 μg/ml) (*A* and *B*). Titrations of these reagents showed that these levels were saturating. All cultures contained 11B11 anti-IL-4 (10 μg/ml), except those in which IL-2 or IL-4 was added (*A*). All values are expressed as a percentage of the IFN-γ levels produced in the absence of ppCSIF, antibodies, and cytokines.

tigen plus APC. When anti-IL-2 antibody was present, IL-4 enhanced IFN-γ production slightly, and excess IL-2 (overcoming the effect of the anti-IL-2 antibody) could strongly enhance IFN-γ levels. Although both IL-2 and IL-4 antagonized the effect of CSIF by increasing the amount of IFN-γ secreted, CSIF was still active in the presence or absence of saturating amounts of IL-2 or IL-4 (Fig. 6 *A*).

Partially purified anti-TGF-β antibodies slightly enhanced the synthesis of IFN-γ, whereas exogenous TGF-β slightly inhibited IFN-γ production (Fig. 6 *B*). These results suggest that IFN-γ synthesis can be inhibited by TGF-β, and that TGF-β is produced in the assay under normal conditions. CSIF inhibited IFN-γ production in the presence or absence of TGF-β or anti-TGF-β, showing that CSIF is not TGF-β.

Partial Purification and Biochemical Characterization of CSIF. CSIF-containing D10 supernatants were chromatographed on two media. On both columns, CSIF activity eluted in a single area. On the hydroxylapatite-based support, 95% of the applied activity was recovered; also shown are the elution positions of other proteins (Fig. 7 *A*). On the gel filtration column, 63% of the applied activity eluted broadly, with the fractions of high activity corresponding to apparent molecular masses of 27–50 kD (Fig. 7 *B*). The width of the peak may be the result of microheterogeneity similar to that observed with other cytokines (e.g., IL-5; [33]). CSIF could be sequentially chromatographed on both columns in either order with no significant alteration of relative mobility or activity yield (data not shown). These results suggest that CSIF may be a single entity, and we have not yet observed any evidence for directly synergistic interactions of CSIF with any other cytokine. ppCSIF (2,100 U in 0.25 ml 10 mM phosphate buffer, pH 7.4) was carefully acidified to pH 2 with HCl, allowed to stand for 1 h at room temperature, neutralized with 1 M Hepes (sodium salt), and immediately assayed; <5% of the activity remained.

CSIF Is Distinct from Known Th1 and Th2 Cytokines. Various blocking anticytokine monoclonal and polyclonal antibodies were tested for the ability to inhibit CSIF ac-

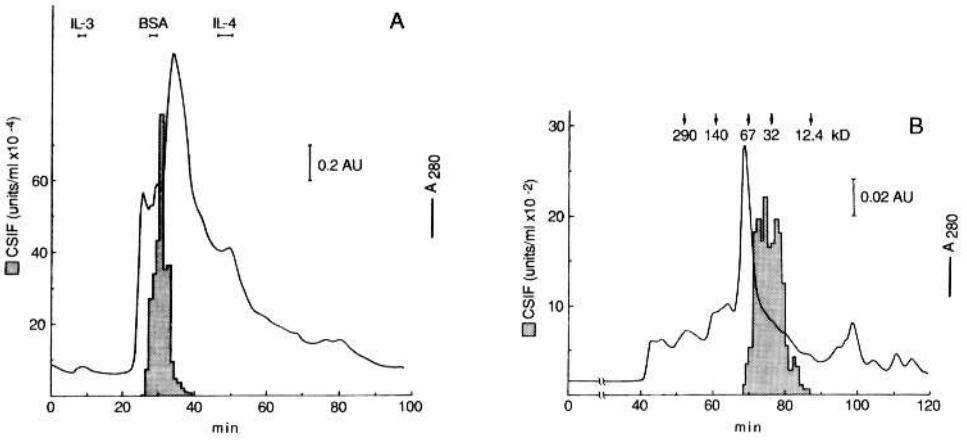

FIGURE 7. Column chromatography of CSIF-containing D10 supernatant. 10 ml of 27-fold concentrated D10 supernatant applied to a Bio-Gel HPHT column in 10 mM sodium phosphate, pH 7.4, 0.12 mM $CaCl_2$, 0.01% Tween-20 at a flow rate of 0.5 ml/min was eluted with a 60-min gradient to 0.35 M sodium phosphate, pH 7.4, 3.5 μM $CaCl_2$, 0.01% Tween-20. The elution positions of other proteins are indicated by horizontal bars (*A*). Gel filtration of 0.2-ml 30-fold concentrated D10 supernatant on TSK-G 3000 SW was performed as previously described (33) (*B*). The elution positions of proteins of known molecular weight are marked by the arrows. In both cases the absorbance at 280 nm was monitored (*curved line*) and aliquots from the 1-min fractions were assayed for CSIF activity (*shaded areas*).

tivity. CSIF activity was not reduced by mAbs specific for IL-2, IL-3, IL-4, and IL-6 (10 μg/ml), IL-5 (2 μg/ml), and GM-CSF (20 μg/ml), or by polyclonal anti-TGF-β antibodies (50 μg/ml). Several cytokines were also tested in the CSIF assay and found to be inactive, either as agonists or antagonists. The synthesis of IFN-γ was enhanced or inhibited by <11% by IL-1 (10^7 U/ml), IL-5 (2.4 ng/ml), IL-6 (25 ng/ml), or IL-7 (500 U/ml). IFN-γ synthesis was enhanced by IL-2 and IL-4 (Fig. 6), slightly enhanced by P40 (results not shown), and inhibited by TGF-β (Fig. 6).

Experiments using recombinant cytokines and mAbs, as well as knowledge of its biochemical properties and presence in Th2 but not Th1 supernatants, distinguish CSIF from many of the known cytokines (Table III). In general, we required at least two nonidentical properties before each cytokine was considered distinct from CSIF. According to these criteria, CSIF is not IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, IFN-γ, LT, TNF, TGF-β, P40, or met-enkephalin, and is unlikely to be soluble immune response suppressor (SIRS) (38).

Discussion

Several lines of evidence show that antibody and DTH responses are often mutually exclusive (16–18, 39). If these responses are mainly due to Th2 and Th1 cells, respectively, this suggests that Th1 and Th2 cells reciprocally inhibit the growth and function of the other cell type. Some of the known mechanisms include the inhibition of growth of Th2 clones by IFN-γ (19, 20), the inhibition of all IL-4 effects on B cells by IFN-γ (40), and the inhibition of Th1 proliferation by a Th2-derived factor (21). Although the activity in the last study was identified as an autocrine

TABLE III

Evidence for Nonidentity of CSIF with Known Cytokines

Cytokine	Present in Th2 sup	Absent in Th1 sup	Ab blocks CSIF activity	Cytokine has CSIF activity	Biochemically similar to CSIF
IL-1	No	Yes	-	No	No: separates by size
IL-2	No	No	No	No	-
IL-3	Yes	No	No	-	No: separates on HPHT
IL-4	Yes	Yes	No	No	No: separates on HPHT, size
IL-5	Yes	Yes	No	No	-
IL-6	Yes	Yes	No	No	No: separates by size
IL-7	-	-	-	No	No: not acid labile
GM-CSF	Yes	No	No	-	No: not acid labile
IFN	No	No	-	-	-
LT	No	No	-	-	-
TNF	Yes	No	-	-	No: not acid labile
TGF-β	-	-	No	Yes (partial)	No: not acid labile
P40	Yes	-	-	No	No: not acid labile
Met-enk	Yes	No	-	-	No: separates by size
SIRS	-	-	-	-	No: separates by size

growth inhibitor for Th cells, the results need to be reinterpreted in light of recent information (1, 3, 5). The Th2-derived growth inhibitory activity inhibited the growth of a Th1 clone ~1,000-fold more effectively than D10 growth (21), but it appears to be distinct from CSIF since the two activities have different apparent molecular masses, and ppCSIF does not inhibit proliferation of Th1 clones.

In addition to IFN-γ and the D10-derived proliferation inhibitor, the CSIF identified in this study could be another of the crossinhibitory regulators of Th1 and Th2 cells. Th1 function should be inhibited by CSIF, since IFN-γ is an important mediator of several Th1 functions (7, 12, 41). There may also be an indirect enhancement of Th2 growth, since the decrease in IFN-γ levels should reduce inhibitory effects on Th2 growth (19, 20). CSIF inhibition of IL-2 but not IL-4 synthesis may also slightly reduce the relative growth rates of Th1 cells, since Th2 cells respond better than Th1 cells to the proliferative effects of IL-4 (3). We have suggested previously (3) that these crossinhibitory cytokine effects may be important early in immune responses, whereas the production of specific Ig isotypes may counterbalance this effect later in the response.

CSIF could also have a more subtle role in the regulation of immune responses. In vitro, Th1 clones are often very poor B cell helpers (8, 9), unless the cytokine levels are adjusted by decreasing IFN-γ and increasing IL-2 levels (7). Since CSIF inhibits IFN-γ production, often more effectively than IL-2 production, and freshly isolated Th1 clones tend to produce more IL-2 than long-term Th1 clones (2), CSIF may modify the functions of Th1 cells so that they become more effective B cell helpers. If true, this effect would only be seen in responses in which at least some Th2 cells were activated. This may explain the apparently contradictory observations that Th1 cells can be good B cell helpers under some circumstances, but that a strong DTH response (presumably due to strong Th1 activation) is often unaccompanied by significant antibody synthesis.

CSIF appears to selectively inhibit Th1 cytokine synthesis, rather than generally

inhibit cell growth or viability. CSIF treatment did not reduce the levels of γ-actin mRNA, nor did it inhibit proliferation in response to exogenous IL-2. Although IL-2 enhances IFN-γ production (42, and Fig. 6), and CSIF often inhibits IL-2 production, the results in Fig. 6 also show that CSIF exerts its effect in either the presence or absence of IL-2. This indicates that CSIF does not inhibit IFN-γ synthesis simply by inhibiting IL-2 production. Although TGF-β also inhibits IFN-γ synthesis (43, and Fig. 6), CSIF was able to inhibit IFN-γ synthesis more effectively than saturating amounts of TGF-β. This indicates that CSIF does not act only by inducing TGF-β.

The action of CSIF on Th1 cytokine synthesis may be indirect, since (*a*) CSIF inhibits IFN-γ synthesis in response to antigen plus APC but not Con A; (*b*) IFN-γ synthesis is inhibited only at later times after stimulation; and (*c*) the volume of the assay is critically important to the degree of inhibition obtained. Two possible mechanisms are the induction by CSIF of an effector molecule synthesized by a cell in the APC population, or the modification of CSIF to an active form by the APC population. Preliminary experiments (unpublished data) show that APC pretreated with CSIF are only marginally reduced in their ability to subsequently induce Th1 cells to produce IFN-γ. This indicates that, if CSF acts primarily on the APC population, the effect is mostly reversible, or requires the presence of T cells.

Analysis of IFN-γ mRNA levels showed that the effect of CSIF is mediated at the mRNA level, in agreement with intracellular protein measurements. Possible mechanisms include reduction of transcription, accelerated degradation, or altered mRNA processing. IFN-γ mRNA levels were slightly reduced at 8 h, and strongly reduced by 12 h, consistent with IFN-γ secretion data showing that CSIF inhibition is detected only at late times. The delayed inhibition of IFN-γ production may also explain why saturating CSIF never completely inhibits cytokine synthesis; the residual cytokine levels may represent the amounts of cytokines synthesized at early times after stimulation.

Although the Th1 and Th2 phenotypes are displayed by the majority of long-term mouse Th clones, other cytokine secretion patterns exist among normal Th cells and short-term clones (2, 13, 20, and Street, N. E., J. H. Schumacher, T. A. T. Fong, H. Bass, D. F. Fiorentino, J. A. Leverah, and T. R. Mosmann, manuscript submitted for publication). These other phenotypes may be precursors of Th1 and Th2 cells, and the details of the functions of such precursors are not yet known. It will be important to assess CSIF production and effects in these other Th types, particularly in cells secreting both Th1 and Th2 cytokines.

The interpretation of the role of CSIF is complicated by the fact that Th2 cells also produce IL-4, which partially counteracts the effects of CSIF. This situation is analogous to the production of IL-2 and IFN-γ by Th1 cells, since these two cytokines have opposing effects on Th2 cells (19, 20). It is possible that these pairs of cytokines are produced in different ratios under different conditions, or that the local clearance rates of each cytokine are very different, leading to temporal differences in the effects observed. Resolution of these questions awaits improved methods for detecting and manipulating cytokine levels in vivo.

Summary

A cytokine synthesis inhibitory factor (CSIF) is secreted by Th2 clones in response to Con A or antigen stimulation, but is absent in supernatants from Con A–induced

Th1 clones. CSIF can inhibit the production of IL-2, IL-3, lymphotoxin (LT)/TNF, IFN-γ, and granulocyte-macrophage CSF (GM-CSF) by Th1 cells responding to antigen and APC, but Th2 cytokine synthesis is not significantly affected. Transforming growth factor β (TGF-β) also inhibits IFN-γ production, although less effectively than CSIF, whereas IL-2 and IL-4 partially antagonize the activity of CSIF. CSIF inhibition of cytokine synthesis is not complete, since early cytokine synthesis (before 8 h) is not significantly affected, whereas later synthesis is strongly inhibited. In the presence of CSIF, IFN-γ mRNA levels are reduced slightly at 8, and strongly at 12 h after stimulation. Inhibition of cytokine expression by CSIF is not due to a general reduction in Th1 cell viability, since actin mRNA levels were not reduced, and proliferation of antigen-stimulated cells in response to IL-2, was unaffected. Biochemical characterization, mAbs, and recombinant or purified cytokines showed that CSIF is distinct from IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IFN-γ, GM-CSF, TGF-β, TNF, LT, and P40. The potential role of CSIF in crossregulation of Th1 and Th2 responses is discussed.

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Note added in proof: The identity of CSIF as a novel cytokine has recently been confirmed by the isolation of a cDNA clone that expresses CSIF activity when transfected into COS cells (Moore, K. W. et al., manuscript in preparation). mAbs specific for CSIF react with both the recombinant protein and the D10-derived factor (Mosmann, T. R. et al., manuscript in preparation). The sequence of the cDNA clone is different from all previously known cytokines. At the time of submission of this cDNA clone sequence for publication, we will request the Nomenclature Committee of the International Union of Immunological Societies to assign an IL designation for this factor.

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