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Identification of a 24-kDa cytokine that is required for development of cytolytic T lymphocytes

(dendritic cells/interleukin 2/cytotoxic differentiation factor/lymphokines)

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ABSTRACT **It is known that the production of cytolytic T lymphocytes requires growth factors such as interleukins 2 and 4 (IL-2 and IL-4). Elsewhere we have described bioassays that detect a cytokine that operates in concert with growth factor to generate cytolytic T lymphocytes. The factor that is termed cytolytic T-lymphocyte differentiation factor (CDF), together with IL-2 and lectin, mediates the formation of $CD8^+$ killer cells in 2 days from thymocyte or peripheral lymphoid precursors. CDF is not mimicked by natural or recombinant sources of interferons, colony-stimulating factors, and IL-1 to IL-4. Here we use these bioassays to isolate and further characterize a single 24-kDa CDF protein from the conditioned medium of stimulated human blood mononuclear cells. CDF is first enriched by three successive chromatographic procedures that utilize anion exchange, hydroxyapatite, and phenyl-Superose. A single 24-kDa band with CDF activity is then isolated on 12% $NaDodSO_4$/PAGE and clearly distinguished from the 17-kDa band of IL-2. The apparent molecular mass is similar under reducing and nonreducing conditions. After elution from $NaDodSO_4$/PAGE the cytokine is maximally active at 0.25 nM in the CDF assay and has no growth factor activity for T lymphoblasts. To generate cytolytic $CD8^+$, $CD4^-$ cells from spleen and lymph node T lymphocytes, IL-2 and small numbers of accessory dendritic cells must be applied together with CDF.**

The development of cell-mediated immunity requires polypeptides termed cytokines or lymphokines. These proteins operate at subnanomolar levels to bring about the growth and/or differentiation of the different cell types that participate in the immune response. The production of killer or cytolytic T lymphocytes (CTL), the subject of this paper, requires a 17-kDa growth factor termed interleukin 2 (IL-2) *in vivo* and *in vitro* (1–5). The availability of a rapid and reliable bioassay allowed IL-2 to be purified by biochemical approaches (6–10). Recent evidence indicates that a second growth factor, IL-4, can mimic IL-2 in the production of CTL (11).

There are several models (reviewed in ref. 12) describing the need for supplementary factors in conditioned medium, in addition to IL-2, for the production of CTL. These factors, often termed CTL differentiation factors (CDF), have yet to be purified. We recently have identified two rapid bioassays in which CDF and IL-2 are both required for the development of CTL (12). One assay utilizes thymocytes from select strains of mice, and the other utilizes peripheral T cells cultured in the presence of hydrocortisone. In each assay $CD8^+$ killer cells arise in 2 days in the presence of lectins, IL-2, and CDF. A large panel of previously defined cytokines has been tested and found to lack CDF activity. Here we use these bioassays to identify a 24-kDa protein with CDF activity from the conditioned medium of stimulated human blood cells.

MATERIALS AND METHODS

Preparation of Human Leukocyte-Conditioned Medium (H-CM). Human blood mononuclear cells were isolated from leukocyte buffy coats by using Ficoll/Hypaque columns and suspended at 10^7 cells per ml in RPMI 1640 medium (GIBCO) supplemented with gentamicin (20 μg/ml, GIBCO), phorbol 12-myristate 13-acetate (20 ng/ml, Sigma), and phytohemagglutinin (5%, GIBCO). Twenty-five milliliters of the suspension was applied to 150-mm plastic Petri dishes (Falcon) to which the cells adhered because of the absence of serum proteins. After 2 hr, the monolayers were washed twice. The cells were recultured 38 hr in serum-free and mitogen-free RPMI 1640 medium with gentamicin. The conditioned medium was collected, concentrated 10-fold with a YM10 Amicon ultrafiltration membrane, and stored at −20°C.

Bioassays. CDF was assayed in two systems (12). One was used to monitor the purification of CDF. Thymocytes (5×10^5) from Swiss mice were cultured in RPMI 1640 medium supplemented with test cytokines, gentamicin, 5% fetal calf serum (Sterile Systems, Logan, UT), and 1 μg of Con A per ml (Miles) in flat-bottomed microtest trays (Costar, no. 3596, Cambridge, MA). After 42 hr, 10^4 ^{51}Cr-labeled P815 cells were added as targets in the presence of 10 μg of phytohemagglutinin per ml (Difco). After 4 hr, the supernatants were harvested to measure specific isotope release. Under these culture conditions, the observed cell-mediated lympholysis was due to $Thy\text{-}1^+$, $CD8^+$, $CD4^-$ cells (12). We also tested enriched fractions of CDF in a second bioassay that used peripheral T cells from lymph node or spleen. The culture conditions were identical to the thymocyte assay except that the lymphocytes were nylon wool-nonadherent, Ia^- cells, and the medium was supplemented with 0.1 mM hydrocortisone acetate (Sigma). Hydrocortisone acetate ablated the formation of killer cells in response to lectin and IL-2 alone (see *Results*). As accessory cells, we tested bulk plastic-adherent, spleen cells as well as the adherent, dendritic cell subpopulation enriched as described (13). IL-2 activity was tested in a 1-day bioassay in which one measures the growth of lectin-primed mouse spleen T blasts (8, 12).

Purified Cytokines. We were generously provided with several purified cytokines that were not available at the time of our initial studies of CDF (12). These cytokines, none of which proved to have CDF activity (see *Results*), were murine recombinant granulocyte/macrophage colony-stimulating factor (rGM-CSF) (S. Gillis, Immunex, Seattle, WA; 4×10^7 units/mg), human recombinant granulocyte

Abbreviations: CTL, cytolytic T lymphocyte(s); TGF, transforming growth factor; IL, interleukin; r, recombinant; CDF, CTL differentiation factor(s); IFN, interferon; CSF, colony-stimulating factor; G-CSF, granulocyte CSF; GM-CSF, granulocyte/macrophage CSF; H-CM, human leukocyte-conditioned medium.

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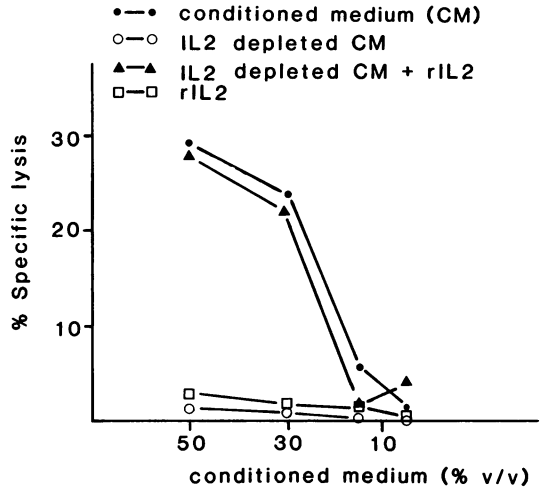

FIG. 1. Bioassay for a CDF in H-CM. Thymocytes were cultured for 2 days in the presence of Con A and H-CM (●), H-CM depleted of IL-2 on anti-IL-2 columns (○), 30 units of human rIL-2 per ml (□; Biogen, Cambridge, MA), or IL-2 with IL-2-depleted H-CM as a source of CDF (▲). Lysis of P815 targets in the presence of additional lectin was measured (12). The immunoglobulin fraction of a rabbit anti-human IL-2 antiserum, R135 (kindly provided by R. Robb, DuPont, Glenolden, PA), was coupled to CnBr-activated Sepharose (Pharmacia). Depletion of IL-2 was performed and monitored as described (12).

CSF (rG-CSF) (L. Souza, Amgen Biologicals, Thousand Oaks, CA; 8×10^7 units/mg), purified mouse IL-3 (Genzyme, Norwalk, CT; 5000 colony-forming units/ml), and purified porcine transforming growth factor, type β (TGF-β) (R & D Systems, Minneapolis, MN; 10^6 half-maximal units/mg).

Biochemical Methods. All chromatographic procedures were performed at room temperature on a Waters HPLC system and are described in detail in the figure legends. NaDodSO$_4$/PAGE was done with a Laemmli system with a 12% separating gel (14). Marker proteins were low molecular mass standards of Pharmacia (Uppsala, Sweden). Gels were silver stained as described (15). Protein determinations were based on UV absorption at 280 nm or were estimated with known amounts of ovalbumin or lactalbumin on the silver-stained 12% NaDodSO$_4$/PAGE.

Table 1. Effect of different cytokines in the CDF bioassay

Factor added	Concentration	% specific lysis: Without rIL-2	% specific lysis: With rIL-2 (30 units/ml)
None		0.3	1.7
H-CM	30% (vol/vol)	58.9	NT
	15% (vol/vol)	44.2	NT
Murine rGM-CSF	10 ng/ml	0	3.1
	3 ng/ml	0.1	1.7
Human rG-CSF	400 units/ml	1.0	2.0
	160 units/ml	0.1	1.3
Murine purified IL-3	50 units/ml	0.8	3.8
	20 units/ml	0.5	3.0
Porcine rTGF-β	1 ng/ml	0.1	0.7
	0.3 ng/ml	1.1	2.1

Thymocytes (3×10^5) were cultured 2 days with 1 μg of Con A per ml and the factors indicated. Half of the cultures in addition received human rIL-2. At 44 hr, 5×10^3 ^{51}Cr-labeled P815 cells and 1 μg of PHA per ml were added to each well. Release of radiolabel was determined 4 hr later. NT, not tested.

Anti-IL-2 Affinity Chromatography. IL-2 was removed from H-CM by passage over a rabbit anti-human IL-2 column as described (12).

RESULTS

Bioassay for a Distinct CDF. The bioassay that was used to identify CDF is summarized in Fig. 1. When mouse thymocytes from select strains of mice were cultured in H-CM and lectin, cytotoxic activity was generated within 2 days. CTL did not develop if the H-CM was depleted of IL-2 by anti-IL-2 affinity chromatography (Fig. 1; ref. 12). Recombinant IL-2 (rIL-2) by itself did not induce CTL but did reconstitute H-CM that had been depleted of IL-2 (Fig. 1). CDF is defined as that factor or factors in H-CM that mediated the development of CTL in concert with IL-2. CDF is also present in the CM of stimulated mouse leukocytes but not in the CM of macrophages stimulated with lipopolysaccharide or EL-4 thymoma cells stimulated with phorbol 12-myristate 13-acetate (12).

A large panel of purified mouse and human cytokines was shown to lack CDF activity in prior work. The inactive molecules included IL-1, IL-3, and IL-4; lymphotoxin and

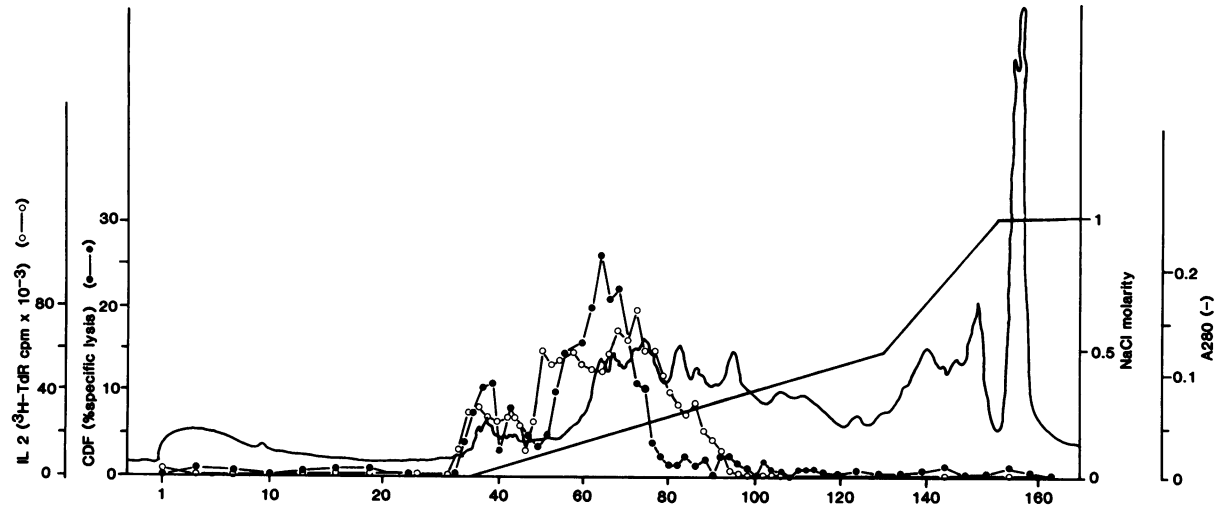

FIG. 2. Elution of CDF and IL-2 from HPLC Mono Q anion-exchange columns. A Mono Q HR 10/10 column (Pharmacia) was equilibrated with 10 mM Tris·HCl (pH 7.4). Concentrated H-CM in the same buffer was applied and eluted with a linear gradient of NaCl to 1 M at 4 ml/min. Fractions (2 ml) were collected, and aliquots were assayed for CDF (●, lytic activity in the thymocyte assay with exogenous human rIL-2 at 10 units/ml), IL-2 (○, proliferation of T lymphoblasts; ^{3}H-TdR, [^{3}H]thymidine), and protein content (—, A_{280}).

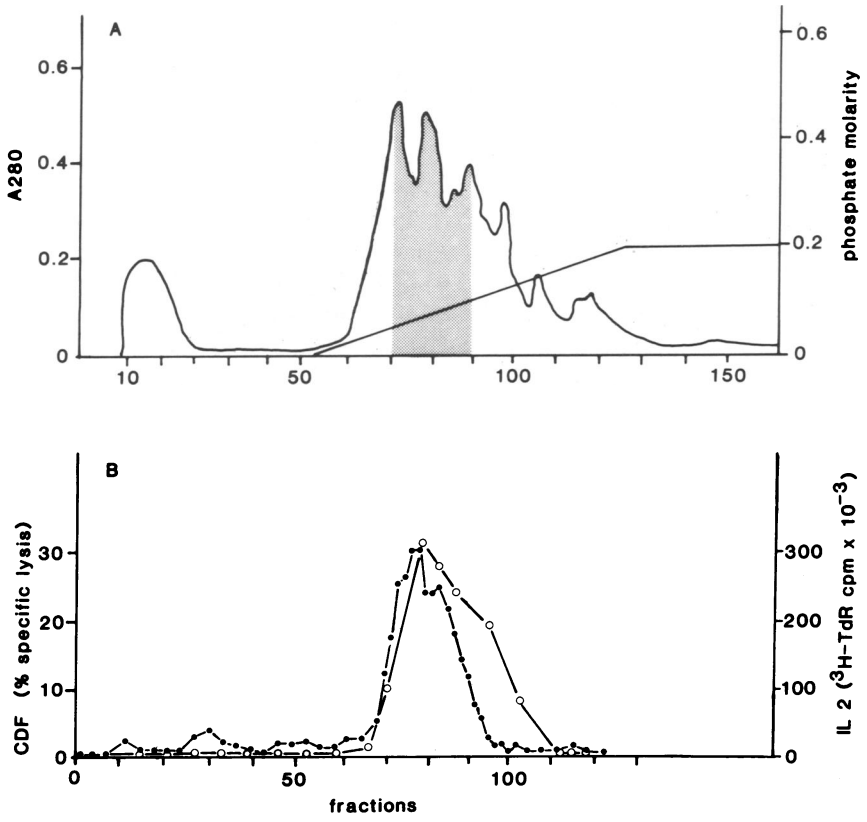

FIG. 3. Elution of CDF and IL-2 from HPLC hydroxyapatite columns. A Rainin HPLC HCA column was equilibrated with 5 mM phosphate buffer at pH 7.0. The active fractions from the Mono Q columns (Fig. 2) in the same buffer were applied and eluted with a linear gradient of sodium phosphate from 5 mM to 0.2 M at 1 ml/min (*A*). Fractions (0.3 ml) were collected and tested for protein content (*A*) and CDF (●) and IL-2 (○; ^{3}H-TdR, [^{3}H]thymidine) activity (*B*). The shaded area in *A* shows the fractions that were pooled for further purification.

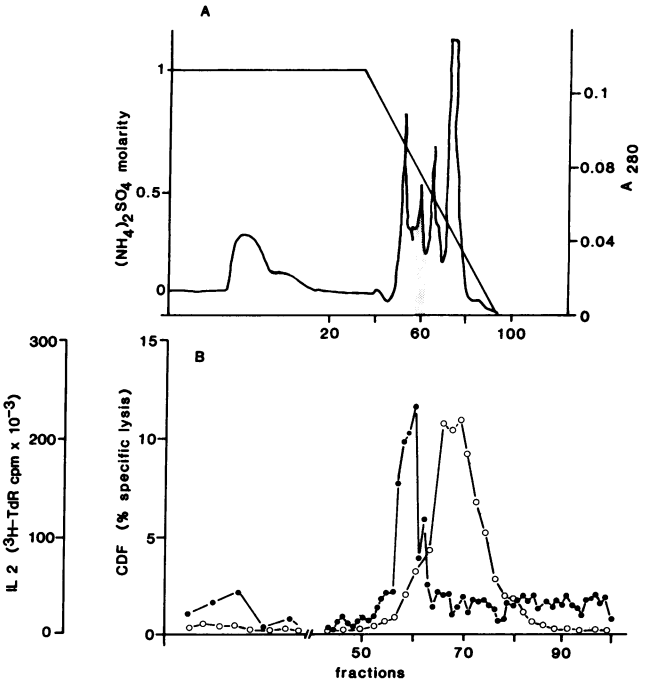

FIG. 4. Elution of CDF and IL-2 from HPLC phenyl-Superose columns. A Pharmacia HPLC phenyl-Superose HR 5/5 column was equilibrated with 50 mM phosphate (pH 7.0) in 1 M ammonium sulfate. The active fractions from the hydroxyapatite column (Fig. 3) were made 1 M with ammonium sulfate, applied, and eluted with decreasing ammonium sulfate to 0 M (50 mM phosphate). The elution rate was 0.5 ml/min. Fractions (0.3 ml) were collected. Following dialysis into phosphate-buffered saline, the fractions were tested for CDF (●) and IL-2 (○; ^{3}H-TdR, [^{3}H]thymidine) activities. The shaded area in *A* shows the fractions that were pooled for subsequent purification.

cachectin/tumor necrosis factor; macrophage CSF; and interferons α, β, and γ (IFN-α, IFN-β, and IFN-γ) (12). Here we tested preparations of mouse rGM-CSF, human rG-CSF, rTGF-β, and purified mouse IL-3. All were inactive in mediating CTL development alone or in combination with IL-2 (Table 1). These results indicate that a different factor is required for the development of at least some CTL from noncytolytic precursors.

Purification of CDF. Since a short reliable assay was available, we attempted to purify CDF to better define its biologic role and biochemical characteristics. We performed four cycles of purification. In each we started with 1600 ml of H-CM that was obtained by stimulating 16 × 10^9 human blood mononuclear cells with phorbol 12-myristate 13-acetate and PHA in serum-free medium. The H-CM was concentrated 10-fold and dialyzed against the appropriate buffer to provide 150–190 mg of starting protein. The purification protocol that we developed involved three chromatographic steps followed by a final separation on $NaDodSO_4$/PAGE.

HPLC anion-exchange chromatography. The concentrated H-CM was loaded on a HPLC Mono Q column and eluted with a linear NaCl gradient. CDF activity eluted between 0.06 and 0.16 M NaCl and substantially overlapped with IL-2 activity (Fig. 2). The active fractions were pooled and contained 11–24 mg of protein.

HPLC hydroxyapatite chromatography. This step provided a substantial concentration of the CDF-containing material that was obtained from the Mono Q column. Following elution with a linear sodium phosphate gradient, CDF was recovered at about 50 mM. Again, CDF and IL-2 activities overlapped (Fig. 3). The positive fractions were pooled and contained 3.3–3.7 mg of protein.

HPLC hydrophobic interaction chromatography. Material from hydroxyapatite columns was applied to phenyl-Superose and eluted with a decreasing gradient of 1–0 M $(NH_4)_2SO_4$. CDF eluted at about 0.5 M. Phenyl-Superose

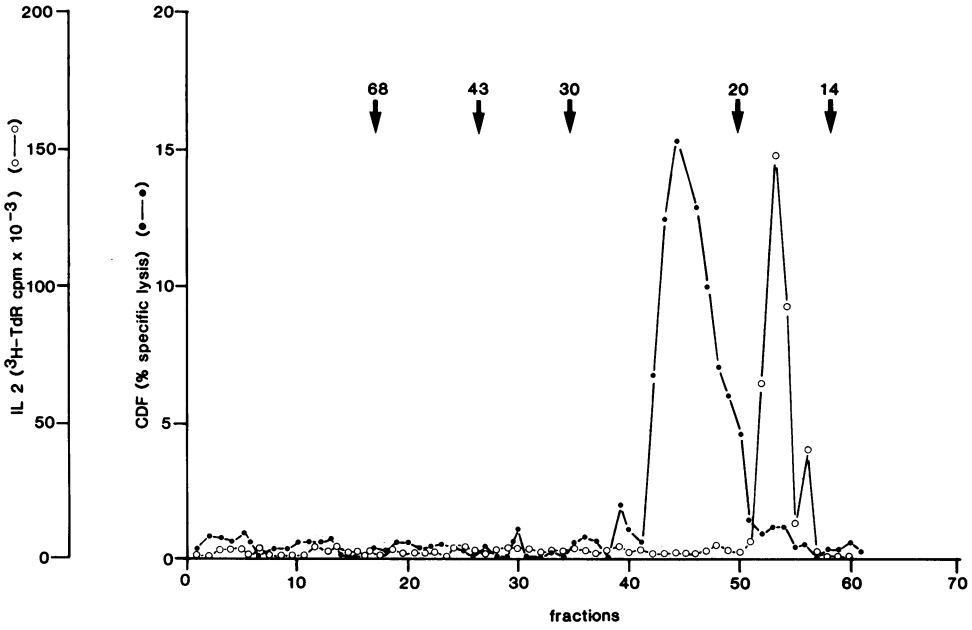

FIG. 5. Isolation of CDF and IL-2 on NaDodSO$_4$/PAGE. Positive fractions from the HPLC phenyl-Superose columns were dialyzed against NaDodSO$_4$, lyophilized, and reconstituted in sample buffer to give a final concentration of 1% NaDodSO$_4$. Following electrophoresis in a 12% polyacrylamide gel, 1-mm slices were prepared and eluted into 1:10 phosphate-buffered saline. Samples were assayed for CDF (●) and IL-2 (○; ^{3}H-TdR, [^{3}H]thymidine). The molecular masses of the positive fractions (shown in kDa) were estimated by comparison with standards.

resolved quite nicely the peaks of CDF and IL-2 activity (Fig. 4). The pooled fractions had 0.3–0.4 mg of protein with CDF activity.

NaDodSO$_4$/PAGE. The active fractions from phenyl-Superose were separated on 12% acrylamide gels. Lanes were then cut in 1-mm slices, eluted, and assayed for CDF and IL-2 activities. CDF was recovered as a single peak, with an apparent molecular mass of 24 kDa. IL-2 was completely distinct and migrated at 17 kDa (Fig. 5). Sixteen to 25 μg of purified CDF was obtained on each of the four cycles of purification that we performed. Fractions of each step of the purification were separated on NaDodSO$_4$/PAGE and analyzed by silver staining (Fig. 6). Each step, particularly phenyl-Superose, led to a progressive reduction of protein bands. A single band at 24 kDa was evident after the final step under reducing (Fig. 6*A*) and nonreducing (Fig. 6*B*) conditions.

Biological Effects of Purified CDF. Dose–response studies with purified CDF in the thymocyte bioassay indicated that maximal cytotoxic responses were obtained at 0.25 nM (Fig. 7). The activity of CDF could be higher given the facts that some denaturation may occur during the final separation in detergent and some contaminants may comigrate with CDF.

The purified CDF was also tested on peripheral T cells (12). Bulk or CD8$^+$ (L3T4$^-$), spleen, or lymph node T cells were obtained by passage over nylon wool and depletion of residual Ia$^+$ accessory cells. For peripheral T cells, it was essential to add 0.1 mM hydrocortisone acetate to block the development of lytic cells that was observed with IL-2 alone (12). In the presence of hydrocortisone acetate, substantial levels of lytic activity developed in the combined presence of lectin, CDF, IL-2, and accessory cells (Fig. 8). The lytic activity was ablated by treatment with anti-CD8 but not anti-CD4 monoclonal antibody and complement (data not shown), indicating that the lytic cells developing in response to pure CDF were primarily CTL rather than natural killer cells. The purified CDF had no mitogenic activity for peripheral T cells (not shown).

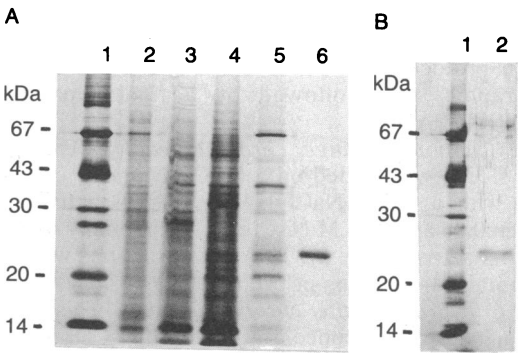

FIG. 6. NaDodSO$_4$/PAGE of active fractions from the purification protocol. (*A*) Silver staining of samples run under nonreducing conditions. Lane 1, molecular mass standards; lane 2, concentrated H-CM; lane 3, CDF fractions from the Mono Q column; lane 4, CDF fractions from the hydroxyapatite column; lane 5, CDF fractions from phenyl-Superose; lane 6, the final CDF fraction from the preparative gel. (*B*) Silver staining of samples run under reducing conditions. Lane 1, molecular mass standards; lane 2, the CDF fraction from the preparative gel.

DISCUSSION

CTL represent an important form of adaptive immunity. CTL are able to eliminate cells bearing foreign antigens in an antigen-specific and major histocompatibility complex-restricted fashion. The known targets of CTL include transplants bearing major and minor transplantation antigens, cells infected with virus, and tumors. By using different bioassays, we have substantially purified a CDF that is

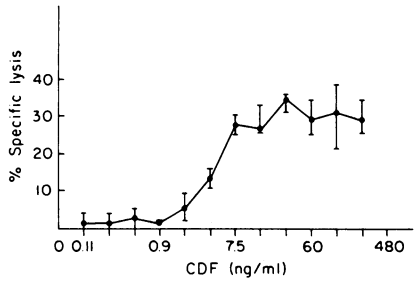

FIG. 7. Activity of purified CDF in the thymocyte bioassay. Purified CDF at the indicated doses were added to the thymocyte bioassay, with additional human rIL-2 at 20 units/ml, and CTL were measured 2 days later. No CTL developed in the absence of IL-2 (not shown).

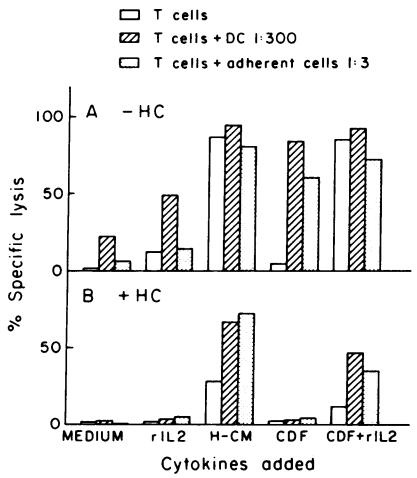

FIG. 8. Activity of purified CDF in the peripheral T-cell bioassay. Nylon wool-nonadherent spleen lymph node cells were depleted of residual accessory cells by panning with a combination of anti-Fc receptor and anti-Ia monoclonal antibody. Accessory cell depletion was evident in the fact that CTL did not develop in the presence of lectin and there was no cell proliferation (not shown). Enriched T cells (2.5×10^5) were cultured in microtest wells with lectin in the absence (*A*) or presence (*B*) of 0.1 mM hydrocortisone acetate and in the absence or presence of accessory cells. The accessory cells were bulk adherent populations added at a submaximal dose of 1 adherent to 3 T cells or enriched dendritic cells (DC) added at a submaximal dose of 1:300. Results are shown for CDF (60 ng/ml), CDF with human rIL-2 (10 units/ml), rIL-2 alone, or H-CM [30% (vol/vol)].

required for the development of CTL in a polyclonal model. Activity is associated with a single protein band in Na-DodSO$_4$ with an apparent molecular mass of 24 kDa. The CDF has been enriched about 1000-fold from serum-free conditioned medium. The protocol described here involves anion exchange, adsorption and elution from hydroxyapatite, and hydrophobic interaction chromatography. Taking advantage of the stability of CDF in the presence of detergent, we used an electrophoretic step in polyacrylamide gels as a major purification step (Fig. 6). Additional procedures will be required to determine if the single 24-kDa band is contaminated with other proteins. If we assume that the protein is pure and that denaturation did not occur, then CDF is fully active at 0.25 nM (Fig. 7).

CDF has no detectable growth factor activity since we observe neither blastogenesis nor DNA synthesis with resting T cells or primed T lymphoblasts (Fig. 5 and data not shown). However, CDF mediates CTL development only in concert with IL-2 and accessory dendritic cells (Fig. 8). Given the role of dendritic cells in triggering lymphokine release and responsiveness (16, 17), these accessory cells may allow the T cell to become responsive to CDF or may induce the release of yet another required factor.

One defined cytokine that has been reported to have CDF activity has been IFN-γ (18, 19). However, we have repeatedly found no enhancing effect of mouse or human rIFN-γ in our bioassays over a dose range of 1–100 units/ml (12). Recently, a T-cell-derived stimulatory factor for B lymphocytes termed IL-5 has been shown to have CDF activity in a different bioassay (20). Human and mouse IL-5 have a molecular mass of 45 kDa (20, 21). A large panel of known cytokines (12) (Table 1) lacks CDF activity in the bioassays we have employed. Therefore we suspect that CDF is a previously unreported entity.

Further studies (as with specific neutralizing antibodies) should clarify if CDF is an essential cytokine for antigen-specific CTL differentiation. Many precedents exist for the requirement of distinct differentiation and growth factors in T-cell-mediated immunity. The production of microbicidal and tumoricidal macrophages requires cytokines such as IFN-γ that act as "activation" rather than growth factors (22). Production of specific B-cell isotypes in T-dependent antibody responses also requires cytokines such as IL-4 and IFN-γ (23–25), but these cytokines do not act as growth factors on the primed B lymphoblast (26).

Preliminary experiments indicate that the CD4$^+$ subset of blood T cells is much more active than the CD8$^+$ subset for the production of CDF. Additional evidence that CDF is a T-cell product or lymphokine comes from prior work with the immunosuppressive drug cyclosporin A. A major effect of this drug is to block lymphokine production at the transcriptional level. We have noted that the mRNA for CDF could *not* be detected in oocyte translation systems following application of cyclosporin A (27). Glucocorticoids also suppress lymphokine production (28). We find that it is essential to add steroid to our peripheral T-cell cultures to observe the requirement for *exogenous* CDF in CTL development. It is possible that the steroid acts to block the production of endogenous CDF from helper or other cells.

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