Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6808–6812, September 1990
Immunology

Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells

(lymphokine-activated killer cells/interleukin 2/T-cell growth factor/natural killer cell/tumor necrosis factor)

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Communicated by John J. Burns, June 11, 1990 (received for review April 20, 1990)

ABSTRACT **A cytokine that can synergize with interleukin 2 to activate cytotoxic lymphocytes was purified to homogeneity. The protein, provisionally called cytotoxic lymphocyte maturation factor (CLMF), was isolated from a human B-lymphoblastoid cell line that was induced to secrete lymphokines by culture with phorbol ester and calcium ionophore. The purification method, utilizing classical and high-performance liquid chromatographic techniques, yielded protein with a specific activity of 8.5×10^7 units/mg in a T-cell growth factor assay. Analysis of the purified protein by sodium dodecyl sulfate/polyacrylamide gel electrophoresis demonstrated that CLMF is a 75-kDa heterodimer composed of disulfide-bonded 40-kDa and 35-kDa subunits. Determination of the N-terminal amino acid sequences of the two subunits revealed that both subunits are not related to any previously identified cytokine. Purified CLMF stimulated the proliferation of human phytohemagglutinin-activated lymphoblasts by itself and exerted additive effects when used in combination with suboptimal amounts of interleukin 2. Furthermore, the purified protein was shown to synergize with low concentrations of interleukin 2 in causing the induction of lymphokine-activated killer cells.**

The potential utility of cytokines in the treatment of neoplasia and as immunoenhancing agents has recently been demonstrated in studies using human recombinant interleukin 2 (rIL-2) (1–6). However, the clinical use of rIL-2 has been complicated by the serious side effects that it may cause (2, 3). One approach to improving the efficacy of cytokine therapy while reducing toxicity is to use two or more cytokines in combination. For example, synergistic antitumor activity has been shown to result when rIL-2 is administered to tumor-bearing mice together with recombinant interferon α (rIFN-α) (7, 8) or with recombinant tumor necrosis factor α (rTNF-α) (9). The antitumor effects of rIL-2 are thought to be mediated by host cytotoxic effector lymphocytes, which are activated by rIL-2 *in vivo* (10). rIFN-α (11) and rTNF-α (12, 13) have been shown to synergize with rIL-2 in activating cytotoxic effector cells *in vitro* as well as to exert synergistic antitumor effects when given in combination with rIL-2 *in vivo* (7–9). Hence, a cytokine was sought that could synergize with rIL-2 to activate cytotoxic lymphocytes *in vitro* and thus might also have utility as an antitumor agent when administered in combination with rIL-2 *in vivo*.

Previously we demonstrated that IL-2-depleted lymphokine-containing cell supernatant solutions from cultures of human peripheral blood lymphocytes activated with phytohemagglutinin (PHA) or in mixed lymphocyte cultures contained such a factor, provisionally called cytotoxic lymphocyte maturation factor (CLMF) (14, 15). However, the quantities of human CLMF produced by peripheral blood lymphocytes were too low to permit its purification to homogeneity. Therefore, human lymphoid cell lines were screened for the production of cytokines that could synergize with rIL-2 to activate cytotoxic lymphocytes *in vitro*. The NC-37 B lymphoblastoid cell line was identified that, when stimulated with phorbol ester and calcium ionophore, secreted factor(s) mediating CLMF-like activity. The present study reports a procedure for the purification of NC-37-derived human CLMF to homogeneity and its chemical characterization.

MATERIALS AND METHODS

Source of CLMF. Human NC-37 B lymphoblastoid cells (American Type Culture Collection) were used for production of CLMF. High producer sublines of NC-37 cells were derived by limiting dilution cloning in liquid microcultures. Three sublines, NC-37.89, NC-37.98, and NC-37.102, were identified that routinely produced CLMF at titers 4-fold greater (≥800 units/ml) than that produced by the parent NC-37 cell line. Bulk production of CLMF was carried out in roller bottle cultures. Cell suspensions were prepared containing 1–1.5 × 10^6 NC-37.89, NC-37.98, or NC-37.102 cells per ml of RPMI 1640 supplemented with 1% Nutridoma-SP (Boehringer Mannheim), 2 mM L-glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, 10 ng of phorbol 12-myristate 13-acetate per ml (PMA), and 20–25 ng of calcium ionophore A23187 per ml (Sigma). Two hundred fifty to 350-ml samples of the cell suspensions were incubated at 37°C in Falcon 3027 tissue culture roller bottles with continuous rolling for 3 days. At the end of this time, the culture supernatant solutions were harvested. EDTA and phenylmethylsulfonyl fluoride were added to the culture supernatant solutions at final concentrations of 1 mM and 0.1 mM, respectively, to retard proteolytic degradation. The supernatant solutions were stored at 4°C until concentration.

Concentration of Cell Supernatant Solutions. Crude human CLMF supernatant solutions prepared from several batches of induced NC-37 cells were pooled and concentrated 30-fold using the Pellicon cassette system (30,000 NMWL

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Abbreviations: IL, interleukin; r, recombinant; IFN, interferon; TNF, tumor necrosis factor; PHA, phytohemagglutinin; CTL, cytolytic T lymphocyte; LAK, lymphokine-activated killer; CLMF, cytotoxic lymphocyte maturation factor; PMA, phorbol 12-myristate 13-acetate; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; TGF, T-cell growth factor; NK, natural killer; TCM, tissue culture medium.

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PTTK00005; Millipore). After concentrating to the desired volume, a buffer exchange was performed with 10 mM Mes, pH adjusted to 6.0 with 10 M NaOH. The concentrate was centrifuged at 10,000 × g for 10 min at 4°C and the precipitate was discarded.

Ion-Exchange Chromatography on NuGel P-SP Column. The supernatant solution was applied at a flow rate of 120 ml/hr to a NuGel P-SP (Separation Industries, Metuchen, NJ) column (5 × 5 cm), equilibrated in 10 mM Mes (pH 6.0). Adsorbed proteins were eluted with a 500-ml salt gradient from 0 to 0.5 M NaCl/10 mM Mes, pH 6.0, at a flow rate of 2 ml/min. Fractions containing T-cell growth factor (TGF) activity were pooled and dialyzed (Spectra/Por 7; Fisher Scientific) against 50 vol of 20 mM Tris·HCl (pH 7.5).

Dye-Affinity Chromatography on Blue B-Agarose Column. The dialyzed sample was centrifuged at 10,000 × g for 10 min at 4°C and the precipitate was discarded. The supernatant solution was applied at a flow rate of 20 ml/hr to a blue B-agarose (Amicon) column (2.5 × 10 cm) equilibrated in 20 mM Tris·HCl (pH 7.5). Adsorbed proteins were eluted with a 500-ml salt gradient from 0 to 0.5 M NaCl/20 mM Tris·HCl, pH 7.5, at a flow rate of 15 ml/hr. Fractions containing TGF activity were pooled and dialyzed against 100 vol of 20 mM Tris·HCl (pH 7.5)

Ion-Exchange Chromatography on Mono Q Column. The dialyzed sample was filtered through a 0.45-μm cellulose acetate filter, and the filtrate was applied at a flow rate of 60 ml/hr to a Mono Q HR 5/5 (Pharmacia LKB Biotechnology) column (5 × 5 mm) equilibrated in 20 mM Tris·HCl (pH 7.5). Adsorbed proteins were eluted with a 1-hr linear salt gradient from 0 to 0.25 M NaCl/20 mM Tris·HCl, pH 7.5, at a flow rate of 60 ml/hr. Aliquots of fractions were assayed for TGF activity.

Reversed-Phase HPLC. The chromatographic system has been described (16). Reversed-phase HPLC was carried out using Vydac C_{18} or diphenyl columns (4.6 × 20 mm; The Separations Group). Further chromatographic details are given in the figure legends.

Protein Analysis. Protein purity of the final preparation was assessed by SDS/PAGE with and without reduction (17). Gels were silver stained (18) to visualize protein.

Sequence analysis was performed using an Applied Biosystems model 470A gas-phase sequencer (19). Phenylthiohydantoin (PTH) amino acid derivatives were identified "on-line" with an Applied Biosystems model 120A PTH analyzer.

Lymphokine-Activated Killer (LAK) Cell Induction Assay. The assay used to measure synergy between rIL-2 and CLMF-containing solutions in the induction of human LAK cells was similar to the assay previously used to detect synergy in the induction of human cytolytic T-lymphocyte (CTL) responses (14, 15). Human peripheral blood mononuclear cells were isolated from the blood of normal volunteer donors, depleted of accessory cells, and fractionated by centrifugation over a discontinuous Percoll density gradient as described (15). Mononuclear cells recovered from the 38%, 41%, and 45% Percoll layers were pooled and used as a source of LAK cell precursors in the assay. These cells were washed and suspended in tissue culture medium (TCM) composed of a 1:1 mixture of RPMI 1640 and Dulbecco's modified Eagle's medium, supplemented with 0.1 mM nonessential amino acids, 60 μg of arginine hydrochloride per ml, 10 mM Hepes buffer, 2 mM L-glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, 0.05 mM 2-mercaptoethanol, 1 mg of dextrose per ml, and 5% human AB serum (Irvine Scientific). The cells were incubated in 24-well tissue culture plates in 1-ml cultures (7.5×10^5 cells per culture) to which 0.1 mM hydrocortisone sodium succinate (Sigma) was added to minimize endogenous cytokine production (20). Some cultures also received human rIL-2 at a final concentration of 5 units/ml and/or supernatant solutions to be assayed for CLMF activity. All cultures were incubated for 3–4 days at 37°C.

At the end of this incubation, the contents of each culture were harvested by centrifugation and resuspended in 0.5 ml of fresh TCM. One-tenth milliliter samples of these cell suspensions were mixed with 0.1-ml samples of ^{51}Cr-labeled target cells and tested for their lytic activity in 5-hr ^{51}Cr release assays. The methods for labeling target cells with ^{51}Cr and performing the cytolytic assays have been described (21). The percent specific ^{51}Cr release was calculated as $[(e - c)/(100 - c)] \times 100$, where e is the percentage of ^{51}Cr released from target cells incubated with lymphocytes and c is the percentage of ^{51}Cr released spontaneously from target cells incubated alone. All lymphocyte populations were assayed in quadruplicate for lytic activity.

TGF Assay. The ability of culture supernatant solutions and chromatographic fractions to stimulate the proliferation of PHA-activated human T lymphoblasts was measured as follows. Human peripheral blood mononuclear cells were isolated (21) and cultured at 37°C at a density of 5×10^5 cells per ml in TCM containing 0.1% PHA-P (Difco). After 3 days, the cultures were split 1:1 with fresh TCM, and human rIL-2 was added to each culture to give a final concentration of 50 units/ml. The cultures were then incubated for an additional 1–2 days, at which time the cells were harvested, washed, and resuspended in TCM at 4×10^5 cells per ml. Heat-inactivated goat anti-human rIL-2 antiserum (prepared by R.C.; final dilution, 1:200) was added to this cell suspension to block any potential IL-2-induced cell proliferation in the assay. This antiserum was shown to cause 50% neutralization of 2 units of natural or recombinant IL-2 per ml at a serum dilution of 1:20,000. Fifty-microliter samples of the cell suspension containing anti-IL-2 antiserum were mixed with 50-μl samples of serial dilutions of culture supernatant fluids or fractions in the wells of Costar 3596 microplates. The cultures were incubated for 1 day at 37°C, and 50 μl of [^{3}H]thymidine (New England Nuclear; 10 μCi/ml in TCM; 1 Ci = 37 GBq) was then added to each well. The cultures were further incubated overnight. Subsequently, the culture contents were harvested onto glass fiber filters by means of a cell harvester (Cambridge Technology, Cambridge, MA), and [^{3}H]thymidine incorporation into cellular DNA was measured by liquid scintillation spectroscopy. All samples were assayed in triplicate.

To measure units of CLMF activity, a partially purified preparation of human cytokines produced by coculturing PHA-activated human peripheral blood mononuclear cells with NC-37 cells was used as a standard. Several dilutions of this preparation, which was assigned an arbitrary titer of 2000 units/ml, were included in each TGF or LAK induction assay. From the results obtained for the standard preparation, a dose–response curve was constructed and used to calculate the activity of each unknown sample in units/ml.

Anti-CLMF Antibodies. A rat immunized with ≈4 μg of partially purified human CLMF produced serum antibodies that neutralized CLMF bioactivity. Hybridoma cells secreting monoclonal antibodies to CLMF were produced by fusing NSO cells with splenocytes recovered from this rat. Antibodies were purified from ascites fluid by affinity chromatography on a GammaBind G-agarose column (Genex). Monoclonal anti-CLMF antibody 7B2 was found to be an IgG2a antibody that immunoprecipitated radiolabeled CLMF heterodimer and reacted with the 40-kDa subunit of CLMF, as demonstrated by immunoblot analysis (R.C. and T.T., unpublished data).

RESULTS

Purification of CLMF. IL-2-depleted supernatant solutions from cultures of activated NC-37 cells were found to synergize

Table 1. Purification of CLMF

Fraction	Total protein,* mg	Total volume, ml	Total activity,† units	Specific activity, units/mg of protein	Purification, fold	Yield, %
Conditioned medium	8000	60,000	1.6×10^8	2.0×10^4	1.0	100
Ultrafiltered concentrate	3550	1,940	3.0×10^8	8.5×10^4	4.3	188
NuGel P-SP	63	90	1.8×10^8	2.8×10^6	140.0	113
Blue B-agarose	11	45	1.4×10^8	1.3×10^7	650.0	87.5
Mono Q	0.480	6	4.1×10^7	8.5×10^7	4250.0	25.6‡
Vydac diphenyl	0.010	1	5.2×10^5	5.2×10^7	2600.0	0.33‡

*Protein concentration of the first four fractions was determined by the bicinchoninic acid protein assay (Pierce) according to the manufacturer's instructions. In all other instances, absolute protein determination was performed by amino acid analysis (22).
†Units of activity measured by TGF assay.
‡Eluted fractions from the previous chromatography containing the highest specific activity were rechromatographed. Yields reflect only the recovery in the highest specific activity Mono Q and diphenyl fractions. The total recovery of activity from the Mono Q and diphenyl columns was 54% and 12%, respectively.

with low concentrations of rIL-2 in the induction of cytolytic LAK cell activity. Fractionation of the crude supernatant solution by Mono Q ion-exchange FPLC revealed the presence of at least two distinct cytokines in the NC-37 culture supernatant solution capable of mediating this activity (data not shown). One of these cytokines likely was identical to TNF, whereas the second, which was retained longer on the Mono Q column, did not mediate TNF bioactivity. Further efforts were therefore directed at the purification of this second cytokine, designated NC-37-derived CLMF.

Fractions from the Mono Q column that contained CLMF were also found to stimulate the proliferation of human PHA-activated T lymphoblasts in the TGF assay (data not shown). In all subsequent chromatographic procedures, it was found that LAK cell induction and TGF activities invariably eluted in the same fractions, and, as will be shown below, both activities were mediated by purified CLMF. Since the TGF assay was more quantitative and reproducible than the LAK cell induction assay and was of shorter duration, the TGF assay was used for routinely monitoring the yield and specific activity of CLMF recovered from the various purification steps.

CLMF was purified from 60 liters of supernatant solution from cultures of NC-37.89, NC-37.98, and NC-37.102 lymphoblastoid cells stimulated with PMA and calcium ionophore A23187. The CLMF was partially purified as described under *Materials and Methods* and summarized in Table 1. The active fractions from the Mono Q column were pooled and further fractionated by reversed-phase HPLC on a Vydac diphenyl column (Fig. 1). This procedure yielded a symmetrical peak of protein coinciding with the major peak of TGF activity. Recovery of biological activity in this step was typically 10–50%. A second peak of biological activity eluted 23 min later in the chromatogram. The fractions (219–223 min) containing the major peak of TGF activity were pooled and protein purity was assessed by SDS/PAGE under nonreducing conditions, using a 10% slab gel (Fig. 2, −2ME). These fractions were of greater than 95% purity and revealed protein of 75 kDa. Under reducing conditions, the 75-kDa CLMF was separated into two subunits of 40 and 35 kDa (Fig. 2, +2ME). Thus, CLMF is a 75-kDa heterodimer composed of disulfide-bonded 40-kDa and 35-kDa subunits.

The overall purification of CLMF that was achieved is shown in Table 1. A specific activity of 8.5×10^7 units/mg and 5.2×10^7 units/mg for Mono Q- and Vydac diphenyl-purified material, respectively, was obtained. The fact that the diphenyl-purified protein has a slightly lower specific activity than the Mono Q-purified material may be due to inactivation or denaturation of some of the molecules of CLMF in the HPLC elution solvents (i.e., acetonitrile in 0.1% TFA). Alternatively, it may simply reflect the variability inherent in the biological assay.

Chemical Characterization. The ability to prepare homogeneous CLMF allowed for the determination of the N-terminal sequence of the naturally occurring CLMF protein. Sequence determination was attempted by automated Edman degradation on 100 pmol of CLMF. Data from the first 23 cycles indicated two sequences present, as would be expected from the heterodimeric structure of CLMF.

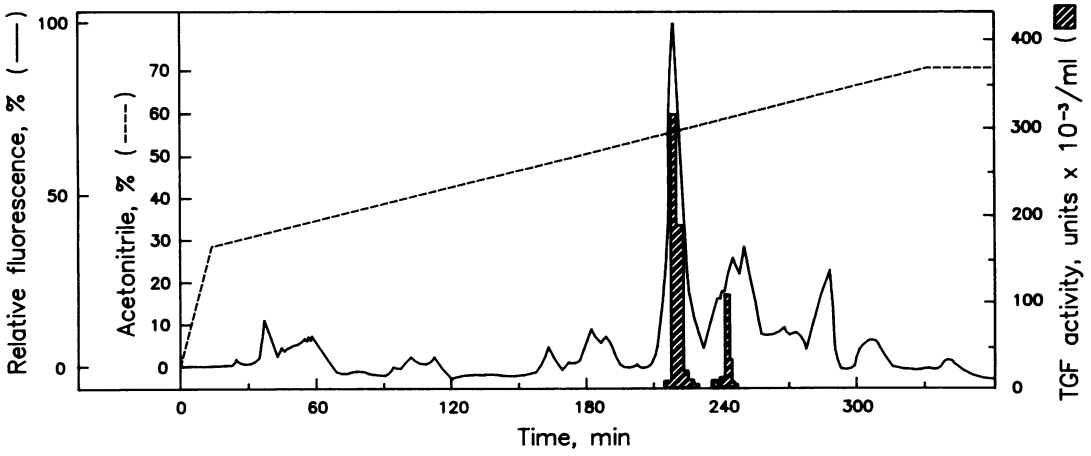

FIG. 1. Purification of CLMF by reversed-phase HPLC. Pooled fractions from the Mono Q ion-exchange FPLC containing 7.6×10^6 units of activity and a total of 100 μg of protein were diluted 1:1 (vol/vol) with 8 M urea. The diluted sample was then applied to a Vydac diphenyl column (5 μm, 4.6 × 20 mm) at a rate of 30 ml/hr. The column was washed with 5 ml of 0.1% trifluoroacetic acid (TFA) and eluted with a gradient of acetonitrile (---) in 0.1% TFA at a rate of 20 ml/hr. Four percent of the column effluent was diverted to the fluorescamine monitoring system for protein detection (16). Aliquots of each fraction (2.5 min) were assayed for biological activity. ▨, TGF activity; ——, relative fluorescence.

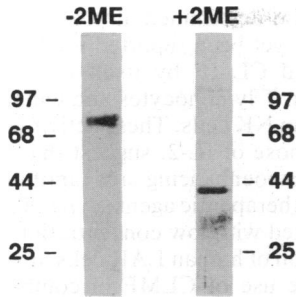

FIG. 2. SDS/PAGE of purified CLMF. Aliquots of pooled fractions containing the major peak of TGF activity eluting from the diphenyl column were analyzed under nonreducing (−2ME) and reduced (+2ME) conditions in the presence of 2-mercaptoethanol using a 10% slab gel. Visualization was by silver staining. The molecular masses indicated in kDa were estimated from standards electrophoresed in a parallel lane.

Therefore, in order to obtain the N-terminal sequences of the individual subunits, 140 μg of CLMF was reduced in 5% 2-mercaptoethanol in the presence of 4 M urea and heated for 5 min at 95°C. The sample was applied onto a Vydac C_{18} column, and the column was then washed with 5 ml of 0.1% TFA. Adsorbed proteins were eluted over 5 hr with a gradient of 0–70% acetonitrile in 0.1% TFA (data not shown). SDS/PAGE analysis of fluorescamine-positive fractions under nonreducing conditions revealed a diffuse band at 35 kDa that was greater than 95% pure (data not shown). The 40-kDa subunit was strongly adsorbed to the C_{18} column and could only be eluted with a solution of 42% formic acid/40% 1-propanol.

N-terminal sequence determination was attempted by automated Edman degradation on 100 pmol of the C_{18}-purified 35-kDa subunit. Results were obtained for the first 26 cycles and the residue assignments were as follows: NH_2-?-Asn-Leu-Pro-Val-Ala-Thr-Pro-Asp-Pro-Gly-Met-Phe-Pro-?-Leu-His-His-Ser-Gln-Asn-Leu-Leu-Arg-Ala-Val, where ? represents an undetermined residue.

With the N-terminal sequences of the heterodimeric CLMF and the 35-kDa subunit of CLMF determined, the N-terminal sequence of the 40-kDa subunit of CLMF was deduced. The N-terminal sequence of the 40-kDa subunit can be summarized as follows: NH_2-Ile-Trp-Glu-Leu-Lys-Lys-Asp-Val-Tyr-Val-Val-Glu-Leu-Asp-Trp-Tyr-Pro-Asp-Ala-Pro-Gly-Glu-Met.

Computer searches of GenBank and National Biomedical Research Foundation protein data bases revealed no significant homology to any currently available sequences.

Biological Characterization. Purified CLMF stimulated the proliferation of human PHA-activated lymphoblasts in the TGF assay (Fig. 3). When suboptimal concentrations of purified CLMF and human rIL-2 were tested in combination in the TGF assay, additive proliferation was observed, up to the maximum proliferation caused by rIL-2 alone (data not shown). However, proliferation caused by rIL-2 could be distinguished from proliferation due to CLMF in that the former was totally inhibited in the presence of a neutralizing goat anti-human IL-2 antiserum but the latter was not affected (Fig. 3).

The ability of purified CLMF to activate cytotoxic effector cells was examined in a 4-day LAK cell induction assay. In this assay, purified CLMF at concentrations as high as 125 units/ml had little activity in the absence of added IL-2 (Fig. 4). However, CLMF synergized with a low concentration (5 units/ml) of human rIL-2 in causing LAK cell induction. In

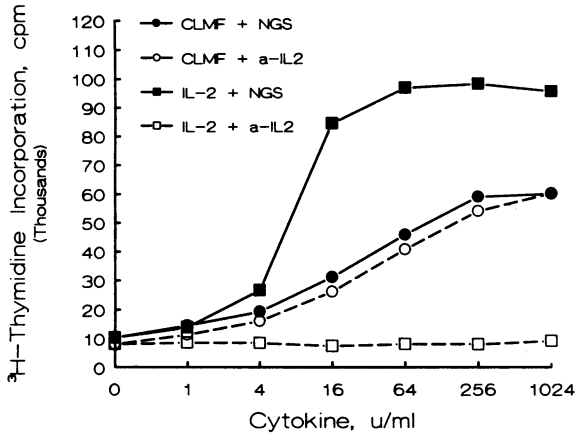

FIG. 3. Purified CLMF causes IL-2-independent proliferation of human PHA-activated lymphoblasts. Human PHA-activated lymphoblasts were cultured in various concentrations of human rIL-2 (□, ■) or purified, NC-37-derived human CLMF (○, ●), and [^{3}H]thymidine incorporation was measured after 2 days as described in the text (TGF assay). Goat anti-human rIL-2 (a-IL-2; □, ○) or normal, preimmune goat serum (NGS; ■, ●) was used at a dilution of 1:200.

the presence of rIL-2, purified CLMF was active at concentrations as low as 1 unit/ml.

Evidence that the biologic activities attributed to CLMF were, in fact, mediated by the heterodimeric CLMF protein was provided by experiments in which monoclonal anti-CLMF antibodies that immunoprecipitated the heterodimeric protein were shown to immunodeplete and, in some cases, to neutralize the bioactivity of CLMF. An example of such an experiment is shown in Table 2, in which the monoclonal anti-CLMF antibody 7B2 specifically inhibited proliferation of human PHA blasts induced by purified CLMF but had no effect on rIL-4-induced lymphoblast proliferation. Similar results were obtained in the LAK cell induction assay (data not shown).

DISCUSSION

In this study, a B lymphoblastoid cell-derived cytokine, tentatively named CLMF, was purified to homogeneity using classical and high-performance chromatographic procedures. The purified cytokine is a 75-kDa heterodimer composed of disulfide-bonded 40-kDa and 35-kDa subunits. Furthermore, several lines of evidence indicate that CLMF is a glycoprotein

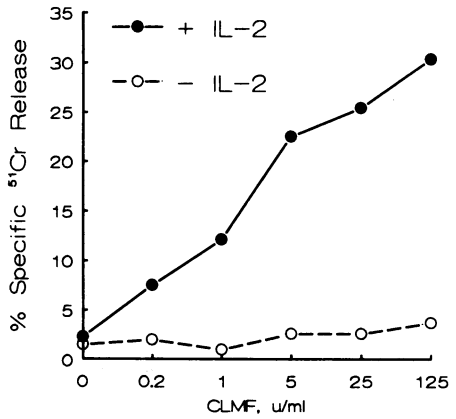

FIG. 4. Purified CLMF synergizes with low concentrations of rIL-2 to induce LAK cells. Human LAK cells were generated in the presence of various concentrations of purified, NC-37-derived human CLMF with (●) or without (○) human rIL-2, 5 units/ml. Lytic activity was measured on natural killer (NK)-resistant Daudi target cells at an effector:target ratio of 25:1. The spontaneous ^{51}Cr release was 11%.

Table 2. Neutralization of CLMF bioactivity by monoclonal anti-CLMF antibody

Assay contents		[^{3}H]Thymidine incorporation,	
Cytokine*	Antibody†	cpm	% inhibition‡
None	None	10,227 ± 331	—
CLMF	None	24,813 ± 1391	—
CLMF	Anti-CLMF IgG	10,763 ± 878	96
CLMF	Control rat IgG	27,654 ± 1086	0
rIL-4	None	25,328 ± 1638	—
rIL-4	Anti-CLMF IgG	26,256 ± 707	0
rIL-4	Control rat IgG	28,103 ± 2597	0

*Purified CLMF and rIL-4 (Genzyme) were used in the TGF assay at concentrations of 20 and 100 units/ml, respectively.
†Purified 7B2 monoclonal anti-CLMF antibody or a control rat IgG2a antibody was added at a final concentration of 200 μg/ml.
‡Reduction of [^{3}H]thymidine incorporation to the level seen in the absence of added cytokines was considered to be 100% inhibition.

with the 35-kDa subunit more extensively modified than the 40-kDa subunit (A.S.S. and F.J.P., unpublished data). Purified CLMF was shown in these studies to mediate two distinct biologic activities. It caused IL-2-independent proliferation of human PHA-activated lymphoblasts, and it synergized with low concentrations of human rIL-2 to stimulate the induction of human LAK cells. In both assays, purified CLMF displayed significant activity at concentrations <1 pM. That the 75-kDa heterodimer was, in fact, responsible for mediating these activities was strongly suggested by studies in which monoclonal rat anti-human CLMF antibodies were found to immunoprecipitate the 75-kDa heterodimer and to block the biological activities attributed to CLMF. Several cytokines unrelated to CLMF have previously been reported to augment human LAK cell generation in the presence of IL-2. These include IFN-γ (23), TNF (13), IL-1 (24), and, under certain circumstances, IL-4 (25). Of these cytokines, only TNF was active in our assay, in which we attempted to minimize endogenous cytokine production and the activation of cytokine cascades by rigorous depletion of accessory cells and inclusion of hydrocortisone in the culture medium.

The heterodimeric structure of CLMF is unique compared to previously characterized cytokines. Interestingly, we observed that the 40-kDa subunit of CLMF, as identified on SDS/PAGE and confirmed by immunoblotting, was present in NC-37 cell supernatant solutions in relatively large amounts free of the 35-kDa subunit (A.S.S. and F.J.P., unpublished data). This leads one to speculate that the 40-kDa subunit may be a carrier protein for a set of cytokines, and CLMF may be one of a family of multi-subunit immunomodulators. Precedent for this concept can be found in the family of adenohypophysial glycoprotein hormones (26) and in the inhibins (27). Members of each of these hormone families are heterodimeric proteins that share a common α subunit with other family members but possess distinct β subunits.

Kobayashi *et al.* (28) have recently identified a cytokine that also is composed of two chains with molecular masses of 40 and 35 kDa. This cytokine, called natural killer cell stimulatory factor (NKSF), was isolated from supernatant solutions from cultures of activated RPMI 8866 B lymphoblastoid cells. NKSF, alone and in synergy with IL-2, was shown to induce normal lymphocytes to produce IFN-γ. In addition, NKSF was found to augment the NK cell-mediated cytotoxicity of peripheral blood lymphocytes and to enhance the proliferative response of T cells to mitogenic lectins and phorbol diesters (28). We have found that purified NC-37-derived CLMF is capable of inducing the production of IFN-γ and enhancing NK activity in an overnight assay in the absence of added IL-2 (M.K.G., unpublished data). However, whether NKSF and CLMF are identical or closely related cannot be determined at present since no sequence information has yet been reported for NKSF.

NC-37-derived CLMF by itself caused proliferation of activated human T lymphocytes and enhanced the cytolytic activity of human NK cells. These activities of CLMF, which are similar to those of IL-2, suggest that CLMF, like IL-2, may have immunoenhancing and antitumor effects when used as a single therapeutic agent *in vivo*. In addition, purified CLMF synergized with low concentrations of rIL-2 in causing the generation of human LAK cells *in vitro*. These results suggest that the use of CLMF in combination with rIL-2 might constitute a more optimal antitumor therapy than using either cytokine alone.

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