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Biochemical Characterization and Purification of HILDA, a Human Lymphokine Active on Eosinophils and Bone Marrow Cells

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We previously described a lymphokine termed HILDA (for human interleukin DA) produced by T-lymphocyte alloreactive clones after antigenic stimulation. This factor sustains the growth of a murine IL3-sensitive cell line (DA2). In addition, HILDA is a potent activator of eosinophils and displays a burst-promoting activity on human bone marrow. In the present study, HILDA was purified to homogeneity from T-cell clone supernatant using successively sequential concentration, concanavalin A (ConA) affinity chromatography with differential elution (α-D glucopyranoside and α-D mannopyranoside), high-performance liquid chromatography (HPLC) gel filtration and reverse-phase HPLC. The pure material appeared as a 38-kd glycoprotein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or nonreducing conditions. Biologic activity could be recovered from SDS-PAGE gel slices corresponding to the 38-kd band. We conclude from the specificity of the DA-2 cell line and biochemical characteristics described that this lymphokine is different from other known factors produced by human T lymphocytes.

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CLASSICALLY, eosinophilia has been related to allergic conditions and parasitic infections. However, several reports have described graft-infiltrating eosinophils as well as blood eosinophilia early in the course of rejection crisis.[1] Some time ago, blood eosinophilia was also reported to be under T-cell control.[2]

Recently, we showed that culture supernatants of human T-cell clones derived from lymphocytes infiltrating rejected kidney allograft were able to trigger the proliferation of an interleukin-3 (IL-3)–sensitive murine cell line, DA-2. This activity, human interleukin DA (HILDA) was purified from such T-cell clone supernatants. Using this purified preparation, we also showed that HILDA as identified by the DA-2 proliferative assay had strong chemotactic and activating properties on mature eosinophils in mouse and human. On the other hand, this material was proven to have no colony-stimulating factor (CSF) activity on murine and human bone marrow, but exhibited in synergy with recombinant erythropoietin a burst-promoting activity.[3]

Although the knowledge about the biologic activities of this lymphokine is far from complete, we believe that HILDA may represent a new link between the immune system and specifically the eosinophils among mature granulocytes. In this article, we first describe the purification to homogeneity of the active molecule from a human T-lymphocyte clone conditioned medium; second, by testing a wide panel of mouse and human recombinant molecules in the DA-2 assay, we provide strong evidence that HILDA is distinct from many previously characterized growth factors.

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Submitted April 6, 1987; accepted January 25, 1988.

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0006-4971/88/7106-0013$3.00/0

MATERIALS AND METHODS

Cell Cultures.

Supernatants of human alloreactive T-lymphocyte clones (ATLCs) were harvested 4 days after specific antigenic stimulation in RPMI 1640 culture medium supplemented with 0.94 nmol/L pure recombinant IL-2 (courtesy of Biogen, Geneva) and 5% human agammaglobulinemic sera (Centre de Transfusion Sanguine, Lille, France) as already described in detail.[4] Supernatants were kept sterile and stored at 4°C until used. The HILDA biologic activity of all batches was assessed as indicated below and is given in units per milliliter. Only supernatants containing >100 U/mL were further used.

Assays of HILDA and Recombinant Cytokines

This assay, previously reported in detail,[3] is based on the proliferation of the murine DA2 cell line induced by HILDA. In the proliferation assay, 20×10^3 DA2 cells (in 0.05 mL) were added in duplicates to 0.05 mL of threefold dilutions of the HILDA containing supernatant to be tested or in IL-3 WEHI-3 conditioned medium (CM) for positive control in 96-well microtiter plates (Falcon, Oxnard, CA). After 36-hour incubation (37°C, 5% CO_2), 0.01 mL phosphate-buffered saline (PBS)-MTT solution (MTT [Sigma, St Louis] 5mg/mL of PBS) was added to the wells. Four hours later, 0.15 mL propanol-1-HCl 0.04 N was added according to the colorimetric assay previously described.[5] Microplates were then read in a Multiskan Titertek (Flow). Results are reported in optical density at 570 nm. The standard ATLC-CM was arbitrarily designated 100 U/mL (aliquoted and kept frozen at −80°C) and titrated in each experiment to determine maximum of absorbance arbitrary units. The dilution giving a response equal to 50% of the maximum was then determined (optical density 50% max). Each unknown sample was also titrated, and the dilution corresponding to the optical density 50% max was similarly determined. The ratio of dilutions of sample divided by standard and multiplied by 100 equals the number of units of HILDA per milliliter. Human and murine IL-4 were purchased from Genzyme (Boston). Human IL-2 was obtained from Biogen, pure interferon-γ (INF-γ) was obtained from Roussel-Uclaf (Paris), and pure tumor necrosis factor (TNF-α) was obtained from Biogen.

The remaining cytokines were a gift from G.G. Wong and S. Clark from Genetics Institute (Cambridge, MA).

Supernatant Filtration-Concentration

Crude supernatants (2 L) of ATLCs were first filtered through a PTHK cassette (100,000 mol wt retention, Millipore Minitan System, Strasbourg, France) to remove large molecular complexes and

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then concentrated ~60-fold using a PTGC cassette (10,000 mol wt retention).

ConA Ultrogel Chromatography

All chromatographic procedures were carried out at room temperature. ConA ultrogel (IBF) was packed in a 25-mL chromatographic column (IBF 25, ID 2.5 cm) and equilibrated with 40 mmol/L Tris-HCl buffer, pH 7.4, containing 110 mmol/L NaCl, 1 mmol/L MnCl2, 1 mmol/L MgCl2, and 1 mmol/L CaCl2 (buffer A). The concentrated HILDA-containing material (40 mL) was loaded on the column at a flow rate of 15 mL/h. The column was then washed with 100 mL buffer A before being eluted at a flow rate of 25 mL/h with different concentrations of α-methyl D-glucopyranoside (10 to 100 mmol/L) and α-methyl D-mannopyranoside (300 mmol/L). Fractions of 4 mL were collected and assayed for protein content and HILDA activity.

Ion-Exchange High-Performance Liquid Chromatography (HPLC)

Concentrated material (4 mL) eluted by α-methyl D-glucose or α-methyl D-mannose from the ConA ultrogel column was applied after extensive dialysis (against 20 mmol/L Tris-HCl buffer, pH 7.4) on an anion-exchange column [LKB ultropac diethylamino ethanol (DEAE) 5 PW, 7.5 × 75 mm, Beckman apparatus]. The elution was performed with 25 mL 20 mmol/L Tris-HCl buffer, pH 7.4, followed by a gradient of 0 to 0.5 mol/L NaCl in 20 mmol/L Tris-HCl, pH 7.4. Fractions of 1 mL were collected and assayed for HILDA bioactivity.

Gel-filtration HPLC

HILDA-active material not bound on the DEAE column and eluted in the void volume was concentrated and further applied to a preparative gel-filtration column (LKB ultropac TSK G 2000 SWG, 21.5 × 600 mm, Waters apparatus). Samples of 2 mL were run at 4 mL/min and eluted at the same rate with 0.12 mol/L NaCl in 20 mmol/L phosphate buffer, pH 7.2. Fractions of 4 mL were collected and assayed for HILDA activity. The column was calibrated with bovine serum albumin (BSA) (67 kd), ovalbumin (43 kd), chymotrypsinogen (25 kd), and ribonuclease (13.7 kd).

Reverse-Phase HPLC

Fractions of gel filtration with the highest HILDA activity were pooled, concentrated with polyethylene glycol (PEG) 35,000 (FLUKA), dialyzed against 0.1% trifluoroactic acid (TFA) in water, pH 2.5, and applied to the column.

Reverse-phase chromatography was performed with an LKB System and a TMS 250 column (4.6 × 75 mm), with 0.1% TFA in water as starting solvent. The column was eluted with increasing concentrations of acetonitrile in 0.1% TFA using the following gradient: 0 to 20% acetonitrile for 5 minutes, 20% to 60% for 40 minutes and 60% to 100% for 5 minutes. The flow rate was 0.8 mL/min.

Fractions of 0.8 mL were automatically collected, directly evaporated to dryness in a Speed Vac Concentrator-Evaporator system (Savant, Hicksville, NY), resuspended in a PBS buffer, pH 7.4 (1 mL), and assayed for HILDA activity.

Radiolabeling of Purified HILDA

The most active fraction from reverse-phase chromatography (2,000 U) was dialyzed, evaporated to dryness, and resuspended in 0.05 mL 50 mmol/L sodium-phosphate buffer, pH 7.6. This 0.05 mL was transferred to a glass tube coated with iodogen as described by,[6] and 0.002 mL (100 μCi) of (^{125}I)-Na (C.E.A., Saclay, France) were added. After 20 minutes of agitation at room temperature, the reaction was quenched by addition of 0.45 mL PBS. The mixture was then transferred to another glass tube, and 0.05 mL PBS containing 1% BSA was added to stabilize the protein. Radiolabeled proteins were separated from free iodine by chromatography on a Dowex AX1 (Biorad) column. Radioactive fractions were pooled, dialyzed against 10 mmol/L Tris-HCl buffer, pH 7.2, and chromatographed on a 1-mL anion-exchange column (DEAE Ultrogel, LKB) to separate radioactive HILDA, which eluted in the void volume, from contaminating radiolabeled BSA, which eluted when 1 mol/L NaCl was applied. The peak HILDA fraction (0.045 mL = 10,000 cpm) was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiographed for 4 days at −70°C. The same material (0.40 mL) was concentrated and run on the same gel for detection of HILDA activity after elution from the gel.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli[7] on linear 5% to 15% acrylamide gradient slab gels (1.5 mm thick). Before loading, lyophilized samples were heated for 30 minutes at 56°C in sample buffer under reducing or nonreducing conditions.[8] After electrophoresis (3 hours at 45 mV), the acrylamide gels were either stained by the silver-nitrate method of Merril[9] or cut in thin slices of 1 mm that were each eluted overnight at 4°C in RPMI 1640 containing 10% human serum (agammaglobulinemic) and 10 mg/mL BSA and assayed after centrifugation for HILDA activity. Apparent mol wt was determined with protein standards: phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and lactalbumin (14,400).

Protein Assays

For all steps up to HPLC gel filtration, protein content of fractions was estimated by the method of Lowry,[10] with BSA as standard. After this step, the protein content was too low to be determined with accuracy by any common protein assay method, so that protein concentration was estimated either from the absorbance profile at 280 nm, assuming an average OD 280 of 1 for a protein sample of 10 mg/mL, or from the intensity of silver-stained bands on polyacrylamide gels using BSA as standard. These estimates must, however, be considered approximate values.

RESULTS

General Characteristics

The proteinic nature of HILDA was indicated by the disappearance of its biologic activity after trypsin treatment. HILDA activity remained unchanged after 30-minute heating at 56°C and, after 15-minute heating of 100°C, 30% of the activity was still recovered. The molecule also retained its activity after 30 minutes under pH conditions ranging from 2 to 10.

The first filtration-concentration step, using 100,000 and 10,000 mol-wt retention membranes, allowed 80% of the protein content of the crude supernatant to be eliminated while retaining ~60% of the initial biologic HILDA activity. This first step already indicated a mol wt between 10,000 and 100,000 for HILDA and, although the isoelectric point (pI) of the molecule has not yet been determined, at pH 7.4 HILDA activity was not retained on DEAE-HPLC, whereas

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at pH 6.00 it bound on cation-exchange column (Mono-Q-FPLC), thus suggesting a rather basic nature of the molecule. In addition, retention of HILDA, activity on ConA-coupled affinity supports (Fig 1) indicated that it is a glycoprotein.

Purification

ConA affinity chromatography. As shown in Fig 1, the HILDA activity contained in the filtrated-concentrated material was completely retained on a ConA-coupled affinity column. Elution of the bound activity could be achieved either with α-methyl D-mannose (0.3 mol/L) or α-methyl D-glucose (0.01 and 0.1 mol/L). However, to take advantage of the differential affinities of ConA for D-glucopyranoside and D-mannopyranoside residue, we tested the ability of different concentrations of α-methyl D-mannose and α-methyl D-glucose to elute HILDA activity. An elution buffer containing 10 mmol/L α-methyl D-glucose was chosen (Fig 1) because of its ability to elute efficiently most of the HILDA activity and not the bulk of the protein bound to the lectin column. Further elutions with 0.1 mol/L α-methyl D-glucose and 0.3 mol/L α-methyl D-mannose yielded only minimal amounts of HILDA activity while eluting most non–HILDA-bound glycoproteins. The 10 mmol/L α-methyl D-glucose elution step therefore allowed a substantial gain as compared with a classical single α-methyl D-mannose elution step.

Ion-Exchange HPLC. When run at pH 7.4, the TSK-DEAE column did not bind HILDA biologic activity recovered in the void volume (Fig 2), whereas most serum proteins were bound to the column and eluted after exposure to increasing concentrations of salt.

Gel-filtration HPLC. Figure 3 shows that HILDA activity eluted in fractions 14 through 19, indicating that the active molecule has an apparent mol wt between 40 and 45 kd.

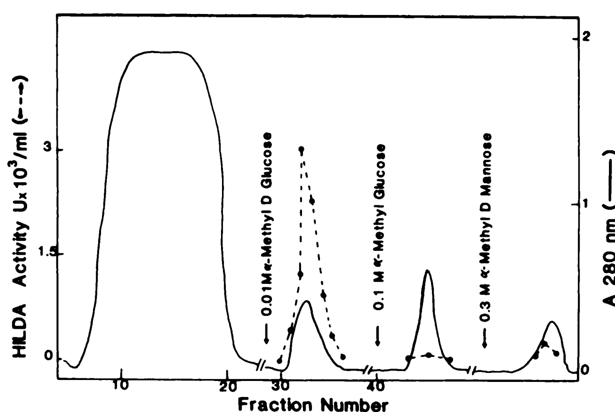

Fig 1. ConA Ultrogel chromatography. Concentrate (50 mL) was applied (15 mL/h) to a ConA ultrogel affinity column equilibrated with a 40 mmol/L Tris-HCl buffer, pH 7.4, containing 110 mmol/L NaCl, 1 mmol/L $MnCl_2$, 1 mmol/L $MgCl_2$, and 1 mmol/L $CaCl_2$ (buffer A). The column was washed with buffer A (100 mL) and eluted successively with 0.01 mol/L, 0.1 mol/L α-methyl D-glucose and 0.3 mol/L α-methyl D-mannose in buffer A (75 mL). Eluted fractions were assayed for HILDA activity.

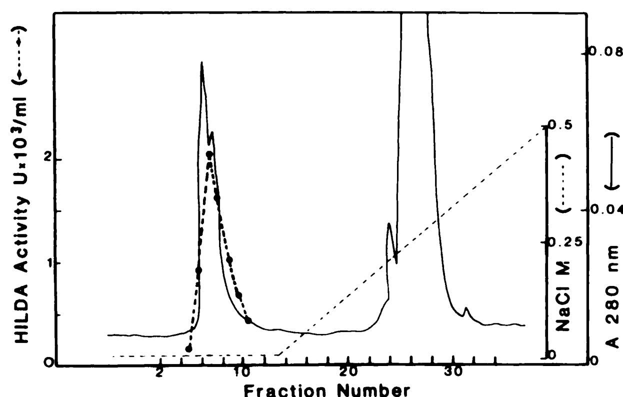

Fig 2. DEAE HPLC. The concentrated HILDA-active material dialyzed against 20 mmol/L Tris-HCl, pH 7.4, was applied to an anion-exchange column (LKB TSK DEAE 5 PW). Elution was performed at 1 mL/min using 20 mmol/L Tris-HCl buffer, pH 7.4, followed by a gradient of 0 to 0.5 mol/L NaCl. Eluted fractions (1 mL) were assayed for HILDA activity.

Reverse-phase HPLC. Figure 4 shows a typical elution profile obtained with a LKB TMS 250 column. HILDA-active fractions eluted between 25% and 30% acetonitrile.

Table 1 summarizes the results of the increase of specific activity and each step in the purification. The specific activity of HILDA increased significantly through each step, resulting in an overall purification of >85,000-fold with a yield of 15% of the biologic activity. Because the final yield of HILDA was too small to permit accurate protein determination, we could only estimate the specific activity of the purified factor to be $>3 \times 10^7$ U/mg.

Analysis of purity and mol-wt determination. The purified material had previously been analyzed on SDS-PAGE,[3] and the intensity of silver-stained bands was compared to the active biologic material eluted from a comparable gel. A visualized band that was at the threshold of detection corresponded to 1,000 U elutable biologic activity. In these

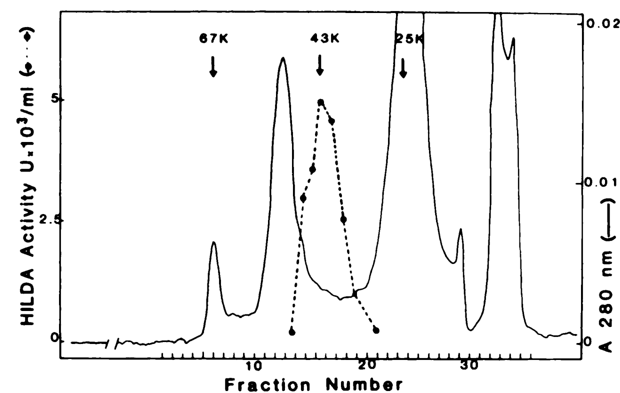

Fig 3. Gel-filtration HPLC. HILDA-active concentrated material was applied to a preparative gel-filtration HPLC column (TSK G 2000) previously calibrated with BSA (67 kd), ovalbumin (43 kd), chymotrypsinogen (25 kd), and ribonuclease (13.7 kd). Eluted fractions (4 mL) in 20 mmol/L phosphate, 120 mmol/L NaCl buffer, pH 7.2, were collected and tested for HILDA activity.

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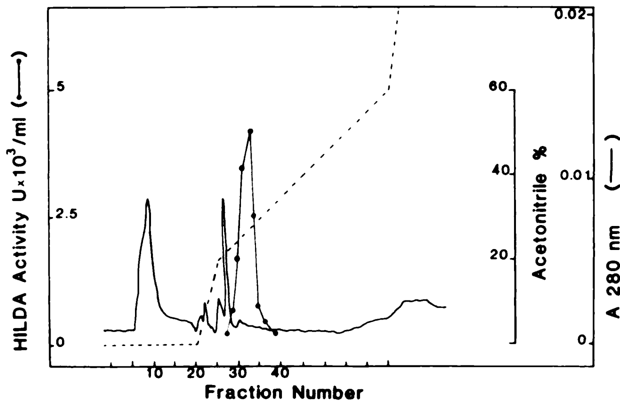

Fig 4. Reverse-phase HPLC. Fractions of gel filtration with the highest HILDA activity were pooled, concentrated, equilibrated with 0.1% TFA, pH 2.5, and applied to an LKB TMS 250 column. Reverse-phase HPLC was performed at 0.8 mL/min with 0.1% TFA as starting solvent followed by increasing concentrations of acetonitrile in 0.1 TFA (10% to 30% for 10 minutes, and then 30% to 70% for 40 minutes). Eluted fractions were collected, evaporated to dryness, resuspended in a small volume of PBS, and assayed for HILDA activity.

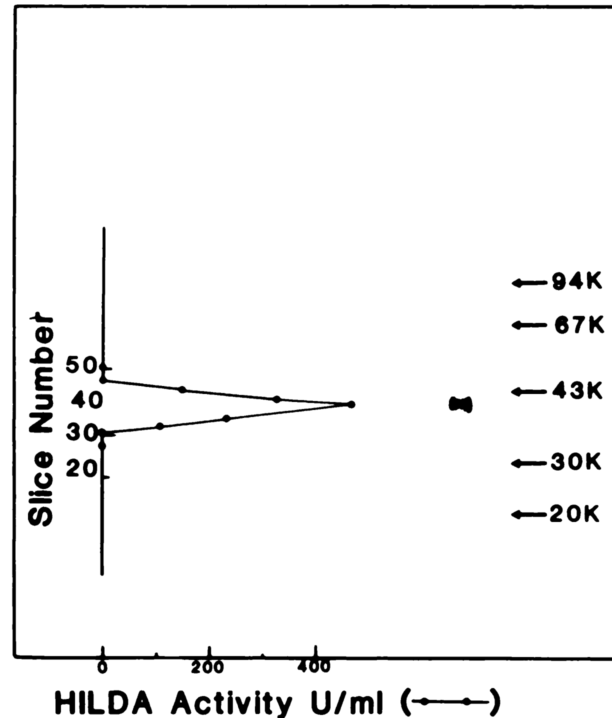

Fig 5. Analysis of the purity of HILDA. After boiling for 4 minutes in reducing SDS sample buffer, purified ^{125}I-labeled HILDA was submitted in two lanes to SDS-PAGE analysis. The first lane (A) was sliced, and slices of the gel were eluted overnight into 0.1 mL culture medium containing 10 mg/mL PSA carrier protein before being analyzed in the DA-2 proliferation assay for biologic activity. The comparison lane (B) was autoradiographed after staining and drying. The other track (b) was autoradiographed after staining and drying.

experiments, the percentage of total recovery in terms of biologic activity appeared to be ~10%. From control experiments with BSA standards (described in the Materials and Methods section), we estimated that the detection limit of the silver-stain method was ~10 ng protein; therefore, we deduced that 10 ng pure HILDA corresponded to 1,000 to 10,000 U. This analysis suggests that the protein eluted from the active band in the gel was active in the HILDA assay at concentrations as low as 1 to 10 pmol, consistent with identification of the silver-stained band as HILDA.

To detect minor contaminants and trace amounts of HILDA, we decided to radiolabel the most active peak fraction of the purified material recovered from reverse-phase HPLC (2,000 U). One-tenth of the iodinate material (10,000 cpm) was loaded on SDS-PAGE for autoradiography, and the remainder was loaded on the same gel for biologic analysis. As shown in Figure 5, autoradiography of the gel revealed a single band at 38,000 Kd without detectable contaminants. Similarly, from the companion lane, the biologic activity eluted at the same level, clearly demonstrating that a single molecule could be responsible for the triggering of the DA-2 proliferation and also that the biologic activity of HILDA was impervious to iodination and boiling in SDS.

Effect of Recombinant Mouse and Human Cytokines on DA-2 Proliferative Response

Table 2 summarizes the effects of the various recombinant cytokines on DA-2 cells. Although these cells proliferated well in the presence of murine IL-3, IL-4, and granulocyte-macrophage colony-stimulating factor (GM-CSF), they failed to respond to any of the corresponding human homologues. Moreover, among the large panel of other cytokines tested, only human G-CSF reacted with DA-2 cells to trigger their proliferation.

DISCUSSION

We recently characterized HILDA as a human factor produced by T-cell clones triggering proliferation of a

Table 1. Purification of Human HILDA

Purification Step	Total Protein (mg)	Total Activity (U × 10³)	Specific Activity (U/mg Protein)	Purification Factor	Yield (%)
Crude supernatant	6,200	220	35.5	1	—
Concentrated material	1,100	132	120	3.4	60
ConA affinity	11	110	10^4	282	50
DEAE HPLC	3	95	$3.2.10^4$	892	43
Gel-filtration HPLC	0.6	66.5	$11.1.10^4$	3,122	30
Reverse-phase HPLC	<0.01	30.1	$>300.10^4$	>85,000	14

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Table 2. Effect of Recombinant Mouse and Human Cytokines on DA-2 Proliferative Response

Cytokines	Positivity on DA-2 Test	Origin	Concentration*	Maximal Concentration Tested	Reference
A: Human and gibbon recombinant factors					
Pure IL-2	0	*Escherichia coli*	—	Up to 60 nmol/L	11
Pure GM-CSF	0	CHO cells	1/1,000	1/6	12
G-CSF	+	COS supernatant	1/1,000	1/6	13
M-CSF	0	COS supernatant	1/1,000	1/6	14
Pure IL-α	0	COS supernatant	1/1,000	1/6	15
Gibbon IL-3	0	COS supernatant	1/1,000	1/6	16
Pure IL-4	0	*E coli*	20,000 U/mL	3,300 U/mL	17
IL-5	0	COS supernatant	1/1,000	1/6	18
IL-6	0	COS supernatant	1/1,000	1/6	19
INF-γ	0	*E coli*	100,000 U/mL	33,000 U/mL	20
TNF-α	0	*E coli*	—	Up to 5 nmol/L	21
B: Murine recombinant factors					
Pure IL-3	+	COS supernatant	1/1,000	1/6	—
Pure IL-4	+	*E coli*	20,000 U/mL	3,300 U/mL	—
GM-CSF	+	COS supernatant	1/2,000	1/6	—

*Concentration of active protein is expressed either by the last dilution still giving a maximal response or in arbitrary units both determined in appropriate assays as described in references given.

murine IL-3–sensitive cell line. HILDA has also proved to be a potent chemoattractant and activator of peripheral eosinophils and a growth burst-promoting factor for marrow-derived erythroid progenitors.[3]

Several other groups have reported the existence of growth-promoting activities for IL-3–sensitive murine cell lines in various culture supernatants, including those of autologous mixed lymphocyte culture[22] and activated T lymphocytes.[23,24] These factors have in several instances been termed "human IL-3"[4] because of their activity on IL-3–sensitive murine target cell lines, although no functional or biochemical characterizations were reported. Recently, a cDNA sequence encoding human IL-3 was isolated, and the recombinant human protein proved to be inactive in supporting the proliferation of murine target cells.[25] Thus, HILDA, as well as the other human factors that support the proliferation of murine IL-3–dependent lines are distinct from human IL-3.

This report describes the purification of HILDA to apparent homogeneity using a five-step procedure. We characterized the purified HILDA as a hydrophobic glycoprotein of 38 kd with specific activity of $\sim 10^8$ to 10^9 U/mg. Our standardized protocol typically gives an 85,000-fold purification with a recovery of 15% of the biologic activity of the starting material.

The DA-2 cell line has been analyzed for its proliferative response with a wide panel of recombinant human and murine growth factors. Of the human factors tested, only G-CSF and HILDA supported DA-2 cell proliferation. DA-2 cell does not respond to human IL-1-α, IFN-γ, GM-CSF, M-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, and TNF-α. However, the DA-2 cells do respond to the murine IL-3, GM-CSF, and IL-4, all factors that are relatively species restricted. In this regard, HILDA and G-CSF appear to cross the species barriers very well.

The biochemical and biologic properties of HILDA were distinct from those of G-CSF. First, G-CSF and HILDA have significantly different mol wt. Second, HILDA does not bind to DEAE resins at pH 7.4, sharing this property only with human IL-3.[26] Third, our HILDA step preparations after the gel filtration are incapable of supporting hematopoietic progenitor cell colony formation using either human or murine bone marrow cells. Finally, the 32Dcl cell line, which responded to recombinant G-CSF, is unresponsive to crude CM from our T-cell clones; neither will it proliferate in the presence of the highly purified HILDA. Therefore, we believe that HILDA represents a novel lymphokine, but definitive proof of this conclusion awaits the molecular cloning of the corresponding gene.

We previously ascribed to the purified HILDA (at the gel filtration step[3]) biologic effects with erythroid progenitor cells and with mature eosinophils. Recently, we confirmed that at least with eosinophils, purified HILDA is responsible for the previously described biologic effects (unpublished data, 1987).

That HILDA has been produced by most of the T-lymphocyte alloreactive clones that we have tested[4] suggests that this molecule may play a role as a mediator in the rejection process. This possibility is reinforced by the fact that an increase of the peripheral eosinophil count in allograft recipients' blood precedes rejection episodes.[1] Furthermore, nude mice fail to develop eosinophilia in response to parasite infection, suggesting that eosinophil production is controlled in part by T lymphocytes and their secreted lymphokines such as HILDA or IL-5.[2] Thus, HILDA may prove useful in further elucidating the host-defense mechanisms against parasites as well as the role of T-lymphocyte activation in triggering eosinophils in various immunopathologic models.

ACKNOWLEDGMENT

We thank the Biogen Company, Geneva, for providing us with recombinant IL-2, Drs G.G. Wong and Y.C. Yang for their gift of recombinant cytokines, and Dr S. Clark for a critical reading of the manuscript.

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