Identification of a human non-interferon lymphokine activating monocyte complement biosynthesis

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A monocyte-stimulating activity produced by mitogen-induced mononuclear cells has been defined by its ability to enhance the synthesis *in vitro* of complement C1 subcomponents, C2 and C3. A lymphokine responsible for this activity was purified from culture supernatants of peripheral blood mononuclear cells activated by staphylococcal enterotoxin A. From 0.5 litre of supernatant the purification procedure [(NH₄)₂SO₄ precipitation, phenyl-Sepharose chromatography and preparative electrofocusing] yielded about 100 pmol of purified lymphokine. Its pI is 7.9 and its M_r, estimated by SDS/polyacrylamide-gel electrophoresis, is 14600, 27000 and 56000, the high-M_r species representing oligomeric forms of the M_r-14600 molecule. Its amino acid analysis reveals a high percentage of hydrophobic amino acids (34 %); the absence of histidine residues suggests that it is a novel monocyte-activating lymphokine. It enhances C1r and C1s biosynthesis at a pretranslational level. From its structure and activity this lymphokine appears different from γ -interferon.

INTRODUCTION

Although the bulk of plasma complement proteins is produced by hepatocytes, it is now established from experiments *in vitro* that monocytes are able to produce most components of the system (Cole & Colten, 1988). Local production of complement by phagocytes in the tissues is likely to be of significance in host defence (Colten *et al.*, 1979), as it depends on the activation state of the macrophage, at difference with proteins, such as lysozyme, which are secreted constitutively (Cole *et al.*, 1982; Takemura & Werb, 1984). As previous studies indicated that conditioned media are able to increase the production of C2 (Littman & Ruddy, 1977) and of C1 subcomponents (Bensa *et al.*, 1983) by monocyte cultures, we chose these media as a source for the purification of a new activation factor. γ -Interferon (IFN- γ) is the best documented lymphokine known to modulate mononuclear-cell functions (Schreiber & Celada, 1985). It has been shown to increase synthesis of C2 and factor B by monocytes (Strunk *et al.*, 1985; Hamilton *et al.*, 1987) and to decrease the synthesis of C3 (Takemura *et al.*, 1986); however, as demonstrated by Sanders & Littman (1986), IFN- γ is not the only lymphokine accounting for the enhancing activity in supernatants (conditioned media) of mitogen-stimulated mononuclear cell cultures.

We report here the purification and characterization of a human lymphokine secreted by mitogen-stimulated mononuclear cells. Our studies indicate that the enhancement of complement secretion is supported by a 14 kDa protein distinct from other lymphokines which activate macrophage functions, such as IFN- γ or IL-4 (Schreiber *et al.*, 1983; Crawford *et al.*, 1987).

MATERIALS AND METHODS

Media and reagents

Ficoll-Hypaque, RPMI 1640 medium and fetal-calf serum (FCS) were purchased from Flow Laboratories, Puteaux, France. FCS was heat-inactivated for 30 min at 56 °C. Calf thymus DNA was obtained from Sigma. Staphylococcal enterotoxin A (SEA) was from Serva, Heidelberg, Germany. IgG Sorb was from the Enzyme Center, Malden, MA, U.S.A. Affinity-purified human γ -interferon (1.2 $\times$ 10⁶ units/mg) was from Cellular Products Inc., New Jersey, NJ, U.S.A. L-[³⁵S]Methionine (specific radioactivity > 800 Ci/mmol) was from New England Nuclear, and Na¹²⁵I (~ 2000 Ci/mmol), [α -³²P]-dATP (~ 300 Ci/mmol) and [α -³²P]dCTP (~ 3000 Ci/mmol) from Amersham International. Other chemicals and reagents were of analytical grade.

Complement proteins and antibodies

C1q, C1r and C1s were purified from human plasma as described by Arlaud *et al.* (1979). C2 and C3 were purified according to Thielens *et al.* (1982) and Al Salihi *et al.* (1982) respectively. Antisera to human C1 subcomponents, C2 and C3 were raised in rabbit and immunoglobulins were prepared by Na₂SO₄ precipitation of sera (Prah & Porter, 1968).

Separation and culture of monocytes

Blood from healthy donors (Centre de Transfusion Sanguine, Grenoble, France) was collected in the presence of heparin (15 units/ml). Monolayers of adherent monocytes were established from Ficoll-separated human

Abbreviations used: FCS, fetal-calf serum; IL-1, -2, -3 and -4, interleukin-1, -2, -3 and 4; IFN- γ , γ -interferon; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; SaCl, *Staphylococcus aureus* (Cowan 1 strain); SEA, staphylococcal enterotoxin A; TNF, tumour necrosis factor.

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peripheral blood mononuclear cells (PBMC) and maintained as previously described (Bensa *et al.*, 1983). Briefly, PBMC ($d = 1.077$) were layered on to 24-well plates for 1 h in a humidified atmosphere of CO₂/air (1:19) at 37 °C, in RPMI 1640 medium supplemented with 15% (v/v) heat-inactivated (30 min, 56 °C) autologous serum. The monolayers were washed three times in RPMI 1640 medium alone to remove platelets and non-adherent cells. After 8–10 days in culture with the same supplemented medium as above, the monolayers were washed three times. Cells were considered as macrophage-like secreting cells at this time and were cultivated further in RPMI 1640 medium containing 10% (v/v) heat-inactivated FCS instead of autologous serum.

Preparation of conditioned media

Non-adherent PBMC were centrifuged (15 min, 150 g, r_{av} , 14.7 cm) to remove most of the remaining platelets and suspended in RPMI 1640 medium containing 5% (v/v) heat-inactivated FCS. Cells were cultivated in the presence of 0.71 nM-SEA for 3 days. Culture supernatants were stored at –30 °C.

Measurements of secreted C1 subcomponents, C2 and C3

Secreted proteins in supernatants were quantified by a radioimmunoassay as follows. Medium (500 μ l) was diluted with 500 μ l of SaCl buffer [150 mM-NaCl, 0.5 mM-EDTA, 50 mM-Tris/HCl (pH 7.4), containing 0.25% Nonidet P40, 0.02% NaN₃ and 0.5% ovalbumin] and incubated overnight at room temperature with 75 μ l of ¹²⁵I-radiolabelled antigen (C1q, C1r, C1s, C2 or C3) and 100 μ l of the corresponding antibody at half-saturating concentration. Then 100 μ l of a 10% (w/v) suspension of IgG Sorb in SaCl buffer was added, and the mixture was further incubated for 1 h. After centrifugation (15 min, 9000 g, r_{av} , 8.6 cm), ¹²⁵I radioactivity was measured on the SaCl pellet. For C1q measurements, NaCl concentration in SaCl buffer was raised to 0.2 M and the medium was adjusted to 80 mM-MgCl₂ just before SaCl addition in order to prevent non-specific binding of C1q to SaCl. Purified components were used as standards.

DNA determination in cell lysates

After harvesting of supernatants, adherent cells were washed once with 1 ml of PBS (137 mM-NaCl, 2.7 mM-KCl, 8 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, pH 7.2), covered by a lysis buffer, and DNA content in frozen and thawed lysate was determined according to West *et al.* (1985). Standards were prepared from purified calf thymus DNA (0.5–4 μ g). An average value of 1.1 μ g of DNA corresponds to 10⁵ mononuclear cells.

Interferon assays

Antigen IFN- γ was assayed by the IMRX kit from Centocor Inc. (Malvern, PA, U.S.A.). Interferon activity was titrated by cytopathic inhibition in MRC-5 cells with vesicular-stomatitis virus as challenge virus (Dean & Virelizier, 1983).

¹²⁵I-labelling of proteins

Complement proteins were ¹²⁵I-labelled to a specific radioactivity of 0.2–1.0 Ci/mmol by using the Iodogen method (Salacinski *et al.*, 1981). The purified lymphokine was labelled using the Bolton and Hunter reagent. ¹²⁵I-

labelled proteins were separated from free iodine by gel filtration on Sephadex G-50 fine as described by Penefsky (1977); for the lymphokine the gel was equilibrated in 4 M-urea/50 mM-Tris/HCl, pH 7.4, keeping a ratio of 1:40 (v/v) between sample and bed gel.

SDS/polyacrylamide-gel electrophoresis

Gel electrophoresis was performed in 12.5% (w/v) polyacrylamide/0.1% (w/v) SDS slab gels as described by Laemmli & Favre (1973), except that samples were reduced (60 min, 37 °C) in the presence of 50 mM-dithiothreitol and 4 M-urea. Marker proteins were BRL low- M_r standards (Gibco-BRL, Cergy-Pontoise, France). After electrophoresis the gels were stained with Coomassie Blue, dried and autoradiographed with Hyperfilm- β max (Amersham International) at –70 °C.

Amino acid analysis

After lyophilization, samples were hydrolysed for 24 h under reduced pressure at 110 °C in constant-boiling HCl containing 0.5% (w/v) phenol. Analyses were carried out by reverse-phase h.p.l.c. of amino acid phenylthiocarbamoyl derivatives with a Pico-Tag amino acid analysis system (Waters Associates–Millipore, St. Quentin-Yvelines, France).

DNA probes

The C1r probe was a 0.50 kb *Bam*H1/*Pst*I fragment of plasmid pHClr/253 (Journet & Tosi, 1986). The C1s probe, a 0.59 kb *Bam*H1 fragment from plasmid pHClS 4.6 (Tosi *et al.*, 1987) was a kind gift from Dr. M. Tosi, Institut Pasteur, Paris, France. Both were radiolabelled using [α -³²P]dATP and [α -³²P]dCTP with the BRL kit 8160 SB (Gibco-BRL). They were heat-denatured (15 min, 95 °C) just before use.

Extraction of total RNA and dot-blot analysis

Total RNA was extracted from monocytes by lysis with 5 M-guanidinium thiocyanate and purified by centrifugation through 5.7 M-caesium chloride at 118000 g (r_{av} , 9.17 cm) for 18 h (Chirgwin *et al.*, 1979). RNA was quantified by u.v. absorbance at 260 nm (1 A_{260} unit = 40 μ g of RNA/ml); 1–1.5 μ g of RNA were isolated from 5×10^5 adherent monocytes, and no significant difference in RNA recovery between stimulated and control monolayers was observed. It was dissolved in 25 mM-Na₂HPO₄/NaH₂PO₄, pH 6.5, and heated (15 min, 60 °C) in the presence of 1.25 M-formaldehyde (White & Bancroft, 1982). Serial 2-fold dilutions (50 μ l) were dotted onto Gene Screen membranes (New England Nuclear) by using a Schleicher und Schuell minifold apparatus. After heating (2 h, 80 °C) *in vacuo*, filters were pre-hybridized and hybridized with nick-translated DNA probes [(0.7–4.0) $\times 10^8$ c.p.m./ μ g] using the dextran sulphate method according to specifications provided by the manufacturer (New England Nuclear), and subsequently submitted to autoradiography.

Translation *in vitro* of total RNA and immunoprecipitation

A translation system using a rabbit reticulocyte lysate provided by Dr. T. Meo, Institut Pasteur, Paris, France, was used for optimized cell-free translation of total RNA as described by Tosi *et al.* (1987). Translation was for 2 h at 30 °C. Immunoprecipitation of the translated proteins was carried out, according to Reboul *et al.*

Table 1. Secretion of C1q, C1s, C2 and C3 by stimulated and non-stimulated monocytes

Monocytes from four cultures were stimulated in the presence of 5% (v/v) conditioned medium. Values for secreted proteins are means $\pm$ S.D. for triplicate samples collected after three consecutive periods of 48 h.

Experiment	Secreted proteins (ng/24 h per μ g of DNA)			
	C1q	C1s	C2	C3
1 ($n = 3$)				
Stimulated monocytes	29.65 $\pm$ 4.90	1.98 $\pm$ 0.22	4.27 $\pm$ 0.081	80.04 $\pm$ 18.50
Controls	8.09 $\pm$ 0.54	0.20 $\pm$ 0.08	0.53 $\pm$ 0.11	19.54 $\pm$ 7.62
2 ($n = 3$)				
Stimulated monocytes	7.54 $\pm$ 0.95	6.91 $\pm$ 1.33	6.84 $\pm$ 1.22	727.12 $\pm$ 96.26
Controls	2.87 $\pm$ 1.46	0.20 $\pm$ 0.09	0.67 $\pm$ 0.10	177.24 $\pm$ 75.02

(1986), with anti-C1s antibodies (Villiers *et al.*, 1982) or commercially available antibodies (anti-lysozyme; Calbiochem). SDS/polyacrylamide (12.5%, w/v) gel electrophoresis was performed under reducing conditions. The gel was impregnated with En³Hance (New England Nuclear) and autoradiographed.

RESULTS

Activation of monocytes by conditioned media: secretion of C1q, C1s, C2 and C3

Cultures of monocytes were incubated for three successive periods of 48 h in medium alone (non-stimulated monocytes) or in the same medium containing 5% (v/v) of a pool of conditioned media previously dialysed against PBS (stimulated monocytes). The supernatants were collected at the end of each period and protein secretion was measured. The mean and the S.D. were calculated from the protein secretion in each culture (Table 1). The same protocol was used to estimate the activity of chromatographic eluates (reported below). The mitogen SEA itself was demonstrated to be without activity on monocyte protein secretion.

Addition of conditioned media to the culture enhanced protein secretion (Table 1). The enhancement was variable among the different proteins in the same culture, and the secretion of each protein varied among several different cultures. The increase of C1s and C2 secretions (10-fold or more) was at least twice the increase of C1q and C3 secretions (about 4-fold). For this reason, and also because of the interest of our laboratory in this protein and the closely related protein C1r, C1s secretion was chosen as a marker of the monocyte activation.

Purification of a monocyte-activating lymphokine from conditioned media

Addition of 5% FCS to culture medium was necessary to prevent loss of lymphokine activity. A pool of conditioned media (0.5 l) was treated with 3.9 M-(NH₄)₂SO₄ (final concentration) for 60 min at 0 °C. After centrifugation for 30 min at 1000 *g* (r_{av} , 8.6 cm) and 4 °C the resulting precipitate was discarded; the supernatant was concentrated by ultrafiltration (Amicon PM 10) to 50 ml and extensively dialysed against 150 mM-NaCl/20 mM-Na₂HPO₄/NaH₂PO₄, pH 7.2. The sample was then filtered through a 0.45 μ m membrane. The filtrate (120 ml) was then applied to a column of phenyl-Sepharose CL4B. Elution was first with a low-ionic-

strength buffer, followed by a linear gradient of ethylene glycol. Fig. 1 shows the elution profile of proteins, the lymphokine activity (based on activation of C1s secretion) and the IFN- γ content. Most of the lymphokine activity eluted at low ionic strength was IFN- γ . A group of active fractions eluted at 25–80% (v/v) ethylene glycol was not related to IFN- γ (Fig. 1b). Two peaks could be distinguished in this group, a minor one [peak 1; 25–40% (v/v) ethylene glycol] associated with high A_{280} values and a major one [peak 2; 60–80% (v/v) ethylene glycol] with low A_{280} values (Fig. 1a). At the present time it is not clear if these two activity peaks are related or represent two distinct monocyte-activating factors. This study focused on the characterization of peak 2, which was demonstrated to contain less than 3 units of INF- γ /ml. Pool 2, corresponding to the active fractions of peak 2, was concentrated to 5 ml on an Amicon YM5 membrane and extensively dialysed against water. It was then fractionated by isoelectric focusing in the presence of ethylene glycol to minimize hydrophobic interactions. The dialysed fraction was supplemented with 20% (v/v) saccharose and 0.8% (w/v) LKB ampholytes (pH 5–10.5), in order to apply it close to its final position. A constant voltage of 600 V was maintained for 48 h, leading to a final current of 0.3 mA; after elution the collected fractions were assayed for biological activity. As shown in Fig. 2, the major monocyte-stimulating activity was detected in fractions from pH 7.6 to pH 8.1. The concentration of pure lymphokine in the pH 7.9–8.0 fraction, deduced from the amino acid composition, was 30 pmol/ml; thus about 100 pmol of pure lymphokine was obtained from 0.5 l of conditioned media.

SDS/polyacrylamide-gel electrophoresis of the purified lymphokine

The final preparation (pH 7.9–8.0 fraction) obtained after electrofocusing was labelled with ¹²⁵I and analysed by SDS/polyacrylamide-gel electrophoresis followed by autoradiography (Fig. 3). Two major protein bands (M_r 14 600 and 56 000) and one minor protein band (M_r 27 000) were seen under both reducing (Fig. 3, lane 1) and non-reducing (not shown) conditions. To analyse the relationship between these three bands, a 0.4 ml fraction of phenyl-Sepharose pool 2 was reduced, alkylated (140 mM-iodoacetamide) and loaded on to an electrophoresis slab gel. Immediately after migration the gel was sliced between the migration front and the M_r -18 000 migration zone. Proteins were electroeluted from the

Fig. 1. Phenyl-Sepharose chromatography of supernatant from $(\text{NH}_4)_2\text{SO}_4$ precipitation

Phenyl-Sepharose column (2.5 cm $\times$ 16 cm) was equilibrated in 150 mM-NaCl/20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2, and used at a flow rate of 0.3 ml/min. A sample of 120 ml (A_{280} 0.9) was applied and the column washed with the bed gel volume of equilibration buffer. Elution was first with 20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2, followed by a linear ethylene glycol gradient (0–80 %, v/v) in the same buffer (total elution volume 160 ml). The stimulating activity on monocytes and the IFN- γ content were assayed on groups of two fractions (2×3 ml) after extensive dialysis against PBS (137 mM-NaCl, 2.7 mM-KCl, 8 mM- Na_2HPO_4 , 1.5 mM- KH_2PO_4 , pH 7.2) and concentration to 2 ml on an Amicon YM5 ultrafiltration membrane. (a) A_{280} (●) and activity (○) evaluated by secretion of C1s (mean $\pm$ s.d., triplicate samples collected after three consecutive periods of 48 h) were tested in each eluted fraction, used at the concentration of 5% (v/v) in monocyte culture medium as described in the Results section. The secretion of non-stimulated monocytes is indicated at the right bottom by ★. (b) IFN- γ (■) was quantified in the same fractions as above (mean $\pm$ s.d., duplicate samples) by an immunoradiometric assay (1 unit corresponds to 0.12 ng). Fractions 59–64 and fractions 71–76 were pooled separately and designated as pool 1 and pool 2 respectively. ----, Ethylene glycol gradient.

slice of gel (3 mA, 150 V; 16 h at 4 °C) inside a dialysis bag buffered by 192 mM-glycine/25 mM-Tris, pH 8.3, and subsequently ^{125}I -labelled. Re-electrophoresis of the proteins eluted from the slice revealed three protein bands after autoradiography, of respective M_r values 14600,

27000 and 56000, identical with that shown in Fig. 3 (Fig. 4). This result shows that the monocyte-stimulating lymphokine is likely to oligomerize and that protein bands of M_r 27000 and 56000 represent oligomers (dimer and tetramer respectively) of the M_r 14600 form.

Fig. 2. Isoelectric-point determination of the monocyte-activating lymphokine

Pool 2 from the phenyl-Sepharose eluate was separated by preparative isoelectrofocusing in a 5–40% (w/v) saccharose gradient containing 0.8% (w/v) ampholytes using a LKB 8100-1 column. Cathodic and anodic solutions were 0.2 M-triethylamine/20% (v/v) ethylene glycol/50% (w/v) saccharose and 0.2 M-citric acid/20% (v/v) ethylene glycol respectively. A constant voltage of 600 V was applied for 48 h. Fraction (1 ml) were collected and the pH tested. Fractions between pH 9.0 and pH 6.0 were pooled in threes, supplemented with 1% (v/v) FCS (final concn.) and extensively dialysed against PBS. Pools were subsequently assayed for the stimulating activity on monocytes (●) as described in Fig. 1(a). The secretion of non-stimulated monocytes is indicated at bottom right by ★. An arrow indicates the fraction used for electrophoretic analysis.

Treatment of the purified lymphokine with peptide *N*-glycosidase F (EC 3.2.2.18) purified from the culture supernatant of *Flavobacterium meningosepticum* (Aude *et al.*, 1988) did not change the electrophoretic mobility of the protein (not shown). This finding suggests the absence of *N*-linked carbohydrates from the lymphokine.

Amino acid composition

The amino acid composition of the purified 14 kDa lymphokine (the pH 7.9–8.0 eluate from electrofocusing) is given in Table 2. The composition of the 14 kDa lymphokine purified by electroelution of a polyacrylamide-gel fragment (see above) was similar. These results, in addition to the electrophoretic results, confirm the identity between the molecules purified by these two different methods.

The amino acid composition is characterized by the presence of two cysteine and seven glutamic acid residues and by the absence of histidine.

Comparison of the 14 kDa lymphokine and IFN- γ activities on C1s secretion by monocytes

Stimulation by the 14 kDa lymphokine was studied with phenyl-Sepharose pool 2 eluate. Addition of 14 kDa lymphokine or IFN- γ to cultures enhanced C1s secretion in a dose-dependent fashion (Table 3) without significant changes in cell number or viability (not shown). A plateau was reached with the two stimulating agents. The secretion of C1s was enhanced by doses of IFN- γ as low as 1 unit/ml (0.03 pmol/ml); maximal stimulation was

Fig. 3. Electrophoretic analysis of electrofocusing-purified lymphokine and recombinant IFN- γ

Electrophoretic separation of samples and autoradiography were as described in the Materials and methods section. Lane 1, 125 I-labelled purified lymphokine (10000 c.p.m.); lane 2, 125 I-labelled recombinant IFN- γ (10000 c.p.m.).

achieved at a concentration of 100 units/ml (3 pmol/ml). Concentrations of 14 kDa lymphokine as low as 1 pmol/ml were active; maximal stimulation was at a concentration of 12 pmol/ml.

Effect of the lymphokine on the transcription of C1r and C1s genes

Monocytes from three cultures were incubated at day 12 with the lymphokine for 24 h. Total RNA was then isolated and subjected to dot-blot analysis. In parallel, culture supernatants were collected to measure C1r and C1s secretions. The results in Fig. 5 indicate that the 14 kDa lymphokine increases similarly C1s and C1r mRNA production. In parallel, secretions of C1r and C1s were both enhanced by a factor of about 12 (results not shown). These results suggest that the modulation of expression of the corresponding genes is co-ordinated.

Cell-free translation of monocytic C1s mRNA, induced or not by 14 kDa lymphokine, was evaluated with a rabbit reticulocyte-lysate system, taking lysozyme mRNA translation as a reference. As shown in Fig. 6, the amount of C1s translated from stimulated-cell mRNA (lane 1) was higher than the amount of C1s translated from non-stimulated-cell mRNA (lane 2). This is clearly visible at the level of A and B chains and at the level of proenzymic form. These results confirm that the increase

Fig. 4. Oligomerization of the 14 kDa lymphokine

The phenyl-Sepharose eluate [60–80 % (v/v) ethylene glycol] was reduced (50 mM-dithiothreitol) and alkylated (140 mM-iodoacetamide) before electrophoresis. After migration, the gel was sliced between the migration front and the M_r -18000 zone; electroelution was as described in the text. The ^{125}I -labelled eluate (30000 c.p.m.) was re-analysed by SDS/polyacrylamide (12.5%, w/v)-gel electrophoresis in reducing conditions. The gel was fixed and autoradiographed.

in C1s secretion due to the stimulation by the 14 kDa lymphokine is related to the increase of C1s mRNA.

DISCUSSION

The cellular origin of the 14 kDa lymphokine reported here cannot be unambiguously ascertained. But, interestingly, several results suggest that T cells should be considered as good candidates for the secretion of the stimulating activity. Conditioned media were prepared from mononuclear cells cultivated with SEA. This molecule has been considered as a strong T mitogen even at low concentration (Carlsson *et al.*, 1988). Stimulation, at a lower level, was also observed using supernatants of lymphocytes depleted from adherent cells by Sephadex G-10 absorption. Furthermore, stimulation of monocyte C1s secretion has been observed in supernatants of alloreactive T cell clones.

Complement secretion by monocytes was demonstrated to be enhanced by lymphokine-containing supernatants (Littman & Ruddy, 1977; Bensa *et al.*,

Table 2. Amino acid composition of the 14 kDa lymphokine purified by two different protocols

Amino acid	Composition (mol%)*		
	Protocol 1 (electro-focusing)	Protocol 2 (electrophoresis)	Residues per M_r -14600 molecule†
Alanine	9.6±0.01	7.75±0.06	16.1
Cysteine‡	1.1±0.05	1.0±0.07	1.8
Aspartic acid	1.5±0.02	2.7±0.35	2.5
Glutamic acid	4.2±0.26	6.0±0.44	7.0
Phenylalanine	1.9±0.01	1.6±0.16	3.2
Glycine	40.8±0.31	42.0±2.33	67.6
Histidine	0	0	0
Isoleucine	3.5±0.01	3.3±0.12	5.9
Lysine	1.6±0.03	1.8±0.05	3.0
Leucine	3.4±0.01	3.9±0.20	5.7
Methionine	1.9±0.01	2.2±0.04	3.2
Proline	6.3±0.14	5.4±0.35	10.5
Arginine	3.61±0.15	3.1±0.49	6.0
Serine	9.1±0.02	6.5±0.51	15.0
Threonine	4.0±0.03	6.0±0.01	6.6
Valine	5.1±0.32	4.9±0.13	8.5
Tryptophan	Not determined		—
Tyrosine	2.4±0.05	1.9±0.14	4.0
Total	100.0	100.0	166.6

* Values (mean ± S.D.) were derived from two analyses of the samples.

† Values were determined from data for pH 7.9–8.0 fraction.

‡ Value calculated from carboxymethylcysteine content of the samples.

Table 3. Dose-response effect of IFN- γ and 14 kDa lymphokine-containing eluate on monocyte C1s secretion

Values for C1s secretion are means ± S.D. for triplicate samples collected after three consecutive periods of 48 h.

Lymphokine	C1s secretion (ng/24 h per μg of DNA)
IFN- γ (units/ml)	
0	0.04±0.013
1	0.10±0.004
5	0.13±0.010
10	0.18±0.014
100	0.31±0.049
1000	0.34±0.034
Phenyl-Sepharose eluate pool 2 (pmol/ml)	
0	0.24±0.158
1.2	1.75±0.907
3	3.04±1.021
6	4.61±1.318
12	5.25±1.471
24	5.58±1.114

1983; Takemura *et al.*, 1986). When we started our experiments IFN- γ was considered as the major monocyte-activating lymphokine, and we took it as a reference. Both IFN- γ and 14 kDa lymphokine stimulate C1s secretion, but the two molecules can be separated by

Fig. 5. Effect of the 14 kDa lymphokine on C1r and C1s mRNA expression

Total cellular RNA was extracted from 12 day monolayers incubated for 24 h in the presence of 14 kDa lymphokine (about 6 nM) or in medium alone; 8 μ g or 6 μ g of RNA were obtained and subjected to dot-blot analysis using 2-fold serial dilutions. Membranes were hybridized with C1r and C1s cDNA probes as described in the Materials and methods section. (a) Lymphokine-stimulated monocytes; (b) non-stimulated monocytes.

phenyl-Sepharose chromatography, and their M_r values and their oligomerization patterns are different.

The 14 kDa lymphokine shares with other lymphokines some characteristic features such as hydrophobicity and oligomerization ability. Calculated according to Hopp & Woods (1981) on amino acid analysis of the entire molecule, a very low hydropathy index value was found (-6.0). It is in the same range as values calculated from the sequence data for IL-2 (-4.0 ; Robb *et al.*, 1984), IL-3 (-10.0 ; Yang *et al.*, 1986) and TNF- α (-15.1 ; Pennica *et al.*, 1984), but lower than the values obtained for IFN- γ ($+59.8$; Rinderknecht *et al.*, 1984) and IL-4 ($+25.9$; Yokota *et al.*, 1986). The oligomeric association of 14 kDa lymphokine is strong, as it is not destroyed in the presence of 4 M-urea/1% (w/v) SDS under reducing conditions. Oligomeric forms have been also reported for IFN- γ (the present paper; Yip *et al.*, 1982), IL-1 α (Dinarelli *et al.*, 1984), IL-2 (Robb, 1984) and TNF- α (Beutler & Cerami, 1986).

The amino acid analysis of the purified 14 kDa lymphokine indicates 34% hydrophobic amino acid residues and is characterized by the presence of two cysteine and seven glutamic acid residues and the absence of histidine. These data clearly distinguish the 14 kDa

Fig. 6. Cell-free translation of C1s and lysozyme from total RNA extracted from stimulated and non-stimulated monocytes

Total cellular RNA was isolated (2 μ g in both conditions) from 12 day monolayers incubated for 24 h in the presence of 14 kDa lymphokine (about 6 nM) or in medium alone. It was translated in a rabbit reticulocyte lysate system; primary translation products were immunoprecipitated from the lysate and analysed as described in the Materials and methods section. (1) Non-stimulated monocytes; (2) lymphokine-stimulated monocytes.

lymphokine from other known macrophage-activating factors such as IFN- γ (0 Cys, 17 Glu, 2 His; Rinderknecht *et al.*, 1984), granulocyte, monocyte colony-stimulating factor (4 Cys, 21 Glu, 3 His; Wong *et al.*, 1985), IL-3 (2 Cys, 20 Glu, 3 His; Pennica *et al.*, 1984), IL-4 (6 Cys, 17 Glu, 5 His; Yokota *et al.*, 1986) or IL-6 (4 Cys, 16 Glu, 2 His; Haegeman *et al.*, 1986).

C1s secretion was taken as a basis of estimation of the 14 kDa lymphokine activity. As C1r is the natural sister protein of C1s in the C1 complex it seemed to us interesting to consider the level of regulation of these two proteins by the 14 kDa lymphokine. Furthermore, this could allow a more refined evaluation of the mechanism of action at the gene level, as C1r and C1s genes are located very close together on chromosome 12 (Tosi *et al.*, 1987). Our results indicate a co-ordinated expression of the two genes in keeping with their linkage, but still raising a problem as the genes have been shown to be in a tail-to-tail arrangement (Kusumoto *et al.*, 1988).

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