Biological and Biochemical Characterization of a Factor Produced Spontaneously by Adherent Cells of Human Immunodeficiency Virus–infected Patients Inhibiting Interleukin-2 Receptor α Chain (Tac) Expression on Normal T Cells

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Abstract

Adherent cells from human immunodeficiency virus (HIV)-infected subjects but not from normal blood donors, patients with Gram-positive or -negative bacteremia, active tuberculosis, toxoplasmosis, pulmonary aspergillosis, and cytomegalovirus infection produce spontaneously an activity which inhibits α chain of interleukin-2 (Tac) expression and interleukin 2 (IL-2) production by normal activated T cells and IL-2 production by these cells. A similar biologic activity was detected in culture supernatants of in vitro HIV-I–infected normal adherent and leukemic U937 cells. Tac-inhibitory activity is not cytotoxic and it could be detected in serum-free conditioned media. Recombinant granulocyte/macrophage colony-stimulating factor and phorbol myristate acetate stimulation of patients' and normal adherent cells did not enhance specifically the production of the Tac inhibitor. Biologically active conditioned media did not contain infectious virus as well as secreted p24, gp120 viral proteins; the biologic activity could not be abolished by anti–p24, anti–gp120, and anti–nef monoclonal antibodies or human purified polyclonal anti–HIV IgG. Gel filtration of conditioned media followed by anion exchange chromatography resulted in a 1,200-fold degree of purification and revealed that the biologically active molecule was cationic. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of this fraction and gel elution of the proteins showed that the biologic activity was associated with a 29-kD protein which was distinct from α- or γ-interferon, tumor necrosis factor-α, and prostaglandin E_2. The above findings demonstrate the production of inhibitory factor(s) during HIV infection, which might be involved in the pathogenesis of the patients' immune defect. (*J. Clin. Invest.* 1991. 87:2048–2055.) Key words: human immunodeficiency virus • interleukin-2 receptor • monokines • T-cell activation

Introduction

The human immunodeficiency virus (HIV) is the etiological agent of the acquired immunodeficiency syndrome (AIDS) (1, 2). HIV selectively infects immunocompetent cells such as CD4 lymphocytes (3, 4) and macrophages (5, 6). Although HIV infection induces a cytopathic effect for CD4 lymphocytes, macrophages are chronically infected (7) and may represent an in vivo "reservoir" of the virus (8).

HIV infection is characterized by a progressive depletion of $CD4^+$ lymphocytes which is related to both the clinical outcome of the disease (9, 10) and the profound impairment of cell-mediated immunity (11–13). However, whether this immunodeficiency is due to the direct cytopathic effect of HIV on $CD4^+$ cells or whether other indirect mechanisms are also involved in its pathogenesis is not yet clear. Indeed, although only a small number of peripheral blood lymphocytes (PBL) of HIV-infected patients are expressing virus at any given time (14), recent studies, using in situ hybridization and gene amplification showed that, at least 1/100 $CD4^+$ cells contains HIV-1 DNA (15), indicating that the in vivo infection rate of $CD4^+$ cells is greater than initially thought.

Previous studies from our group and others have demonstrated that the proliferation and differentiation capacity of T-cell progenitors (T-cell colony-forming cells [T-CFC]) are reduced during HIV infection (16, 17), thus providing an additional mechanism which can lead to CD4 lymphopenia. Moreover, it has been shown that lymphocytes from HIV-infected subjects display a decreased expression of the α chain (Tac) of the interleukin-2 receptor (IL-2R)[1] upon mitogenic stimulation (18, 19), indicating their impaired capacity for activation. Since $CD4^+$ lymphocytes have a key role on the regulation of the immune response, elaborating growth factors for both lymphocytes and myeloid cells (20), their quantitative and qualitative abnormalities after HIV infection might be very important in the pathophysiology of the immunodeficiency.

The qualitative defects of $CD4^+$ lymphocytes induced by HIV infection seems to be due to several causes. Indeed, it has been reported that noninfectious HIV can block antigenic activation of normal T cells through gp120 (21, 22). In addition, humoral factors produced by both lymphocytes (23) and adherent cells (24) of AIDS patients can inhibit normal T-cell activation. We have recently shown that adherent cells from AIDS patients produce spontaneously an activity which inhibits normal T-cell colony formation through decreased expression of membrane IL-2R (Tac molecule) and IL-2 production. Unpublished observations from our laboratory have demonstrated that this inhibitory activity can be detected in the supernatants of adherent cell cultures from 80% of HIV-infected subjects, irrespectively of the clinical stage.

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1. *Abbreviations used in this paper:* ARC, AIDS-related complex; BRMP, Biological Response Modifier's Program; CMV, cytomegalovirus; EIA, enzymo-immunosorbent assay; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL-2R, IL-2 receptor; LAS, lymphadenopathy syndrome; Tac, α chain of IL-2R; T-CFC, T-cell colony-forming cell(s).

Here we report on the partial biological and biochemical characterization of this inhibitory activity and we show that Tac-inhibitory activity is due to a 29-kD protein which is distinct from α- or γ-interferon (IFN), prostaglandin E_2 (PGE_2), tumor necrosis factor α (TNFα), and the p24, gp120, and nef viral proteins.

Methods

Subjects. Heparinized peripheral blood was obtained from 60 HIV-infected patients at different stages of the disease as well as from HIV-seronegative patients suffering from Gram-positive (n = 3) and Gram-negative (n = 2) bacteremia, active toxoplasmosis (n = 3), active tuberculosis (n = 3), pulmonary aspergillosis (n = 1), and active cytomegalovirus (CMV) infection (n = 2). Confirmation of HIV infection was performed by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Moreover, peripheral blood mononuclear cells (PBMC) were obtained from seronegative healthy blood donors (n = 15) and used for control experiments.

Preparation of conditioned media. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation. Adherent cells were separated by incubating 10^6 PBMC/ml in plastic Petri dishes in RPMI-1640 supplemented with 2 mM L-glutamine, and 1% (vol/vol) penicillin-streptomycin (all from Gibco Laboratories, Grand Island, NY) for 2 h in 5% CO_2 in air at 37°C. Nonadherent cells (PBMCA$^-$) were harvested by extensive (three times) washing of the dishes with Hank's balanced salt solution (HBSS). Adherent cells were resuspended in fresh serum-free culture medium supplemented with 10 μg/ml leupeptine, 1 mM phenylmethylsulfonylfluoride (PMSF), and 10^{-7} M pepstatin A (all from Sigma Chemical Co., St. Louis, MO). Cultures were incubated at 37°C in 5% CO_2 in air for 48 h. Media conditioned by stimulated adherent cells were also obtained by preincubating the cells for 2 h in the presence of either phorbol myristate acetate (PMA; 10 ng/ml, Sigma Chemical Co.) or recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF; 10 U/ml, specific activity 10^8 CFU/mg, Genzyme Corp., Boston, MA), extensive washing, and further incubation in fresh medium as above. Supernatants from patients and normal subjects (LCM-A$^+$p and LCM-A$^+$n, respectively) were recovered, ultracentrifuged, filtered through 0.22-μm filters (Millipore Corp., Bedford, MA), and stored at −80°C until use.

In some experiments, media conditioned by T cell–enriched (E$^+$) and T cell–depleted adherent (E$^-$T3$^-$A$^+$) cells were also prepared as above. For this, unfractionated PBMC were subjected to rosetting with 2-amino-ethylisothiouronium bromide–treated sheep red blood cell and Ficoll-Hypaque density centrifugation. E$^+$ cell fraction contained 93–95% CD3$^+$ and < 4% OKM1$^+$ cells as determined by indirect immunofluorescence using the OKT3 and OKM1 MAbs (Ortho Pharmaceutical, Raritan, NJ). E$^-$ cells were further depleted of contaminating CD3$^+$ cells by complement-mediated cytotoxicity using the OKT3 (Ortho Pharmaceutical) and T11 (Coulter, Hialeah, FL) MAbs as described (25) and contained < 3% contaminating CD3$^+$ cells. Plastic adherence of E$^-$T3$^-$ cells was performed as above.

Determination of protein concentration. Protein quantification in each sample was determined according to the Shäffner and Weissmann technique (26) using BSA (Sigma Chemical Co.) solution as a standard. The minimum quantity of protein that could be measured by this method was ~ 10 ng.

Expression of the Tac chain of IL-2R. PBMC (10^6/ml) from normal healthy heterosexuals were incubated in RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, and antibiotics. The cells were activated with phytohemagglutinin-M (PHA-M; 1% vol/vol, Difco Laboratories, Inc., Detroit, MI) in the presence of various concentrations of LCM-A$^+$n or LCM-A$^+$p for 48 h. Cells were washed with HBSS and tested for the expression of the Tac molecule by indirect immunofluorescence using the IOT14 monoclonal antibody (Immunotech, Luminy, France). At least 300 cells were counted under an epifluorescence microscope.

In some experiments, the inhibitory effect of LCM-A$^+$p on the expression of the Tac molecule was studied after incubation with indomethacin (1 μg/ml, Sigma Chemical Co.) or increasing concentrations of anti-IFNα and anti-IFNγ horse polyclonal IgG (25–100 U/ml, Boehringer Mannheim, Federal Republic of Germany; 1 U of IgG neutralizes 1 U of the corresponding IFN in a biological assay [27]) or anti-TNFα (25–100 U/ml, Boehringer Mannheim; 1 U of IgG neutralizes 1 U of TFNα on mouse L929-fibrosarcoma line [28]) for 2 h at 4°C. In addition, the inhibitory effect of both recombinant α-IFN (5,000 U/ml Roche, Basel, Switzerland) and TNFα (2 U/ml, Genzyme Corp.) on the expression of Tac molecule was studied. Moreover, in some experiments TNFα was determined in LCM-A$^+$p and LCM-A$^+$n by a sensitive enzymato-immunoadsorbent assay (EIA) using a commercial kit (Biokine, T Cell Sciences, Inc., Cambridge, MA) according to the manufacturer's instructions. The specific inhibitory activity of each supernatant was expressed as the quantity of protein giving 50% of the maximal dinhibition (ID_{50}).

Detection of virus or/and viral proteins. All LCM-A$^+$p were tested for the presence of HIV-1 by measuring reverse transcriptase activity as reported (29). Moreover NP-40–treated ultracentrifugated pellets of LCM-A$^+$p and LCM-A$^+$n were tested for p24 antigen by an EIA using a commercially available kit (Abbott Laboratories, Irving, TX). In addition, Amicon-concentrated LCM-A$^+$p and LCM-A$^+$n were electrophoresed according to the method of Laemmli (30) on a polyacrylamide gel using 7.5% acrylamide in the presence of 0.1% SDS (Sigma Chemical Co.). The samples were denaturated in 1% SDS and 1% glycerol (Sigma Chemical Co.), then electrotransfered, and blotted using a pool of MAbs against gp120 and p24 (Du Pont Co., Wilmington, DE) or purified human anti-HIV polyclonal IgG (kindly provided by Dr. T. Jouault, Paris [31]) as reported (32). Detection of viral proteins was also assayed by dot blot analysis of concentrated LCM-A$^+$p or LCM-A$^+$n using the above mentioned monoclonal and polyclonal antibodies. Finally, the Tac inhibitory activity was tested after incubating Amicon-concentrated LCM-A$^+$p and LCM-A$^+$n with either anti-gp120 (5 μg/ml), anti-p24 (5 μg/ml), and anti-nef (10 μg/ml, kindly provided by Dr. P. M. Kieny, Transgène, Strasbourg, France) MAbs or increasing concentrations of purified human anti-HIV polyclonal IgG.

Cell infection. Adherent cells from HIV-seronegative normal blood donors were isolated as above and were incubated with 50 tissue culture ID_{50} units of HIV-1/10^6 cells in RPMI-1640 medium containing 2 μg/ml Polybrene (Sigma Chemical Co.) and sheep anti-human α-IFN serum (40 U/10^6 cells) for 1 h at 37°C in 5% CO_2 in air. In control experiments, inactivated virus (heated for 1 h at 56°C) was incubated with adherent cells. Subsequently, the cells were washed three times with complete culture medium, resuspended in fresh culture medium at 10^6 cells/ml, and cultured in the presence of PMA (10 ng/ml) and 5 U/ml GM-CSF. Every third day the cells were counted and the supernatants were tested for p24 antigen and reverse transcriptase activity as reported (1). The remaining cells were recultured in fresh culture medium supplemented with PMA and GM-CSF. Cultures were performed at 37°C in 5% CO_2 in air.

An aliquot of culture supernatants as well as supernatants from productively HIV-1–infected U937 cells maintained in long-term culture (kindly provided to us by Dr. J. C. Gluckman, Hôpital Pitié-Salpêtrière, Paris) were ultracentrifugated, and the supernatants were tested for their capacity to inhibit the expression of Tac molecule. These ultracentrifugated supernatants did not contain reverse transcriptase activity, p24 antigen, or secreted p24 and gp120 proteins as assessed by both p24 capture EIA and dot blot analysis using purified human polyclonal anti-HIV IgG.

IL-2 dosage. IL-2 was determined in media conditioned by PHA-activated normal PBMC in the presence or the absence of inhibitory factor by a colorimetric method using the IL-2–dependent CTLL-2 cell line according to Mosman (33) technique as modified by Tada (34). The results are expressed as Biological Response Modifiers Program (BRMP) units per milliliter.

Partial biochemical characterization of the inhibitory activity. Amicon (YM5 membrane)-concentrated supernatants (×100) were salted out with a G25 gel filtration column (Pharmacia, Uppsala, Sweden; 15 cm 1.5 cm^2), at room temperature. The column was equilibrated with 20 mM Tris-Cl, 1 M NaCl, 4 M urea (Merck, Darmstadt, FRG), 1 mM EDTA (Merck), 1 mM EGTA, pH 7.4 buffer supplemented with the previously mentioned protease inhibitors. The proteins contained in the void volume were Amicon concentrated, and 300 μl of the solution was injected on a fast protein liquid chromatography S_{12} gel filtration column (Pharmacia) equilibrated with the same buffer in the presence of protease inhibitors. The column was run at 20°C with a 0.5 ml/min flow rate. The calibration of the S_{12} column was performed with the Pharmacia calibration kit. The various fractions were Amicon (YM5)-concentrated, salted out on a G25 column equilibrated with 20 mM Tris-Cl, pH 7.4, supplemented with protease inhibitors. All fractions were Millipore filtered and tested for biologic activity. The void volume of the biologically active fractions was run on an anion exchange column (Mono-Q, Pharmacia) equilibrated with the same buffer. The retained proteins were eluted with a linear NaCl gradient (0–3 M NaCl) in the same buffer. The biologically active peaks were analysed by SDS-PAGE as described above.

Elution of the proteins from the gels. Gel electrophoresis was realized according to the Laemmli technique on 12% acrylamide 1.5-mm-thick gels. 50 μl of denaturated (1% SDS at 100°C during 3 min) sample (non-retained fraction of the Mono-Q) were loaded in three contiguous slots, and migrated under 200 V. The distance of the front migration was measured, and the gel was fragmented in 15 3-mm bands. Each fragment was immerged in 400 μl of Tris buffer (Tris-Cl 20 mM, pH 7.4) and homogenized with a homogenizer (Wheaton Instruments, Millville, NJ). The Wheaton apparatus was washed with 400 μl of the same buffer. The homogenate was dialyzed against the same buffer during 48 h at 4°C. The cutoff of the tubing used here was about 8,000 kD (Spectrapor; Spectrum Medical Industries, Inc., Los Angeles, CA). The dialysate was centrifuged rapidly to eliminate the gel particles, and the supernatant was freezed in liquid nitrogen and sublimated in a Speed Vac apparatus. The concentrated fractions were used to test the biological activity.

Results

Cellular origin of Tac-inhibitory activity. To identify the cell producing this activity, we prepared media conditioned by different unstimulated cell fractions. Table I indicates that, in media conditioned by patients' unfractionated PBMC, a slight Tac-inhibitory activity was detected which was always increased more than twofold in media conditioned by patients' adherent cells. Conversely, no Tac-inhibitory activity could be detected in media conditioned by patients' E^+-enriched cells. Moreover, media conditioned by both unstimulated PBMC and $E^-CD3^-A^+$ cells of 15 normal controls were inactive (Table I), as well as media conditioned by normal adherent cells activated either by PMA or recombinant GM-CSF for 48 h (not shown). Finally, complement-mediated lysis of patients' adherent cells with My9 MAb completely abrogated the detection of Tac-inhibitory activity in the LCM-A^+p (not shown). The total protein concentration in the LCM-A^+p and LCM-A^+n was 6.0±4.2 and 2.0±1.4 μg/10^6 cells, respectively (range 1.0–10.5 and 1.0–3.6 μg/10^6 cells).

In order to exclude that LCM-A^+p-induced Tac inhibition is not due to the direct binding of a molecule on the Tac protein, preactivated normal PBMC were incubated with different concentrations of LCM-A^+p, and washed cells were stained with the IOT14 MAb. Table II indicates that cell incubation with active LCM-A^+p did not show any decrease of the proportion of Tac^+ cells.

Trypan blue dye exclusion studies indicated that LCM-A^+p were not cytotoxic even at concentrations mediating maximal inhibition. Expression of the inhibitory activity as ID_{50} and dose-response experiments performed with several LCM-A^+p always showed an hyperbolic curve with a maximal inhibition ranging between 1.2 and 2.0 μg/10^6 cells. A representative curve is shown in Fig. 1. This pattern of dose-response curves was very similar in the 60 cases studied. LCM-A^+ from AIDS-related complex (ARC) and AIDS patients displayed a lower ID_{50} than from asymptomatic and lymphadenopathy syndrome (LAS) patients (Table III) but these differences were not statistically significant. Supernatants displaying an $ID_{50} < 0.4$ μg/10^6 cells were considered to contain Tac-inhibitory activity. Using these criteria, this activity could be detected in 55% of asymptomatic seropositive patients, 70% LAS, 71% ARC, and 100% AIDS patients. Table III also demonstrates that LCM-A^+ from patients with Gram-positive or Gram-negative bacteremia, active toxoplasmosis, tuberculosis, and pulmonary aspergillosis did not display Tac-inhibitory activity. LCM-A^+ obtained from patients with an active CMV infection with IgM anti–CMV antibodies showed an ID_{50} = 0.7±0.2 μg/10^6 cells

Table I. Production of Tac-inhibitory Activity by HIV-infected Subject's Adherent Cells

Origin of LCM	Normal seronegative subjects	Patient No. 1	2	3	4
		%			
PBMC	0	12±3*‡	14±2	13±2	15±2
E^+	0	0‡	7±2	1±1	7±1
$E^-T3^-A^+$	0	36±7	39±7	30±2	31±3

Conditioned media from different cell fractions were prepared as described in Methods and were added (20% vol/vol) to normal PBMC (10^6/ml) in culture medium supplemented with PHA-M (1% vol/vol). Expression of the Tac chain of IL-2R was evaluated 48 h later by indirect immunofluorescence. Control experiments were performed in the absence or conditioned medium.

* The results represent the mean±SD of five experiments.

‡ Results are expressed as the percentage of inhibition of Tac chain expression in comparison to control values.

Table II. Effect of Media Conditioned by HIV-infected Patients' Adherent Cells on the Expressed Tac Molecule

	ID_{50} of LCM-A^+p	Concentration of LCM-A^+ (μg/ml) 0	0.25	0.5	1.0
	μg	%			
Experiment 1	0.25*	62‡	58	60	61
Experiment 2	0.36	75	73	71	70

Normal PBMC were activated with PHA-M for 48 h and extensively washed cells were further incubated with either growth medium or with increasing concentrations of LCM-A^+p, for 1 h at 37°C. Washed cells were stained with the IOT14 MAb by indirect immunofluorescence as described in Methods.

* ID_{50} of LCM-A^+p is expressed as μg of protein/10^6 cells needed to obtain the 50% of maximal inhibition.

‡ Percentage of Tac^+ cells.

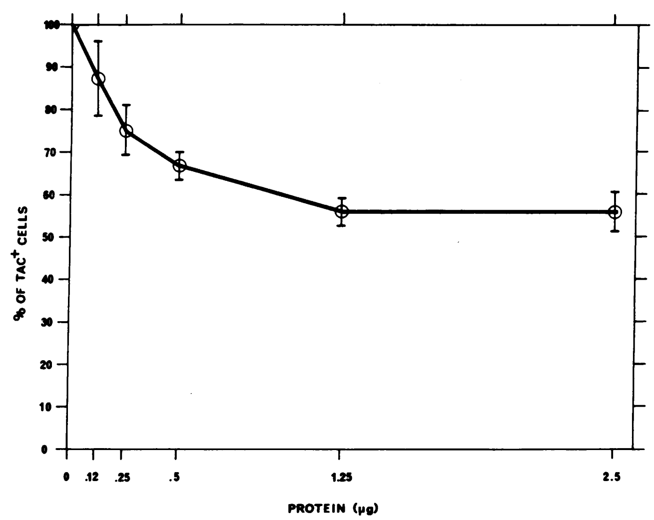

Figure 1. Dose-response curve of the Tac-inhibitory activity detected in media conditioned by adherent cells of HIV-infected patients. The results represent the mean values of Tac$^+$ cells±SD obtained with three different LCM-A$^+$p as described in Methods.

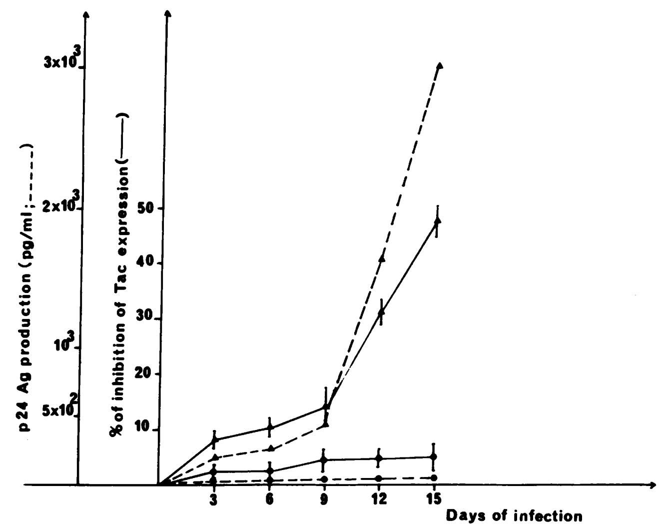

Figure 2. Adherent cells from normal seronegative blood donors were isolated and infected in vitro with either infectious (▲) or heat-inactivated (●) HIV-1 as described in Methods. Culture supernatants obtained at the indicated days were tested for antigen p24 and reverse transcriptase activity, whereas the other ultracentrifugated aliquot of the supernatant was tested for Tac-inhibitory activity. Each experimental point represent the mean±SD values of two different experiments.

(Table III) which was more than twofold higher than the values observed in active LCM-A$^+$p of HIV-seropositive asymptomatic patients (ID_{50} of active LCM-A$^+$p ranging between 0.12 and 0.40 μg/10^6 cells). In addition, normal cells when activated in the presence of 0.25 μg/10^6 cells of Tac-inhibitory activity produced twofold less IL-2 activity (31 BRMP U/ml) than cells activated in the presence of LCM-A$^+$n (67 BRMP U/ml).

Detection of Tac-inhibitory activity in supernatants of in vitro infected adherent cells. To demonstrate that the production of Tac-inhibitory activity is directly related to HIV infection, adherent cells from normal HIV-seronegative blood donors were infected in vitro. Fig. 2 demonstrates a clear time-dependent inhibition of Tac expression induced by virus- and viral protein-free supernatants which peaked at day 15 of culture. This Tac-inhibitory activity production was correlated with viral production as assessed by level of p24 antigen (Fig. 2) and reverse transcriptase activity (not shown) in the culture supernatants. It should be noted that no Tac-inhibitory activity could be detected in culture supernatants of cells incubated with heat-inactivated HIV-1 (Fig. 2).

In addition, a similar inhibition of Tac expression was always observed when virus- and viral protein-free supernatants from chronically HIV-1 infected U937 cells were tested (ID_{50} ranging between 0.25 and 0.35 μg/10^6 cells; mean±SD = 0.35±0.1 μg/10^6 cells of four different experiments). Again, no Tac-inhibitory activity could be detected in media conditioned by U937 cells or U937 cells incubated with heat-inactivated HIV-1.

Production of Tac-inhibitory activity. Kinetic studies revealed that the production of Tac-inhibitory activity became detectable after a 12-h cell incubation, reaching a plateau at 48 h (Fig. 3). However, when the LCM-A$^+$p were prepared in the absence of protease inhibitors, the specific activity declined at 72 h.

Stimulation of adherent cells with rGM-CSF or PMA enhanced both total protein production and the secretion of Tac-inhibitory activity in all but one case (case 3, Table IV). It should be noted that in both PMA- and recombinant GM-CSF-induced and ultracentrifugated LCM-A$^+$p neither reverse transcriptase activity nor p24 and gp120 viral proteins could be detected by dot blot using specific MAbs (not shown). Moreover, no p24 antigen could be detected in these supernatants using a capture EIA.

Tac-inhibitory activity is not infectious HIV or/and HIV gag-, env- and nef-encoded proteins. All LCM-A$^+$p were tested for reverse transcriptase activity and for their capacity to infect the CEM-A_{310} leukemic T cell line. No reverse transcriptase

Table III. Production of Tac-inhibitory Factor by Adherent Cells from Patients Suffering from Various Infections

Infection		n	Tac inhibitory factor
			μg
Gram-positive bacteremia		3	>2*
Gram-negative bacteremia		2	>2
Toxoplasmosis		3	>1.2
Tuberculosis		3	>2
Aspergillosis		1	>2
CMV		2	0.7±0.2‡
HIV	Asymptomatic	20	0.56±0.44
	LAS	10	0.49±0.43
	ARC	8	0.27±0.09
	AIDS	22	0.29±0.1

Media conditioned by unstimulated adherent cells from patients with various bacterial, fungal, or viral infections were prepared and tested for inhibition of Tac expression as described in Methods.
* The results are expressed as the ID_{50} value (μg of protein/10^6 cells giving the 50% of the maximal inhibition).
‡ Mean±SD of the observed individual values.

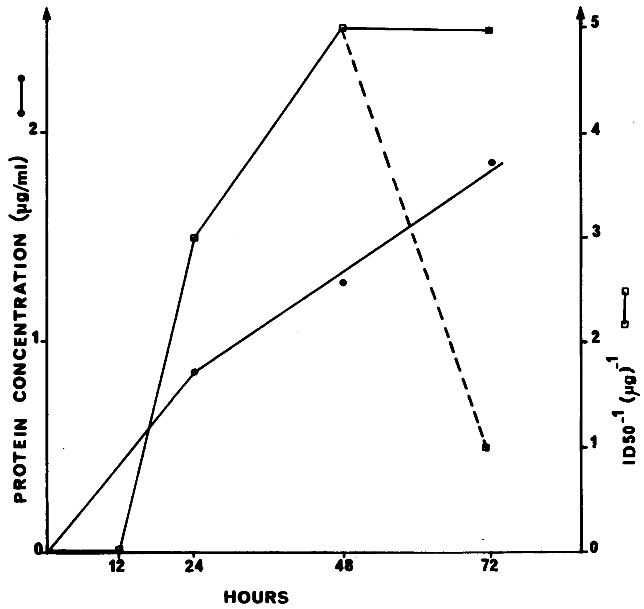

Figure 3. Kinetics of the production of the inhibitory activity. At the same time and during 72 h, the biological activity and the total concentration of protein were measured in the culture supernatants of adherent cells. The dashed line represents the decreasing of the inhibitory activity after 48 h of culture when protease inhibitors were omitted.

activity or infection of the permissive cell line could be detected, as previously reported (24). In some experiments, LCM-A$^+$p were also used to infect PHA blasts. Again, no virus replication could be obtained during the 28-d culture period as assessed by both reverse transcriptase activity and p24 antigen in the culture supernatants. No LCM-A$^+$p were positive for p24 antigen when tested in a capture EIA. To more directly exclude the presence of viral proteins, concentrated LCM-A$^+$p were also tested for the presence of *gag* and *env* proteins by dot as well as by Western blot analysis using either specific monoclonal antibodies or purified human polyclonal anti-HIV IgG. In several experiments performed using different LCM-A$^+$p, no p24 or gp120 proteins could be revealed. In addition, the Tac-inhibitory activity could not be abrogated by either anti-gp120, anti-p24, and anti-nef MAbs, or purified human anti-HIV polyclonal IgG.

Table IV. Effect of Recombinant GM-CSF and PMA on the Production of TAC-inhibitory Activity by Patient's Adherent Cells

Cell origin	Growth medium	Recombinant GM-CSF	PMA
	% of Tac inhibition/2.5 µg of protein		
Patient 1 (LAS)	0 (60 µg)	24 (112 µg)	25 (150 µg)
Patient 2 (LAS)	22 (40 µg)	34 (80 µg)	40 (160 µg)
Patient 3 (LAS)	35 (60 µg)	42 (80 µg)	46 (75 µg)
Patient 4 (LAS)	11 (80 µg)	48 (120 µg)	56 (180 µg)

LCM-A$^+$p were prepared and tested for Tac-inhibitory activity as described in Methods. Adherent cells were incubated with either recombinant GM-CSF (10 U/ml) or with PMA (10 ng/ml) for 2 h, and extensively washed cells were further incubated in fresh serum-free culture medium for an additional 48 h. In parentheses is indicated the quantity of protein produced by the same number (10^6/ml) of seeded cells.

Tac-inhibitory activity is distinct from PGE_2, α- and γ-IFN, and TNFα. We subsequently studied whether media conditioned by HIV-infected subjects' adherent cells contain PGE_2, α- or γ-IFN, or TNFα, which display a known inhibiting effect on cell proliferation. Purified polyclonal horse IgG which has been shown to neutralize α-IFN, γ-IFN, and TFNα in corresponding biological assays (27, 28) could not abbrogate Tac-inhibitory activity of all conditioned media tested even at high antibody concentrations. Moreover, no TNFα could be detected by EIA in 10 out of 10 supernatants tested. In addition, when normal PBMC were stimulated in the presence of recombinant α-IFN (5,000 U/ml) and TNFα (2 U/ml), no inhibition of Tac expression could be detected (not shown). Finally, a concentration as high as 1 µg/ml of indomethacin could not abrogate the inhibitory activity of LCM-A$^+$p (not shown).

Partial biochemical characterization of Tac-inhibitory activity. We have previously shown that Tac-inhibitory activity could be abolished by trypsin and chymotrypsin-treatment or heating of the conditioned media at 56°C for 1 h (24). In order to more precisely characterize the molecule responsible for this activity, LCM-A$^+$p were salted out by chromatography on G_{25} column and proteins were gel filtrated on S_{12} fast protein liquid chromatography column in the presence of 4 M Urea. Fig. 4 *A* shows the elution profile revealing that the biologic activity was co-eluted at 23 kD. The value of ID_{50} of the 23-kD active fraction was decreased 425-fold in comparison to the crude LCM-A$^+$p when tested in the same PBMC.

The 23-kD fraction was, subsequently, salted out on a G_{25} column and subjected to an anion exchange (Mono-Q) column. The biologic activity was detected in the non-retained fraction (Fig. 4 *B*) which represents 5% of the total quantity of protein applied to the column. Again, ID50 value of the non-retained fraction was threefold lower than that of the 23-kD fraction obtained by gel filtration, tested on the same PBMC. Thus, after these two steps of chromatography the calculated degree of purification in terms of ID_{50}, was 1,200-fold (Fig. 5).

In some experiments, both crude conditioned medium and the collected fractions from S_{12} and Mono-Q chromatography were tested using the same target cells. In all cases, the same pattern of dose-response curve of the biologic activity was observed without modification of the maximal inhibitory effect. Conversely, only a progressive decrease of the ID_{50} of the active fractions obtained at each purification step was observed, suggesting that the same biologic effect was detected throughout the whole purification procedure.

SDS-PAGE and elution of the active molecule. Gel electrophoresis of the active fraction obtained after Mono-Q chromatography revealed three groups of proteins at 44, 35, and 29 kD. After elution of the gel, the biologic activity was associated with the 29-kD protein (Fig. 6). No cytotoxicity was detected in the eluted and dialyzed fractions. The biologic activity could be recovered after denaturation in the presence of 1% SDS.

Discussion

The results presented here indicate that adherent but not T cells from HIV-infected subjects produce spontaneously a 29-

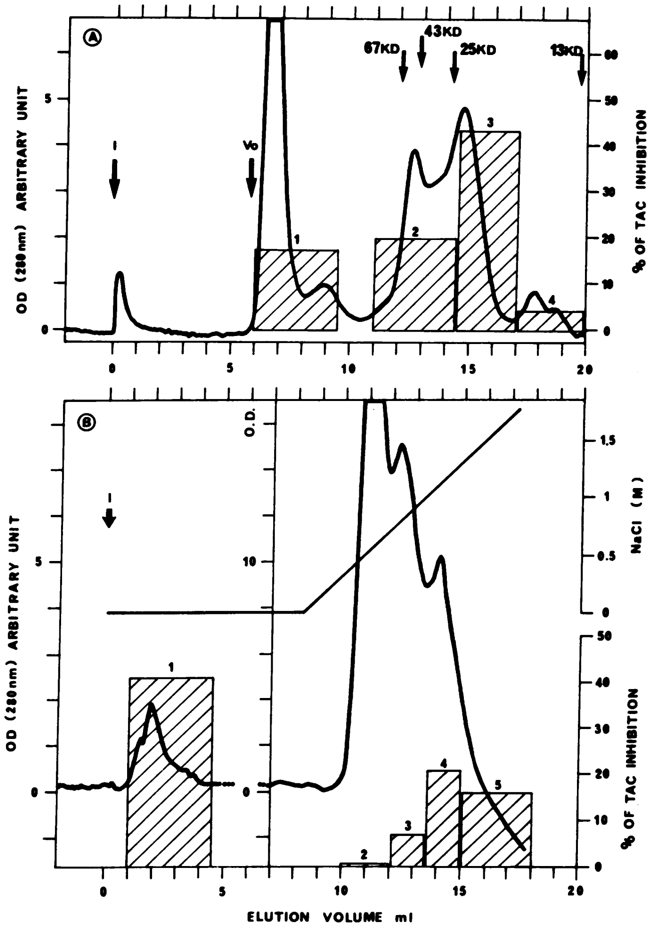

Figure 4. Gel filtration and anion exchange chromatographies. (*A*) Typical gel filtration profile of the conditioned medium in presence of 4 M urea. The biological activity was measured in four pools of fractions 1, 2, 3, and 4. The inhibitory activity was always recovered in the third pool between the 14th and the 17th ml of elution. (*B*) Anion exchange chromatography profile of the active pool characterized in the previous gel filtration step. The biological activity was measured in five pools of fractions 1, 2, 3, 4, and 5. The activity is always associated with the non-retained proteins in the first pool of fractions. I, injection of the sample on the column; Vo, void volume.

kD protein, capable of inhibiting the expression of Tac molecule on normal activated T cells and the production of IL-2 by these cells. In addition, this inhibitory factor is able to inhibit mitogenic- and alloantigen-induced proliferation of normal T cells (manuscript in preparation). This lack of detection of the Tac molecule was shown not to be due to steric hindrance but to active suppression of its expression. The cellular origin of p29 molecule was further confirmed by the observation that complement-mediated lysis with My9 MAb completely abrogated its production. Moreover, recombinant GM-CSF and PMA, which can stimulate macrophages/monocytes increases both the total protein secretion and the production of Tac-inhibitory activity (Table IV). Taken together these findings strongly suggest that p29 is actively secreted by adherent cells and that its release is a time-dependent phenomenon.

Tac-inhibitory factor could not be detected in media conditioned by normal unstimulated or activated adherent cells nor by adherent cells from patients infected with Gram-positive or

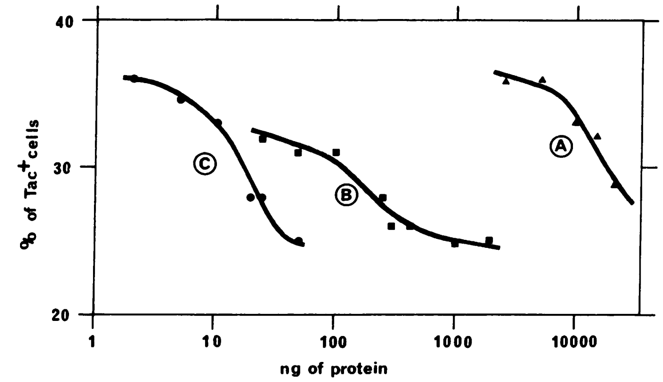

Figure 5. Comparison between the activities of the crude extract (*A*), after gel filtration (*B*) or anion exchange chromatography (*C*). The percentage of Tac$^+$ cells is expressed as a function of the quantity of protein added in the culture media. The same pool of conditioned media and the same target cells were used in these experiments. After the two main steps of the purification protocole the active Mono-Q fraction was calculated to be 1,200 times more active than the crude conditioned medium.

Gram-negative bacteria, and intracytoplasmic microbial agents (Table III). Only a very low production of Tac-inhibitory activity which, according to our criteria could not be considered significant ($ID_{50} > 0.5\ \mu g/10^6$ cells), could be detected in media conditioned by adherent cells from patients with active CMV infection. However, no anti-CMV IgM antibodies, demonstrating a recent CMV primary infection, could be detected in the HIV-infected subjects included in the present study. On the contrary, a production of Tac-inhibitory activity was observed in culture supernatants from in vitro HIV-infected normal adherent and U937 leukemic cells indicating that the production of the inhibitor is clearly related to HIV infection. Thus, these observations seem to indicate that the secretion of Tac-inhibitory factor is specific to HIV infection. However, we cannot completely exclude that other viral infec-

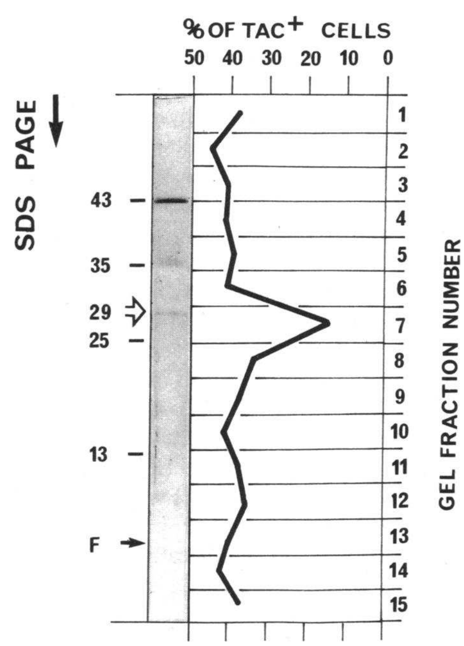

Figure 6. SDS-PAGE, silver staining, and elution of the anion exchange active fraction. The gel presents only three groups of proteins. The biological activity is associated with the 29-kD protein which is contained in the 7th gel fraction. M_r standards are shown at left.

tions could also induce the production of Tac inhibitory activity, thus explaining the higher proportion of patients with more advanced clinical stages of HIV infection producing this activity.

The determination of the ID_{50} of different LCM-A$^+$p always revealed a plateau of the biologic activity. This pattern of activity seems to indicate that the 29-kD inhibitory protein could inhibit Tac expression on only a subset of normal T lymphocytes. Indeed, p29 inhibits Tac expression on CD8$^+$ (up to 60%) but not on CD4$^+$ (up to 15%) cells as determined by two color fluorescence analysis (manuscript in preparation). Alternatively, this molecule could inhibit Tac expression through an indirect mechanism, i.e., inhibiting the production of a factor necessary for cell activation.

As monocytes/macrophages can be infected with HIV (5, 6) and virus replication can be enhanced in the presence of recombinant GM-CSF or cell activation (35), it was critical to define whether Tac-inhibitory activity could be due to released viral particles. This question was important since patients' adherent cells expressed p24 and gp120 viral proteins and contained integrated viral sequences (not shown) indicating their HIV infection. Several arguments are against this possibility: (*a*) no reverse transcriptase activity or HIV p24 antigen could be detected in conditioned media; (*b*) conditioned media could not infect the HIV-permissive CEM-A_{310} leukemic cell line as well as normal PHA blasts as already reported (24); (*c*) no HIV *gag* and *env* proteins could be revealed by both Western and dot blot assays using either MAbs or purified human polyclonal anti-HIV IgG (Fig. 4). The absence of detection of viral proteins does not seem to be due to technical reasons, since dot blot assays were performed using a high quantity (3 μg) of protein which saturated the experimental system; (*d*) finally, Tac-inhibitory activity could not be abolished by both monoclonal antibodies against both the p24, gp120, and nef viral proteins as well as purified human polyclonal anti-HIV IgG. Taken together these observations seem to indicate that Tac-inhibitory factor is of cellular origin, although the possibility of its being a product of a viral regulatory gene cannot be completely ruled out.

Several cellular factors display inhibitory effects on the proliferation of normal cells (36–38). Our findings indicate that Tac inhibitor is not PGE_2 since indomethacin cannot abrogate the biologic effect of LCM-A$^+$p. Moreover, this inhibitor is distinct from α- and γ-IFN and TNFα, since polyclonal neutralizing purified horse IgG against these molecules could not inhibit the biologic activity. In addition, LCM-A$^+$p did not contain detectable amounts of TNFα and recombinant TNFα could not enhance Tac expression on normal activated T cells in the presence of the Tac-inhibitory activity as it does in the absence of the inhibitor as already reported (35); finally, recombinant α-IFN could not inhibit Tac expression on normal activated T cells.

Preliminary biochemical characterization of this activity suggested a protease-sensitive molecule (24). In the present study we confirmed this observation since the detection of Tac-inhibitory activity was decreased at 72 h when LCM-A$^+$p were prepared in the absence of protease inhibitors whereas in the presence of protease inhibitors, a plateau was obtained at 48 h (Fig. 2). Gel filtration revealed that the biologically active molecule was co-eluted with an apparent molecular mass of 23 kD which could not be retained by an anion exchange column suggesting its cationic nature. Our findings concerning the determination of the ID_{50} indicate that with these preliminary purification steps, Tac-inhibitory factor could be purified about 1,200-fold. When this non-retained fraction was subjected to SDS-PAGE, three bands of about 44, 35, and 29 kD were revealed. Protein elution from the gels and study of their capacity to inhibit Tac expression demonstrated that the biologic activity was associated only with the 29-kD protein.

Previous studies have already shown that both unstimulated and mitogen-activated peripheral blood lymphocytes from AIDS patients can inhibit the proliferation of normal lymphocytes (23). Moreover, T-cell hybridomas from AIDS patients also produce constitutively inhibitory activity(ies) for normal cell proliferation (39). Although biochemical studies have not been performed in these reports, the different cell origin of Tac-inhibitory factor suggests that these activities are distinct. In addition, it has been recently reported that AIDS patients' bone marrow cells produce a molecule of 84 kD which inhibits the proliferation of normal GM-CFU (40), which it should be different from p29 inhibitory factor on the basis of their molecular mass.

In conclusion, our findings taken together with previous studies strongly suggest that defective T cell responses in vitro upon mitogenic or antigenic stimulation during HIV infection might be the result of several pathophysiologic mechanisms such as (*a*) impaired proliferation and differentiation of T-cell precursors into mature CD4$^+$ cells due to their infection with the virus (41), (*b*) HIV-induced cytopathic effect on infected and noninfected CD4$^+$ lymphocytes (12), (*c*) direct cell inhibitory effect of the envelope gp120 viral protein (22), and (*d*) production of T-cell activation and proliferation inhibitory factor(s) by infected macrophages-monocytes and T cells (23, 24).

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