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HUMAN IMMUNODEFICIENCY VIRUS INHIBITION BY LYSED LEUKOCYTE ULTRAFILTRATE

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Summary. — The *in vitro* effect of partially purified lysed leukocyte ultrafiltrate (LLU) on LAV 1 strain of the human immunodeficiency virus (HIV) was investigated. Decrease of virus production as a function of LLU concentration was observed while the phenotype of cultured lymphocytes has remained unchanged. We observed the same depletion of helper-inducer T-cells in samples treated with LLU as well as in infected controls. LLU did not influence significantly the growth of cultured lymphocytes. Following the effect of LLU on RNA-dependent DNA polymerase, inhibition of the LAV 1 reverse transcriptase by LLU was observed. This inhibition was shown to be LLU dose-dependent, considering its content of proteins and orcinol-reactive material. Kinetic studies revealed that inhibition is reversible and competitive with the substrate (^{3}HTTP) but not with template-primer (poly rA-oligo dT).

Key words: *human immunodeficiency virus*; *leukocyte ultrafiltrate*; *inhibition*

Introduction

Human immunodeficiency virus (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984) was identified as the aetiological agent of the acquired immunodeficiency syndrome (AIDS). Biological studies on this virus (Coffin *et al.*, 1986) revealed its preferential tropism for T lymphocytes with helper-inducer phenotype (Klatzmann *et al.*, 1984). These experimental data helped to make clear the major immunological abnormality in AIDS patients which manifested itself by quantitative and qualitative defects of the helper-inducer T cell subset (Gerstoft *et al.*, 1982). The defect of cellular immunity in AIDS patients has been indicated by the observations of relative and absolute decreases in T-helper cell number and a changed helper-suppressor ratio, impairment in delayed hypersensitivity to recall skin test antigen, decreased lymphocyte blastogenesis to mitogenes and reduced production of alloreactive cytotoxic lymphocytes *in vitro* (Reuben *et al.*, 1983; Schroff *et al.*, 1983; Gerstoft *et al.*, 1985).

The immunological findings are significant in AIDS and they witness about the underlying central immunodeficiency which can manifest itself also by opportunistic infections and/or unusual neoplasmas. From the point of view of the character of immunodeficiency in AIDS, the experimental testing of immunosupportive drugs appears as an interesting field of investigation.

We payed attention to the immunoactive preparation described by Lawrence in 1955 termed Transfer Factor (TF). The most useful source of TF is dialysable leukocyte extract (DLE) or lysed leukocyte ultrafiltrate (LLU) which represents a complex of cell-liberated low-molecular weight materials. As recently shown (Lawrence and Borkowsky, 1983) DLE contains besides the inducer factor corresponding to the Lawrence's TF, substances with opposite activity; a specific suppressor factor and a non-specific suppressor. All these factors influence the human T cell functions and by this way also the expression of cell-mediated immunity.

In this paper we report the effect of LLU on HIV-1, propagated in cultured lymphocytes and on its reverse transcriptase (RT) activity.

Materials and Methods

Lysed leukocyte ultrafiltrate. LLU was prepared from buffy coats of healthy human donors by repeated freeze-thawing followed by ultrafiltration using an Amicon PM-10 (10 000 m.w. cut-off) membrane. The crude ultrafiltrate was partially purified as was previously described (Mayer *et al.*, 1983; Mayer *et al.*, 1985). Briefly, the ethanol-precipitated material was fractionated on a Sephadex G-15 (fine) column, yielding three peaks. The highest immunobiological activity was found in the second peak. Assuming a functional similarity of human and mouse materials, we used material from this fraction for the experiments described here. Quantification of the material investigated was based on the amounts of protein and orcinol-reactive material (ORM). Protein was determined by the method of Lowry *et al.* (1951), the purine-bound ribose (ORM) by the method of Mejbaum (1939).

Cells and virus. Cultured lymphocytes from the venous blood of healthy individuals were used to investigate the effect of LLU on LAV 1 replication. The cells and virus were propagated as previously described (Barré-Sincussi *et al.*, 1983). We allowed one hour for the virus adsorption. Different amounts of LLU were then added to the individual experimental samples. The extent of virus production was examined every 3 or 4 days by measuring RT activity in 1 ml of cell-free supernatants. The phenotype of cultured lymphocytes was determined by indirect immunofluorescent staining using mouse monoclonal antibodies (Immunotech, Marseille) according to the instructions of the manufacturer.

Enzymes. As the source of reverse transcriptase (RT) we used the LAV 1 isolate propagated in CEM cells. Cell-free supernatant was concentrated 100-times by ultracentrifugation and resuspended in NTE buffer (0.1 mol/l NaCl, 0.1 mol/l Tris, 0.001 mol/l EDTA), pH 7.4.

E. coli DNA polymerase and *E. coli* RNA polymerase were purchased from Boehringer Corp., Mannheim.

Enzyme assay. The reaction mixture for RT assay contained in the final volume of 50 µl: 50 mmol/l Tris pH 7.8; 20 mmol/l KCl; 5 mmol/l $MgCl_2$; 1 mmol/l DTT; 0.1% Triton X-100; 0.05 OD/ml poly rA; 0.05 OD/ml oligo dT_{12-18}; 180 pmol ^{3}HTTP (1.11 TBq/mmol) and 5 µl of viral suspension prepared as mentioned above and corresponding approximately to 0.5 µg of viral proteins. The mixture containing the appropriate amount of LLU was incubated for one hour at 37 °C. The reaction was stopped by adding sodium pyrophosphate and trichloracetic acid. The product was allowed to precipitate at 4 °C for 20 min and subsequently filtrated and counted. Results were expressed as picomoles of tritiated deoxyribonucleotide-monophosphate incorporated into the DNA.

E. coli DNA polymerase assay. The reaction mixture contained in a final volume of 50 µl,

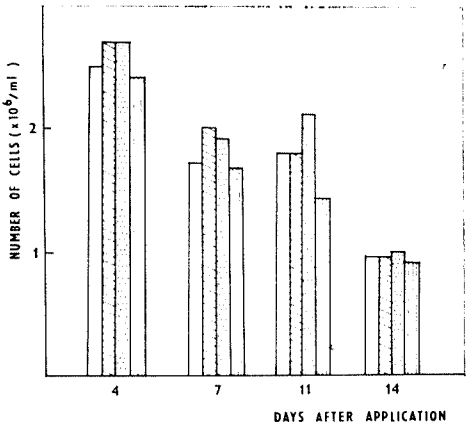

Fig. 1
Effect of LLU on the growth of cultured lymphocytes
Cultured lymphocytes were treated with different amounts of LLU, 0 µl (□), 50 µ (///) 100 µl (::), 200 µl (||||) and every three or four days they were counted after Trypan blue staining. Abscissa: days after application; ordinate: No. of cells (× 10^6/ml).

50 mmol/l Tris pH 7.8; 20 mmol/l KCl; 5 mmol/l $MgCl_2$; 1 mmol/l DTT; 0.004% Triton X-100 0.05 OD/ml poly dA; 0.05 OD/ml oligo dT_{12-18}; 180 pmol ^{3}HdTTP (1.11 TBq/mmol) and 0.3 units of DNA polymerase. The reaction mixture was incubated at 37 °C for 30 min.

E. coli RNA polymerase assay. The reaction mixture contained in a final volume of 50 µl, 40 mmol/l Tris pH 7.9; 4 mmol/l $MgCl_2$; 1 $Mn(CH_3COO)_2$; 20 mmol/l DTT; 0.005% Triton X-100; 4 mmol/l ATP; 0.25 OD/ml poly d(A—T); 200 pmol ^{3}HUTP (1.11 TBq/mmol) and 0.3 units of RNA polymerase.

Results

Effect of LLU on the growth of cultured lymphocytes

In order to know if LLU did influence the growth of cultured lymphocytes we treated them with three different amounts of LLU: 50 µl, 100 µl and 200 µl per 4×10^6 cells, corresponding to 0.6 µg; 1.2 µg, and 2.4 µg of LLU proteins per 10^6 cells, respectively. The results presented in Fig. 1 show

Table 1. Phenotype of cultured lymphocytes after LAV 1 infection and LLU application

	Days after infection					
	0		7		14	
	T4 %	T8 %	T4 %	T8 %	T4 %	T8 %
Control without virus	53	38	53	34	51	44
Infected control	55	24	26	39	3	66
LLU100	51	21	27	28	4	56
LLU 200	45	25	32	28	3	50

100 µl of LLU (LLU 100) and 200 µl of LLU (LLU 200) were added to the cultured lymphocytes 1 hr after infection and the T4 and T8 phenotypes were examined by immunofluorescent method on days 0.7 and 14 after infection.

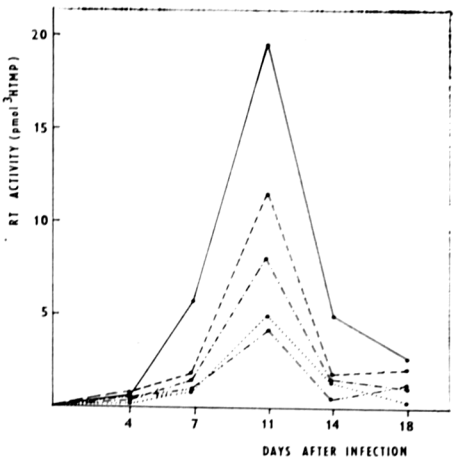

Fig. 2
Effect of LLU on the LAV 1 production by cultured lymphocytes
Different amounts of LLU, 0 µl (—), 25 µl (— — —), 50 µl (—·—·—), 100 µl (····) and 200 µl (—··—) were added to the cultured lymphocytes 1 hr after infection and the virus production was measured every 3 or 4 days by measuring the RT activity in cell-free supernatants.
Abscissa: days after infection; ordinate: RT activity (pmol of ³HTMP).

that LLU did not influence significantly the growth of the cells under the conditions described.

Effect of LLU on HIV-1 production by cultured lymphocytes

Cells were treated with four different concentrations of LLU (25 µl, 50 µl, 100 µl, and 200 µl per 4×10^6 cells) since 1 hour after infection. Virus production, measured by RT activity was assayed every 3 or 4 days. The results summarized in Fig. 2 demonstrate the decrease of virus production as a function of LLU concentration. In other experiments we treated cells with the same amounts of LLU before infection (data not shown). This treatment was less effective. As it was mentioned above, one of the most

Table 2. LLU inhibition of LAV 1 RT activity

LLU		Incorporation of ³HTNP pmol	Inhibition %	ID_{50}[b]	
Proteins µg/ml	ORM[a] µg/ml			Proteins µg/ml	ORM µg/ml
1	7.7	17.1	20	3.7	28.9
2	15.4	14.4	32		
5	38.6	6.9	68		
10	77.2	4.7	78		
15	115.2	2.9	86		
20	154.4	2.3	89		
25	193.0	2.1	90		

[a] orcinol-reactive material
[b] inhibition dose corresponding to the 50% LLU inhibition of RT activity. Appropriate amounts of LLU were added directly to the reaction mixtures. RT activity was assayed as described (Materials and Methods).

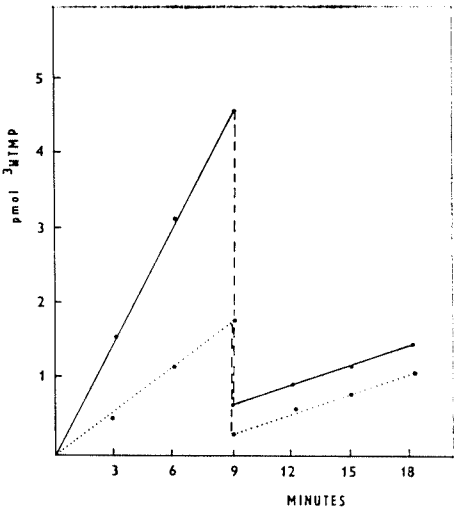

Fig. 3
Reversible inhibition of the reaction in the presence of LLU
Reversibility of inhibition was measured by dilution experiment in which two samples with complete reaction mixture were examined. The first was kept as control (—) and to the second one 50% inhibition concentration of LLU was added (····).
Abscissa: intervals in minutes; ordinate: pmol of ^{3}HTMP.

important characteristics of HIV-1 is its selective tropism for T4 cells. After the observed decrease of virus production (as the result of LLU treatment), we turned our attention to the phenotype of cultured lymphocytes in order to ascertain whether LLU protects the target cells. In spite of the observed decreased virus production, we found similar depletion of T4 cells in the samples treated with LLU as in non-treated infected controls (Table 1).

Inhibition by LLU of LAV 1 reverse transcriptase activity

In the next step we studied the effect of LLU on LAV 1 RT activity. The results, summarized in Table 2, show that the RT activity was inhibited as a function of the dose of LLU, which was added directly to the reaction mixture. For the two components of LLU (i.e. proteins and ORM) which were used as indicators for its quantification, 50 per cent inhibition doses (ID_{50}) were determined (Table 2).

To evaluate the specificity of inhibition we examined the effect of LLU on *E. coli* DNA and RNA polymerases. LLU was added to the reaction mixtures at concentrations achieving 60% and 80% inhibition of RT. No inhibition was seen under this conditions (Table 3).

Reversible inhibition of the reaction in the presence of LLU

Reversibility of inhibition was assessed by dilution experiments in which two samples with complete reaction mixture including the virus were examined. One sample was kept as a control and LLU in a concentration giving 50% inhibition of RT activity was added to the second one. Starting samples were 250 µl in volume. Every three min 50 µl were taken from each sample and the reaction was stopped in order to determine the rate of DNA synthesis. At 9 minutes of incubation the reaction mixtures were diluted

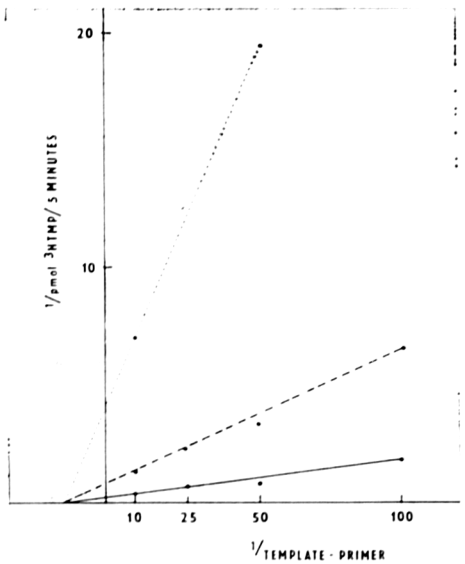

Fig. 4
Influence of template-primer concentration on the inhibition by LLU of HIV-1 RT activity: Lineweaver-Burk plot
Varying concentrations of template-primer poly rA-oligo dT were tested at two LLU concentrations, know to cause 50% (– – –) and 80% (····) inhibition of RT activity. Control was without LLU (—). Abscissa: template-primer concentration (l/template-primer); ordinate: l/pmol ^{3}HTMP/5 min.

tenfold with the complete reaction mixture without LLU and again every three minutes 100 µl were removed and then the reaction was stopped.

The results are summarized in Fig. 3. The rate of DNA synthesis is linear in both samples. After dilution, the rates of DNA synthesis were the same in the control sample as well in sample with LLU indicating the reversibility of inhibition.

Effect of template-primer concentration on LLU inhibition of LAV 1 reverse transcriptase activity

Varying concentration of synthetic template-primer poly (rA)-oligo $(dT)_{12-18}$ at two different LLU concentrations were used. Fig. 4 shows the results of Lineweaver-Burk plot illustrating the non-competitive inhibition mechanism.

Table 3. The effect of LLU on the E. coli DNA polymerase and RNA polymerase

	Incorporation of ^{3}HTMP pmol		
	RT	DNA polymerase	RNA polymerase
Control without LLU	10.0	0.13	75
LLU 6 µl	3.3	0.19	99
LLU 20 µl	1.2	0.56	103

LLU was added to respective reaction mixtures at the concentrations giving 60% (6 µl) and 80% (20 µl) inhibition of RT activity.

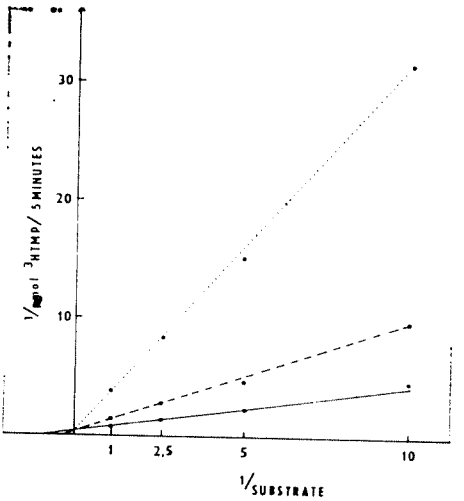

Fig. 5
The influence of substrate concentration on inhibition by LLU of HIV RT activity: Lineweaver-Burk plot
Varying concentrations of substrate ^{3}HTTP were tested at two LLU concentrations, know to cause 50% (— — —) and 80% (····) inhibition of RT activity. Control was without LLU (—).
Abcissa: substrate concentration (1/substrate); ordinate: 1/pmol ^{3}HTMP/5min.

Effect of substrate concentration on the inhibition by LLU of LAV 1 reverse transcriptase activity

The effect of varying concentrations of substrate ^{3}HdTTP at two different LLU concentrations are shown in Fig. 5. The typical competitive inhibition is illustrated.

Discussion

Since the first description of the TF (Lawrence, 1955), major research efforts were directed towards the characterization of the mechanism of its action and towards a posible use of DLE/LLU containing TF in the treatment of immunologic disorders and e.g. of infectious diseases. Results of numerous clinical trials were evaluated recently (Mayer and Borvák, 1987). Findings, relevant for our investigations, were reported by Gottlieb *et al.* (1984). They investigated the effect of a low-molecular weight substance with immunoamplificative effects (IMREG-1), isolated from LLU (Gottlieb and Sutcsliffe, 1982), in patients with AIDS or AIDS-related complex. After its administration a marked improvement — although temporal — of several immunological functions (proliferative response, IL-2 production in response to mitogen and T4 cell number) was noted.

In the described experiments we concentrated our attention on the *in vitro* effect of LLU on HIV-1. After LLU treatment of cultured lymphocytes infected with HIV-1, a significant decrease of virus production was observed without any T4 phenotype modification. The observed depletion of the target T4 cells from infected cultures treated with LLU, where a decreased virus production was observed, could be associated with virus replication, which although decreased, was still cytocidal. Another plausible explanation

is that the HIV-1 genomic determinants of cytopathicity and of those encoding the viral replicative capacity are not intrinsically coupled (Fisher *et al.*, 1986). The events involved in the slowing down of replication must not necessarily influence the pathways of the cell killing. Similar findings, i.e. suppression of viral replication without complete protection of target cells, were described while testing the effect of human interferon-gamma on HIV-1 infected MT4 cells (Nakashima *et al.*, 1986).

The lowered virus production due to the LLU treatment warranted the investigations dealing with possible mechanism(s) of described inhibitory effect. After finding that LLU did not influence the adsorption of the virus (data not presented) we turned our attention to the stage of viral replication in which RT is involved. These experiments characterized LLU as an inhibitor of RT activity. In this context, it seems as important that LLU did not inhibit the activities of the *E. coli* DNA- and RNA-polymerases.

The inhibitory action of LLU on LAV 1 RT was shown as reversible and competitive with substrate. These results suggest an interaction of some components of LLU with the enzyme-substrate complex. We cannot characterize at present which component(s) of LLU is(are) involved in the inhibition of RT activity. As reported (Chase, 1983; Lawrence and Borkowsky, 1983) LLU contains, in addition to TF, factors and substances with nonspecific enhancing or suppressoric functions on cell-mediated immunity also other biochemical entities with different potentialities. This is consistent with our findings demonstrating a novel LLU activity. It seems probable that inhibition of HIV-1 RT activity could be involved in the observed decrease of virus production after *in vitro* treatment of cultured lymphocytes with LLU. However, we cannot exclude that LLU influenced also other components of virus replication.

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