A FE^{3+}/DNA COMPLEX INDUCES AN ANTI-HUMAN IMMUNODEFICIENCY VIRUS FACTOR(S) IN CD4+ LYMPHOCYTE CELL LINES

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Received August 3, 1998; accepted February 4, 1999

Summary. – Numerous cytokines and chemokines are involved in inflammatory and immune response. Whereas some of them inhibit virus replication *in vitro* directly or increase the patients' T4-lymphocyte level, others effects are not so clear. Using human immunodeficiency virus (HIV) and cell cultures we have studied the antiviral effect of complexes of salmon DNA with metals and of a new factor(s) (antiviral factor, AVF) induced in cells by the complexes. The Fe^{3+}/DNA complex possessed the highest antiviral activity. It was found that MT-2, MT-4, CEM and Jurkat cells treated with the complexes secreted AVF which inhibited the replication of nine HIV-1 isolates, was noncytoxic and stimulated cell proliferation. AVF did not inactivate HIV. The molecular mass analysis of AVF showed that its antiviral activity is associated with its fraction of M_r of 3 K. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA from MT-4 cells treated with the complexes showed an increase in the the expression of genes for interleukin-1 alpha (IL-1alpha), tumour necrosis factor alpha (TNF-alpha) and TNF-beta while expression of genes for IL-1-beta, IL-2, IL-4, IL-6, IL-8. IL-10, IL-12; 35p, 40p, IL-13, GMCSF, GSF and RANTES was not detected at all. However, the anti-HIV activity of the cell culture supernatant *in vitro* cannot be explained by mere presence of the inflammantary substances mentioned above, because they do not possess such activity and their M_r is higher than that of AVF. Our findings raise the possibility that AVF(s) may be involved in the mechanism of cell resistance against HIV.

Key words: cytokines; chemokines; HIV; T-lymphoid cells; metal/DNA complex

Introduction

In spite of enormous progress in developing protease inhibitors for treating acquired immunodeficiency syndrome (AIDS) patients a search of new agents with alternative mechanism of action is still urgent (Carpenter *et al.*, 1996). One of possible approaches to this is goal is the use immunomodulators which could suppress HIV replication or enhance the host immune system to combat HIV infection. Several ILs, interferons (IFNs) and IFN inducers inhibit viral replication (Vilcek *et al.*,1979; De Clercq, 1986; Pestka *et al.*, 1987; Nossik *et al.*, 1988; Clerici *et al.*, 1994a; Levy, 1994; Kaye, 1995). Recently it has been also shown that biological response modifiers (BRMs) including chemokines, such as RANTES, MIP1-alpha and MIP1-beta, could suppress HIV replication (Schall, 1991; Mackewicz *et al.*, 1993; Liles and Van Voorhis, 1995; Baier *et al.*, 1995; Cocchi *et al.*,1995; Balter, 1995).

Earlier we have studied immunomodulatory effects of metal/DNA complexes (Nossik *et al.*, 1994). Extending this

Abbreviations: AIDS = acquired immunodeficiency syndrome; AVC = antiviral compound; AVF = antiviral factor; BRM = biological response modifier; CPE = cytopathic effect; DEPC = dietylpyrocarbonate; FCS = foetal calf serum; HIV = human immunodeficiency virus; IFN = interferon; IL = interleukin; MI = multiplicity of infection; MoMLV = Moloney murine leukemia virus; PBL = peripheral blood lymphocyte; p.i. = post infection; RT = reverse transcription, reverse transcriptase; RT-PCR = reverse transcription-polymerase chain reaction

study, several metal/DNA complexes were prepared and studied for their anti-HIV-activity *in vitro*. The analysis of antiviral effect of these complexes enabled to found a new factor(s), AVF, which was induced by them and was a subject of further investigation.

Materials and Methods

Cells. H9, MT-2, MT-4, CEM, CEM-SS and Jurkat cell lines and peripheral blood lymphocytes (PBLs) were used for testing the antiviral activity. The cells were cultivated in RPMI 1640 medium with 10% foetal calf serum (FCS), 100 μg/ml gentamycin, and 5 mmol/l L-glutamine. Cell viability was evaluated by the Trypan Blue uptake method and MTT assay.

Viruses. HIV isolates from the collection of the D.I. Ivanovsky Institute of Virology and reference strains HIV-1/BRU granted by Prof. L. Montagnier, Institute Pasteur, Paris, France, and HIV-1/899A granted by Prof. L Gurtler, Max von Pettenkoffer Institute, München, Germany, were used.

Metal/DNA complexes and antiviral assay. The antiviral activity of the complexes was studied on T-cell lines infected with HIV-1. The cells were cultivated in plastic 24-well plates (Costar) in suspension. Complexes Ag^{1+}/DNA, Co^{2+}/DNA, Zn^{2+}/DNA and Fe^{3+}/DNA, and metal salts AgCl, $ZnSO_4$, $CoCl_2$ and $FeCl_3$ in concentrations of 200 – 1600 mg/ml were present in medium added to cells 1 hr before, 1 hr after and simultaneously with virus, respectively. The multiplicity of infection (MI) was 0.001 $TCID_{50}$/cell. The plates were incubated at 37°C in the atmosphere of 5% CO_2 in humidified incubator for 4 – 7 days. The antiviral activity of the agents was evaluated by inhibition of virus-induced cytopathic effect (CPE), syncytium formation and HIV-1 p24 antigen release in culture fluid measured by an enzyme-linked immunosorbent assay (ELISA) kit (Abbott).

Interferon (IFN) assay was performed in 96-well plastic plates (Falcon Plastics) on monolayer cultures of human fibroblast cells grown in Eagle's medium with 10% FCS and 100 μg/ml gentamycin. The culture fluid was removed and twofold dilutions of samples in Eagle's medium with 2% FCS were added to the cells. The incubation proceeded at 37°C in the atmosphere of 5% CO_2 in humidified incubator for 24 hrs. Then the cells were infected with vesicular stomatitis virus (VSV) at MI of 0.1 PFU/cell. The plates were further incubated for 20 – 24 hrs. The antiviral activity was determined by the method of inhibition of CPE of VSV in cells and the IFN titre was defined as the reciprocal of the highest sample dilution causing 50% CPE inhibition.

Reagents and filters. A native DNA with M_r of 270 – 500 K (hyperchromism >37%, ballast protein content <1.0%) was isolated from salmon milt and was conjugated in a form of 0.5% sodium salt with Ag^{1+}, Co^{2+}, Zn^{2+} and Fe^{3+}. In the case of Fe^{3+} complexes with different Fe^{3+}/DNA ratios (0.005 – 0.1) were prepared. Guanidine isothiocyanate and 2-mercaptoethanol (Wako), sodium N-lauroylsarcosine (Sigma), CsCl (Nakarai), RNase inhibitor (Tokara), 5 x RT buffer and Moloney murine leukemia virus (MoMLV) reverse transcriptase (Life Technologies Gibco BRL), poly(dT)$_{12-18}$ (Pharmacia), gelatin (Perkin Elmer), and Taq polymerase and SYBR Green II (FMC BioProducts) were employed.

Azidothymidine (Retrovir, GlaxoWellcome) was used as the reference antiviral drug. Amicon (Millipore) filters retaining molecules of M_r above 3 K, 5 K, and 10 K, respectively, were used as described by the manufacturer.

Treatment of cells with AVC. Analysing the possible mechanism of antiviral action of AVC (Fe^{3+}/DNA) complex we assumed that it induces in cells some factor (AFV) or factors (AVFs) which could be responsible for the antiviral action of AVC. Taking in account this assumption AVC was added to the suspension of T-lymphoblastoid cells to make different final concentrations (800 –1600 μg/ml) for different periods of time (0.5 – 24 hrs) and the cells were incubated in 5% CO_2 atmosphere at 37°C. Then the medium was discarded, cells were washed by low speed centrifugation (800 rpm for 3 – 4 mins) and were incubated under the same conditions for additional 12 – 24 hrs in fresh RPMI 1640 medium. Then the supernatant was analysed for biological activity as it was described above.

Particle-induced X-ray Emission (PIXE) was used for estimation of concentration of metal/DNA complexes (Miura and Itoh, 1993).

Total RNA extraction and purification. RNA was extracted from cultured MT-4 cells by the guanidine isothiocyanate method. Briefly, cells were lysed by vigorous vortexing in 2 ml of GITC solution (4 mol/l guanidine isothiocyanate, 0.5% sodium N-lauroylsarkosine, 2.5 mmol/l sodium citrate pH 7.5, 0.1 mol/l 2-mercaptoethanol in diethylpyrocarbonate (DEPC)-treated water. The lysate was layered onto 1.5 ml of CsCl solution (5.7 mol/l CsCl and 0.1 mol/l EDTA pH 8.0 in DEPC-treated water) in a 4 ml ultracentrifugation tube. After centrifugation at 35,000 x rpm for 15 hrs at room temperature, the pelleted RNA was dissolved in 200 μl of DEPC-treated water, ethanol-precipitated, pelleted again and washed with 70% ethanol. The dried RNA was dissolved with DEPC-treated water and estimated spectrophotometrically at A_{260}

Cytokine mRNA assay by RT-PCR. For cytokine mRNA-specific RT-PCR we used the primers listed in Table 1. The cDNA was made from the extracted RNA using a reverse transcription (RT) mixture containing 2 μl of 0.1 mol/l dithiothreitol, 0.3 μl (6 U) of RNase inhibitor, 3.2 μl of 2.5 mmol/l dNTPs, 4 μl of 5 x RT buffer and 0.5 μl (100 U) of MoMLV RT. A mixture of 8 μl of sample RNA and 2 μl of 20 μmol/l poly(dT)$_{12-18}$ was heated to 70°C for 10 mins, cooled on ice and added to the RT mixture. After incubation at 37°C for 90 mins the reaction was stopped by heating at 90°C for 5 mins. The obtained cDNA was used for PCR in 20-μl reaction volume consisting of 2 ml of 10 x PCR buffer (100 mmol/l Tris-HCl pH 8.3, 500 mmol/l KCl, 15 mmol/l $MgCl_2$ and 0.01% gelatin), 0.2 μl of each 20 μmol/l primer, 1.6 μl of 2.5 μmol/l dNTPs, cDNA and a 1 U of Taq polymerase. The reaction mixture was first heated for 2 mins at 94°C and the cDNA was then amplified in 35 cycles. Each cycle consisted of 30 secs at 94°C, 45 secs at optimal temperature for the given target cytokine shown in Table 1, and 90 secs at 72°C. Final extention was done for 10 mins at 72°C. Resulting PCR products were subjected to electrophoresis in 1.5% agarose gel and silver-stained with SYBR Green II.

Table 1. PCR primers and annealing temperatures used

	5'-primer sequence	mRNA position (nt)	3'-primer sequence	mRNA position (nt)	PCR product (bp)	°C
Beta-actin	5'TCA CCC ACA CTG TGC CCA TCT ACG A	2141-2165	5'CAG CGG AAC CGC TCA TTG CCA ATG G	2435-2411	295	60
IL-1 alpha	5'GTA AGC TAT GGC CCA CTC CAT	148-168	5'TGA CTT ATA AGC ACC CAT GTC	535-555	408	60
IL-1 beta	5'GGA TAT GGA GCA ACA AGT G	527-545	5'ATG TAC CAG TTG GGG AAC TG	790-771	264	64
IL-2	5'GTC ACA AAC AGT GCA CCT AC	156-175	5'CCC TGG GTC TTA AGT GAA AG	398-417	262	60
IL-4	5'GTC TCA CCT CCC AAC TGC TT	68-87	5'ACG TAC TCT GGT TGG CTT CC	441-460	393	55
IL-6	5'ATG AAC TCC TTC TCC ACA AGC G	34-55	5'CTG GAC TGC AGG AAC TCC TT	647-628	614	60
IL-8	5'ATG ACT TCC AAG CTG GCC GTG	102-122	5'CTC TTC AAA AAC TTC TCC ACA AC	385-363	284	60
IL-10	5'CAC TGC TCT GTT GCC TGG T	44-62	5'AAT AAG GTT TCT CAA GGG GCT	673-653	630	55
IL-12,35P	5'CCA GGA ATG TTC CCA TGC CTT	297-317	5'GGC CTG CAT CAG CTC ATC AAT	710-690	414	60
IL-12,40P	5'GGA CCA GAG GAG TGA GGT CTT	199-219	5'CTC CTT GTT GTC CCC TCT GA	572-553	373	60
IL-13	5'GAG TGT GTT TGT CAC CGT TG	869-888	5'TAC TCG TTG GCT GAG AGC TG	1103-1122	254	55
IL-15	5'CCA TTA GAA GAC AAA CTG TTC TTT GC	317-340	5'ATG AGA ATT TCG AAA CCA CAT TTG	693-670	377	55
GMCSF	5'AGC ATG TGA ATG CCA TCC AG	100-119	5'ATA GTC TGG GTT GCA CAG GA	361-342	262	60
RANTES	5'TCA TTG CTA CTG CCC TCT GC	61-80	5'CTA GCT CAT CTC CAA AGA GTT G	281-302	242	55
INOS	5'CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG	3590-3621	5'GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG G	3848-3817	259	60
TNF-alpha	5'GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A	404-434	5'GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T	847-817	444	60
TNF-beta	5'ATG ACA CCA CCT GAA CGT CTC TTC	141-164	5'CGA AGG CTC CAA AGA AGA CAG TAC T	750-726	610	55
IFN-alpha	5'CAG GAG GAG TTT GAT GGC AAC CAG	187-210	5'GAC AAC CTC CCA GGC ACA AGG GC	501-478	315	60
IFN-beta	5'AAA GAA GCA GCA ATT TTC AGC	167-187	5'CCT TGG CCT TCA GGT AAT GCA	547-527	381	60
IFN-gamma	5'GCA TCG TTT TGG GTT CTC TTG GCT GTT ACT GC	164-195	5'CTC CTT TTT CGC TTC CCT GTT TTA GCT GCT GG	590-559	427	55

Results and Discussion

We observed an increasing level of inhibition of HIV infection with metal/DNA complexes from Ag^{1+} (minimum) to Zn^{2+}, Co^{2+} and Fe^{3+} (maximum) (Fig. 1). It seems that the difference in the inhibitory activity of metal/DNA complexes is connected with different mechanisms of their interaction with DNA.

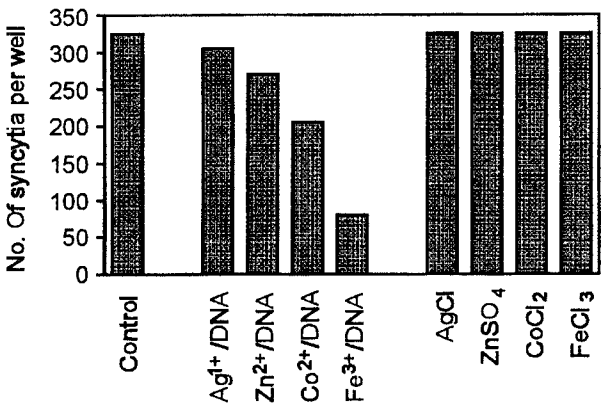

Fig. 1
Effects of metal/DNA complexes and metal salts on syncytium formation in MT-4 cells
The examined substances in the concentration of 800 mg/ml were applied to the cells 1 hr before virus infection at the multiplicity of 0.001 $TCID_{50}$/cell. The number of syncytia was estimated 4 – 7 days p.i.

The solutions of metal salts (AgCl, $ZnSO_4$, $CoCl_2$ and $FeCl_3$) did not exert any antiviral activity. The tested metal/DNA complexes were not cytotoxic in 200 – 800 mg/ml concentrations. Two of them were slightly cytotoxic in 1600 μg/ml concentration (7 – 8% reduction of cell viability). The most active Fe^{3+}/DNA complex was designated as AVC (antiviral compound) and was chosen for further studies.

The concentration of AVC used for induction of cells was 23.854 μg/ml as estimated by PIXE. The size of most DNA in the inducer as estimated by 1.5% agarose gel electrophoresis was below 1 kbp.

The analysis of effects of various Fe^{3+}/DNA ratios showed that the viability of infected cells increased and the syncytium formation decreased with the decrease of the ratio from 0.1 to 0.005 (Table 2). The antiviral effect was seen in all variants of AVC administration (1 hr before, 1 hr after and simultaneously with virus) (data not shown), but the highest effect had AVC when added before virus in concentration of 800 – 1600 μg/ml (Fig. 2).

It was found that the culture fluid of cells induced by a metal/DNA complex possessed anti-HIV-1 activity and we could suppose that it contained some cellular antiviral factor(s) (AVF). The experiments showed that AVF blocked HIV-1 production in naturally infected PBMCs and T-cells infected with HIV-1 (Fig. 3). The culture fluid of cells which were not treated with AVC did not have antiviral activity. The EC_{50}

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