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INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE-1-INDUCED SYNCYTIUM FORMATION AND CYTOPATHICITY BY COMPLESTATIN

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Complestatin, an anti-complement agent, was shown to be a potent inhibitor of human immunodeficiency virus type 1 (HIV-1) infection *in vitro*. It inhibited HIV-1-induced cytopathicity and HIV-1 antigen expression in MT-4 cells; the 50% effective doses for these effects were 2.2 and 1.5 μg/ml, respectively. No toxicity for MT-4 cells was observed at concentrations up to 400 μg/ml. In addition, the agent inhibited the focus formation in HT4-6C cells (CD4-positive HeLa cells); the concentration for 50% focus reduction was 0.9 μg/ml. HIV-1-induced cell fusion in cocultures of MOLT-4 cells and MOLT-4/HTLV-III$_B$ were also blocked by complestatin (the concentration for 50% cell fusion inhibition, 0.9 μg/ml). Complestatin had no ability to inhibit HIV-1 reverse transcriptase activity. When MT-4 cells were pretreated with complestatin for 2 hrs prior to the exposure to HIV-1, the HIV-1-induced cytopathicity was markedly inhibited, while pretreatment of HIV-1 with the agent did not affect the infection. These results suggest that complestatin primarily interacts with cells and inhibits viral adsorption to the cell surface as well as adsorption of infected cells to adjacent cells.

Complestatin ($C_{61}H_{45}N_7O_{15}Cl_6$, MW 1,325) is a peptide-like compound (Fig. 1) isolated from the mycelium of *Streptomyces lavendulae*; it has in its molecular structure two unique amino acids,: D-(-)-4-hydroxyphenylglycine and D-(-)-3,5-dichloro-4-hydroxyphenylglycine (1,2). Complestatin has been shown to inhibit both classical and alternative pathways of complement activation, the former by inhibiting the formation of C4b,2b complex through its binding to C2, and the latter by binding to factor B and hence inhibiting the formation of C3b,Bb complex (3). Factor B and C2 have a specific domain homologous to the I domain of LFA-1 (4) at their N-terminal sides, to which complestatin is supposed to bind (unpublished results).

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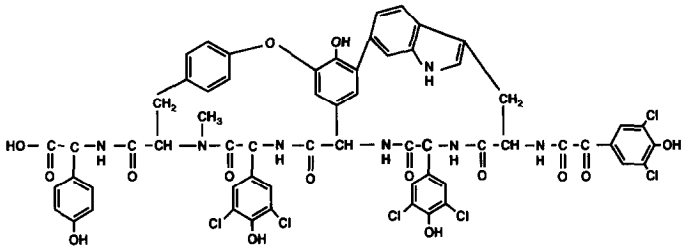

Complestatin : $C_{61}H_{45}N_7O_{15}Cl_6$ (MW 1,325)

Fig. 1. Structural formulas of complestatin.

Cell fusion may be one of the major mechanisms by which HIV-1 infection exerts its cytopathic effect and leads to $CD4^+$ T-lympocyte depletion in individuals with HIV-1. For the syncytium formation of HIV-1-infected cells, interaction of CD4 molecules on permissive cells with HIV-1 gp-120 expressed on HIV-1-infected cells is required (5). However, molecules other than $CD4^+$ have also been proposed to play an important role in HIV-1-induced syncytium formation; Hildreth and Orentas demonstrated that LFA-1 was involved in syncytium formation induced by HIV-1 (6). These observations led us to examine the effects of complestatin on HIV-1-induced syncytium formation and cytopathicity. The results indicate that complestatin binds to permissive cells and inhibits not only the infection of HIV-1, but also the fusion processes of infected cells.

MATERIALS AND METHODS

Cells and viruses: The $CD4^+$ T-lymphoblastoid cell lines, MT-4 (HTLV-1-carring cells) and MOLT-4 (clone 8), and a HIV-1-producing cell line, MOLT-4/HTLV-III_B were kindly provided by Dr. N. Kobayashi and Dr. N. Yamamoto, Department of Virology and Parasitology, Yamaguchi University, Yamaguchi, Japan. HT4-6C, a CD4-expressing HeLa cell line, was kindly provided by Dr. B. Chesebro, Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana, USA. The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics at 37 °C in an atmosphere of 95% air and 5% CO_2. The stock of HIV-1 (strain HTLV-III_B) was prepared from the culture supernatant of persistently HIV-1-infected MOLT-4/HTLV-III_B. The virus stocks were stored in small aliquots at -70 °C until use.

Compounds: Complestatin was isolated from the mycelium of *Streptomyces lavendulae* according to the procedure previously described (1). 3'-Azido-3'-deoxythymidine (AZT) and aurintricarboxylic acid (ATA) were purchased from Sigma Chemical Co. (St. Louis, MO. USA). Dextran sulfate (MW 500,000) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Recombinant HIV-1 reverse transcriptase was purchased from Eiken Kagaku (Tokyo, Japan).

Measurement of cytopathic effect of HIV-1: The procedure for measuring anti-HIV-1 activity in MT-4 cells was performed as described previously (7). Briefly, exponentially growing MT-4 cells were centrifuged for 5 min at 140 x g. The MT-4 cell pellet was infected with HIV-1 stock solution at 100 $CCID_{50}$ in RPMI-1640 medium. After a 1-hr incubation at 37 °C, the MT-4 cells were resuspended at $4x10^5$ cells/ml in

RPMI-1640 medium containing 10% FCS, and then $4x10^4$ cells in 0.1ml were brought into each well of a flat-bottomed 96-well culture plate containing 0.1 ml each of serial two-fold dilutions of each of the test compounds. After a 6-day incubation at 37 °C, the cytopathic effect of HIV-1 was estimated from the viability of HIV-1-infected cells determined by the MTT method (8). In order to estimate the fraction of infected cells, the indirect immunofluorescence assay using HIV-1 infected individual serum was applied (9).

Focus immunoassay: Twenty-four hrs after seeding 2.5 x 10^4 HT4-6C cells in each well of 48-well culture plates (0.5 ml/well), the plates were washed with fresh medium and 0.1 ml of diluted HIV-1 stock solution was added. Immediately after infection, 0.5-ml aliquots of culture medium containing varying concentrations of test compounds were added, and the cells were further incubated at 37 °C . Five days after infection, the medium was removed and the adherent cells were fixed for 5 min with methanol. Focus immunoassay on these plates was carried out according to the method described by Chesebro and Wehrly (10) with slight modifications.

HIV-1-induced cell fusion assay: The extent of cell fusion induced by HIV-1 infection was assayed in cocultures of MOLT-4 and MOLT-4/HTLV-III_B as described previously (11,12).

Reverse transcriptase assay: Activity of recombinant HIV-1 reverse transcriptase was assayed as described previously (13).

RESULTS

On cultivation, MT-4 cells usually form cell clumps (Fig. 2A). Although the clump formation was reduced when MT-4 cells were cultured in the presence of complestatin (Fig. 2 B-D), complestatin by itself showed no cytotoxicity in mock-infected MT-4 cells at concentrations up to 400 μg/ml (Table 1). When MT-4 cells were incubated with 100 $CCID_{50}$ of HIV-1 (HTLV-III_B) for 6 days, almost all cells died due to the cytopathic effect (CPE) of the virus. However, complestatin markedly inhibited the HIV-1-induced cytopathicity in MT-4 cells in a dose-dependent manner; an inhibitory effect on CPE was apparent at 0.39 μg/ml, and complete inhibition was attained at concentrations higher than 6.25 μg/ml (Fig. 3). The 50% inhibitory concentration (IC_{50}) for CPE was 2.2 μg/ml (Table 1). It also inhibited HIV-1 antigen expression in the same concentration range, and to the same extent (Fig. 3). In HT4-6C cells, the number of foci of HIV-1 infection was markedly diminished by the agent; the IC_{50} for focus formation was at 0.9 μg/ml (Table 1) and complete reduction occurred at 3 μg/ml. Furthermore, the agent strongly inhibited HIV-1-induced cell fusion in cocultures of MOLT-4 cells and MOLT-4/HTLV-III_B cells, with a 50% cell fusion inhibitory concentration of 0.9 μg/ml (Table 1).

The effects of several other anti-HIV compounds, including dextran sulfate (14), ATA (15), and AZT (16), were compared with those of complestatin. As shown in Table 1, the IC_{50} of complestatin for HIV-1-induced CPE was comparable to those of ATA and dextran sulfate, which are respectively known to inhibit CD4 receptor function and the

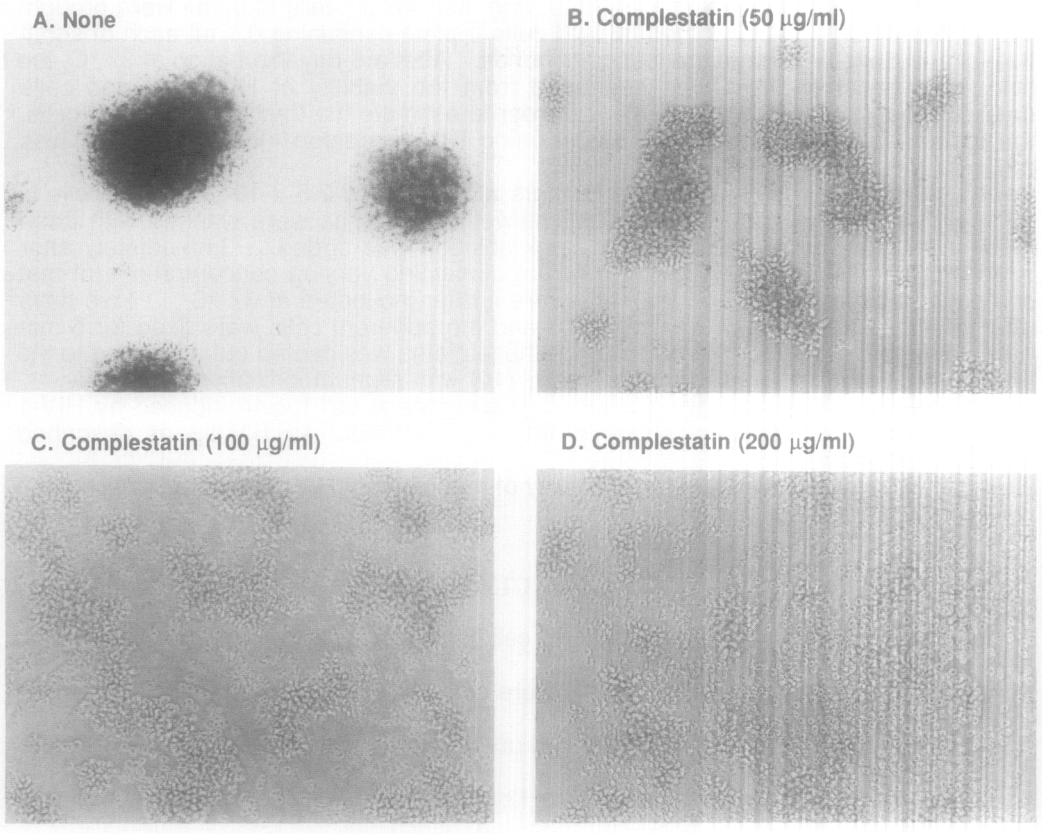

Fig. 2. Morphological changes in MT-4 cells with complestatin. MT-4 cells were cultured without complestatin (A), or with 50 μg/ml (B), 100 μg/ml (C), and 200 μg/ml (D) of complestatin for 6 days.

Table 1. Comparison of anti-HIV-1 activities of complestatin, ATA, dextran sulfate, and AZT in three different assay systems

Compound	IC_{50} for CPE (μg/ml)	IC_{50} for Cell Fusion (μg/ml)	IC_{50} for Focus Formation (μg/ml)	50% Cytotoxicity (μg/ml)	SI
Complestatin	2.2	0.9	0.9	>400	>182
ATA	1.7	1.5	1.2	35	20
Dextran sulfate	4.5	3.2	2.3	>400	>89
AZT	0.03	>10	0.006	25	833

ATA: aurintricarboxylic acid.
AZT: 3'-azido-3'-deoxythymidine.
CPE: cytopathic effect.

Experiments were carried out as described in Materials and Methods. Numbers indicate means for three experiments. Selectivity index (SI) was the ratio of IC_{50} for CPE to 50% cytotoxicity.

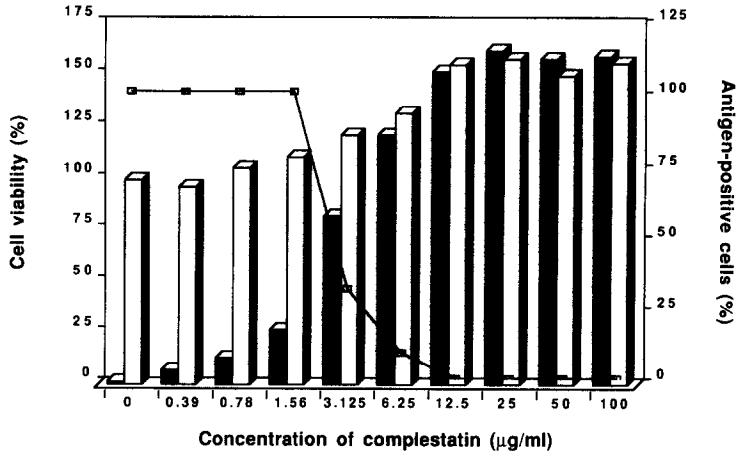

Fig. 3. Inhibitory effects of complestatin on HIV-1-induced cytopathicity and antigen expression in MT-4 cells. MT-4 cells were inoculated with HIV-1 and incubated for 6 days with various concentrations of complestatin. The viabilities of virus-infected cells (closed columns) and mock-infected cells (open columns) were assessed by the MTT method. The viable cells were expressed as a percentage of mock-infected control cells. Viral antigen expression was examined by indirect immunofluorescence assay, and antigen-positive cells (open squares) were expressed as a percentage of virus-infected control cells.

binding of HIV to CD4. Because of the low cytotoxicity of complestatin, the selectivity index for complestatin (>182) was higher than those for ATA and dextran sulfate (Table 1). The IC_{50}s for HIV-1-induced cell fusion and for focus formation were also comparable among complestatin, ATA, and dextran sulfate.

In an attempt to determine the mechanism of the anti-HIV-1 action of complestatin, MT-4 cells were treated with complestatin or other agents at various stages and their anti-HIV-1 activities were compared (Table 2). In experiments designated "Pretreatment," MT-4 cells were preincubated prior to the virus infection with the test compounds at various concentrations for 2 hr at 4 °C in the absence of FCS. Cells were washed and then exposed to HIV-1. The infection of MT-4 cells by HIV-1 was blocked by pretreatment with complestatin and ATA, with respective IC_{50}s for CPE of 35 μg/ml and 25 μg/ml. Pretreatment with dextran sulfate, however, did not protect the cells against the infection at concentrations up to 100 μg/ml. When the cells were first exposed to HIV-1 in the presence of test compounds for 1hr at 37 °C followed by further incubation in fresh growth medium without test compounds (experiment designated "Treatment A"), all compounds, including complestatin, but not AZT, showed a strong anti-HIV-1 effect (Table 2). The experiment designated "Treatment

Table 2. Influence of various treatment periods on anti-HIV-1 activity of complestatin, ATA, dextran sulfate, and AZT in MT-4 cells

Compound	IC_{50} for CPE (μg/ml)		
	Pretreatment	Treatment A	Treatment B
Complestatin	35	12.5	3.0
ATA	25	5.0	2.0
Dextran sulfate	>100	6.0	4.0
AZT	ND	>3.12	0.03

ND: Not determined.
ATA: aurintricarboxylic acid.
AZT: 3'-azido-3'-deoxythymidine.

In experiments designated "Pretreatment," MT-4 cells were incubated with the test compounds in serum-free RPMI-1640 medium at various concentrations for two hrs. The test compounds were removed and the cells were infected with HIV-1. The viability of the washed HIV-1-infected cells was measured by the MTT method.

In experiments designated "Treatment A," MT-4 cells were infected with HIV-1 and exposed simultaneously to the test compounds. After a 1-hr incubation at 37 °C, MT-4 cells were washed and further incubated in the absence of the compounds.

In experiments designated "Treatment B," MT-4 cells were incubated in the test compounds at various concentrations after exposure to HIV-1 for one hr at 37 °C.

Numbers indicate the mean value for two separate experiments. Anti-HIV-1 activities were expressed as the 50% inhibitory concentration, which reduces by 50% the number of cells in a mock-infected culture.

B," shown in Table 2, also revealed that under conditions in which the cells were exposed to HIV-1 for 1hr at 37 °C, followed by removal of unabsorbed viruses and by further incubation in the presence of compounds, AZT was the most effective and the other three had comparably effective in the anti-cytopathic actions. Thus, complestatin showed effects similar as ATA, but not as dextran sulfate and AZT. Neither complestatin nor ATA showed a reduction in virus infectivity, when viruses had been preincubated with the agents for one hr at 4 or 37 °C at various concentrations up to 50 μg/ml (data not shown).

Complestatin did not show any effect on the activity of recombinant reverse transcriptase of HIV-1 at various concentrations up to 100 μg/ml (data not shown).

DISCUSSION

The present study showed complestatin to be a potent inhibitor of HIV-1 infection *in vitro* (Fig. 2, Table 1). In order to analyze the anti-HIV-1 activity of complestatin, we compared the effects of complestatin with those of AZT, dextran sulfate, and ATA, whose mechanisms of a action have been well characterized (14,15). All agents tested showed inhibitory effects on cytopathicity and focus formation induced by HIV.

However, a striking difference was found between complestatin and AZT when their activities to inhibit HIV-1-induced giant cell formation (cell fusion) were assessed (Table 1). That is, complestatin as well as dextran sulfate and ATA, but not AZT, inhibited giant cell formation of MOLT-4 cells cocultured with MOLT-4/HTLV-III_B cells. This observation suggests that complestatin has a mode of action that is different from AZT and rather similar to dextran sulfate or ATA. This notion was further supported by the fact that, in contrast to AZT-triphosphate derivatives, complestatin showed no inhibitory effect on reverse transcriptase activity. The data presented in Table 1 and Table 2 indicate that complestatin, as likely as ATA and dextran sulfate, interferes with an early event in the replicative cycle of the virus, presumably virus adsorption. Complestatin's protective activity was considerable when it was included only during the 1-hr virus adsorption period (Table 2). It was also effective even if the cells were pretreated with it before exposure to HIV-1. On the other hand, the pretreatment of HIV-1 with complestatin showed no effect at all. Thus, it is reasonable to assume that complestatin interacts directly with the cell, but not with HIV-1, and thereby inhibits HIV-1 adsorption and cell fusion. Pretreatment of the MT-4 cells with dextran sulfate did not offer any protection, while complestatin and ATA protected the cells against the infection. Therefore, complestatin seems to inhibit the infection of HIV-1 with a mechanism similar to that of ATA, which was reported to exert its inhibitory effect through interaction with cell surface molecules on target cells.

Many matrix receptors, for example LFA-1, Mac-1, VLA-2, fibronectin receptors, glycoprotein IIb/IIIa, and vitronectin receptors have been characterized (17) and collectively called integrins (18). They are all heterodimers with α and ß chains (18). Among them, LFA-1, VLA-2, and Mac-1 are unique in that at the central sites of their α chain, they have additional inserted (I) domain that is homologous to the region at the N-terminal side of factor B/C2 (4). Hildreth and Orentas recently reported that the monoclonal antibodies against both chains of LFA-1 completely inhibited HIV-1-induced cell fusion, but the antibodies did not block binding of gp-120 to CD4 (6). This observation indicates that, in addition to CD4, cell surface molecules of the LFA-1/integrin family also play an important role in the process of cell fusion induced by HIV-1 infection. We have previously shown that complestatin inhibited the alternative

pathway of complement activation by a direct interaction with factor B (3); complestatin directly bound to factor B and obscured its binding site for C3b. Therefore, the results of the present study may be interpreted that complestatin binds to cell surface molecules related to the integrin family and blocks HIV-1-induced cytopathicity and HIV-1-cell fusion. The inhibition of cell-cell adhesion in MT-4 cells by complestatin shown in the present study (Fig. 2.) may reflect an alteration in the function of integrin molecules.

Complestatin is a unique compound in its way of providing a rapid and selective modulation of both cell-cell interaction and cell-virion interaction. Complestatin and other inhibitors of integrin molecules can be a new category of anti-HIV agents.

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