The Effect of Cyclosporine A on Infection of Susceptible Cells by Human Immunodeficiency Virus Type 1

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The effect of cyclosporine A (CyA) on the ability of the human immunodeficiency virus type 1 (HIV-1) to infect the H-9 T-cell leukemic line, as well as interleukin-2 (IL-2)–grown human peripheral blood-derived lymphocytes, has been studied. Pretreatment of H-9 cells and human lymphocytes with CyA over 24 hours completely prevented viral infection over a 21-day period, whereas the addition of drug at two hours postinfection with HIV-1 had a significant inhibitory effect on viral replication and expression of the virus-specific antigens p17 and p24. However, if CyA was added at later times to these lymphocytic cells, this inhibitory effect was lost. Indeed, the removal of CyA from cultures that had been treated from two hours after infection led to the rapid production of progeny virus. HIV-1 was able to infect peripheral blood lymphocytes obtained from each of four kidney allograft recipients on long-term CyA antirejection therapy, as long as drug was not included in the culture medium. In addition, we asked whether pretreatment with CyA of cells of the U-937 monocytic line and primary cultures of human monocytes/macrophages might have on infection by HIV-1. CyA had no demonstrable effect on the ability of HIV-1 to infect cells of either type.

Aquired immune deficiency syndrome (AIDS) is caused by the human immunodeficiency virus, type 1 (HIV-1). This virus has the ability to infect and replicate in cells that express CD4 antigen on their surface. The primary targets of HIV-1 in the body seem to be T helper lymphocytes. However, it is clear that other cell types such as monocytes, microglial cells, and endothelial cells may also be infected. Indeed, monocytes and macrophages that are infected by HIV-1 seem to be somewhat refractory to the usual cytopathic effects of this virus. For this reason, infected monocytes are thought to constitute an important reservoir for dissemination of HIV-1 throughout the body.

There is no consensus of opinion to account for the fact that numbers of circulating T helper cells in HIV-1–infected individuals are often <10% of normal. This conceptual difficulty is buttressed by the fact that in situ hybridization analysis has revealed the percentage of HIV-1–infected mononuclear cells in the blood to be <0.01%. Accordingly, a number of investigators have postulated that the development of HIV-1–related diseases might include an autoimmune phase in which various types of effector cells, sensitized by infected lymphocytes, might be involved in the destruction of both HIV-1–infected as well as healthy cells. Effector cells in such reactions could include cytotoxic T lymphocytes (CTL), macrophages, cells participating in antibody-dependent cellular cytotoxic reactions (ADCC) and in complement-dependent cytotoxic reactions.

On the basis of speculation that such autoimmune phenomena might be involved, several groups have proposed that some form of immunosuppressive therapy might be able to help restore normal levels of circulating CD4 (+) lymphocytes in AIDS patients or, at the least, might prevent further diminution of the CD4 (+) pool in HIV-1–infected individuals. One compound that has undergone limited clinical testing in this regard is the immunomodulating drug cyclosporine A (CyA). CyA has been used successfully in a variety of clinical settings, as both a prophylactic and therapeutic agent, to prevent graft versus host disease and inflammatory disease. Its effects on the immune system have recently been reviewed.

Therefore, the purpose of our investigation was to determine the effect of CyA on the ability of HIV-1 to infect and to replicate in CD4 (+) lymphocytes and in monocytes. We have found that T cells, but not monocytes, which were treated with CyA, before or during exposure to HIV-1, became relatively resistant to infection by this virus. The removal of CyA from such cells, at times after binding of HIV-1 had already occurred, resulted in active infection and production of progeny virus. The addition of CyA to cells that had already been productively infected by HIV-1 had no effect either on viral replication or on viral antigen expression.

MATERIALS AND METHODS

Cell Lines and Propagation of HIV-1

The human T cell leukemia virus (HTLV)-IIIIB strain of HIV-1 was harvested from culture fluids of the H-9 T cell line (kindly supplied by Dr R.C. Gallo, National Institutes of Health [NIH], Bethesda, MD). This cell line is productively infected by the HTLV-IIIIB strain of HIV-1 and produces large quantities of virus on a continuous basis. Supernatant fluids, harvested three hours after change of medium, were kept frozen at −70°C until use. No loss of infectivity or reverse transcriptase activity was noted during storage of this pool of virus. On the basis of viral dilutions and infectious center assays performed on previously uninfected H-9 cells, the viral suspension used contained about 8 × 10^3 infectious units of HIV-1/mL. A common preparation of virus was used throughout these studies. In some experiments, we employed the

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U-937 monocytic cell line (kindly supplied by Dr J. Hiscott, Jewish General Hospital, Montreal) as a target of HIV-1 infection, either in the presence or absence of CyA.

Lymphocytic Cultures

Blood was obtained on numerous occasions from healthy donors. Mononuclear cells were separated from whole blood by Ficoll-Isoopaque centrifugation, and, after washing, were stimulated by phytohemagglutinin (concentration of 1:1,000) (PHA-P; Difco Laboratories, Detroit) at a concentration of 10^6 cells/mL in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), as previously described. Three days after stimulation, cells were washed and resuspended at a concentration of 10^6 cells/mL in fresh medium, supplemented with 10% FCS and exogenous purified interleukin-2 (IL-2) (2% vol/vol) (Boehringer-Mannheim, Munich). After various times, eg, seven days, ten days, 14 days, the cells were washed by centrifugation and recultured in the presence of supplemented medium and IL-2 at a concentration of 5 x 10^5 cells/mL. These lymphocytes were used in experiments designed to assess the effect of time of addition of CyA on HIV-1 infectivity and in washout experiments in which CyA was removed from cultures that we had attempted to infect by HIV-1. Lymphocytes were also obtained from four renal allograft recipients on long-term CyA therapy and assessed for ability to be infected by HIV-1.

HIV-1 Infection of Target Cells

For purposes of infection, 5 x 10^5 peripheral blood-derived lymphocytes that had been grown in the presence of IL-2 were pretreated with polybrene (2 μg/mL) for 20 minutes in 5 mL RPMI medium. The cells were then washed by centrifugation, resuspended in 0.5 mL of an undiluted, thawed suspension of cell-free infectious H-9 culture fluid, and incubated at 37°C for two hours with constant rotation. The cells were then centrifuged again to remove unbound virus and resuspended in fresh medium (10^6 cells/mL) containing exogenous IL-2. In some experiments, the culture medium also contained CyA at a variety of concentrations, as explained below. Thus, multiple medium changes, accomplished by washing the cells by centrifugation and resuspension in fresh medium, were performed at times when CyA, polybrene, IL-2, and/or HIV-1 needed to be either added or removed from the cell suspension. (The CyA used in these studies was a gift of Sandoz Canada, Dorval, Quebec) Previously uninfected H-9 cells and U-937 cells were exposed to HIV-1 in the same way, but without addition of IL-2.

In some experiments, we decided to investigate the effect of pretreatment with CyA on infection by HIV-1 of fresh cultures of human monocytes/macrophages. Toward this end, 5 x 10^5 peripheral blood mononuclear cells were seeded into each well of 24-well plates and incubated at 37°C for three days. Nonadherent cells were removed by washing with balanced salt solution, and the remaining adherent cells were cultured in FCS-supplemented RPMI medium. Infection of such cultures, which displayed morphologic characteristics of human monocytes/macrophages and which were positive for nonspecific esterase, was carried out in the presence of 2 μg/mL polybrene. Unattached virus was washed free of these cultures after two hours, and the cells were followed for production of infectious progeny virus and reverse transcriptase activity as described below. A variety of concentrations of CyA were employed in this study in order to evaluate dose-dependent effects. In some experiments, CyA was added to the medium only at two and 24 hours after initial exposure to virus. In other studies, a washout of CyA from the culture medium was performed at times after completion of the viral adsorption period.

Quantification of Infection

Indirect immunofluorescence. The percentage of infected cells in these various cultures was determined using a fixed cell indirect immunofluorescence assay (IFA). This involved the fixation of cells onto slides by cytocentrifugation, followed by exposure to a 1:1 acetone-methanol solution for 30 minutes at room temperature. A mixture of mouse monoclonal antibodies against the viral proteins p17 and p24 (kindly supplied by Dr R.C. Gallo, NIH) and a fluorescein-labeled goat-antimouse immunoglobulin conjugate (Miles Laboratories, Elkhart, IN) were used to visualize infected cells. At least six fields, containing a total of not less than 1,000 cells, were examined in each case, and the percentage of fluorescent cells was determined.

Reverse transcriptase activity. Reverse transcriptase activity was assayed by mixing 0.75 mL of clarified culture fluid with 0.25 mL of a 30% solution of polyethylene glycol 6000, as previously described. After overnight incubation at 4°C, virus was pelleted at 3,000 rpm for 30 minutes and then was resuspended in 25 μL of solubilizing buffer (0.8 mol/L NaCl, 0.5% Triton-x-100, 0.5 mmol/L phenylmethyl sulfonil fluoride, 1 mmol/L dithiothreitol [DTT], 20% glycerol, 50 mmol/L Tris-HCl [pH 7.5]). Aliquots of solubilized virus were kept frozen at -70°C until assayed by combining 20 μL of sample with 80 μL of reaction mixture that included 10 μCi thymidine triphosphate (methyl-3H; 40 to 70 Ci/mmol, ICN Inc, St Louis), 62.5 mmol/L Tris-HCl (pH 7.5), 1.25 mmol/L DTT, 0.1 mmol/L ATP, 12.5 mmol/L MgCl2, and 0.05 U poly(rA)p(dT)12,13 (Pharmacia Fine Chemicals, Montreal). After one hour at 37°C, the reaction was stopped in an ice bath, at which point 15 μL Escherichia coli tRNA (5 mg/mL) (Sigma Chemicals Co, St Louis) and 3.5 mL ice-cold trichloroacetic acid (TCA) (10%) was added to each tube. The samples were filtered and washed with ice-cold 5% TCA, after which the filters were dried in ethanol and air and counted.

RESULTS

Effect of Treatment With CyA on Infection by HIV-1 of H-9 Cells

CyA at concentrations ranging between 0.001 μg/mL and 1 μg/mL had no effect on either the viability or replication of either uninfected H-9 cells or H-9 cells that had been chronically infected by HIV-1, as followed over a 28-day period. Furthermore, CyA did not affect levels of CD4 expression by uninfected cells over this time. Virtually 100% of such cells were positive in these studies for expression of CD4 antigen (results not shown).

We were initially interested in assessing what effect exposure to CyA of H-9 cells, chronically infected by HIV-1, might have on either viral antigen expression or viral replication. The results indicated that treatment with concentrations of CyA ranging from 0.01 μg/mL to 1 μg/mL had no effect on the percentage of cells that were positive for the viral antigens p17 and/or p24. A zero time control revealed that roughly 85% to 90% of infected H-9 cells expressed these antigens, as determined in an indirect immunofluorescence assay. Nor did treatment with CyA have any effect on viral replication over a 72-hour period, as determined by assaying pelleted culture fluids for reverse transcriptase activity, and by carrying out infectivity assays, using previously uninfected H-9 cells as targets (results not shown). We reiterate that CyA did not affect cell viability or replication in any of these studies. This is important because of the need
to exclude the possibility that any antiviral effect of CyA might have been due to cellular toxicity.

We next asked what the effect of exposure of previously uninfected H-9 cells to CyA might be on the ability of this virus to enact infection. Toward this end, cells were co-cultivated with HIV-1, as described in Materials and Methods, were washed free of unattached virus after two hours, and were subsequently maintained in supplemented RPMI medium. In one set of cultures, the various medium changes contained CyA (0.1 μg/mL) from 24 hours prior to infection to the end of the viral adsorption period, while, in other cases, CyA (0.1 μg/mL) was only added to the medium at two or 24 hours after initial exposure to virus. Figure 1 shows that if CyA had been included in the culture medium for 24 hours before infection by HIV-1, as well as during infection itself, that no expression whatever of viral antigen took place, as determined by IFA. Figure 1 also indicates that the inclusion of CyA in the culture medium from the end of the viral adsorption period, ie, two hours, was inhibitory to the expression of p24 and/or p17 antigens after each of 5, 12, and 21 days. The addition of CyA to culture medium after 24 hours, however, was without significant effect in this regard. Further supporting data is provided in Table 1, where it can be noted that no production of progeny virus or reverse transcriptase activity occurred under conditions of pretreatment with CyA (0.1 μg/mL). Table 1 also shows, as suggested from the data of Fig 1, that those cells that were exposed to CyA either at the beginning or end of the viral adsorption period displayed restricted production of reverse transcriptase activity and progeny virus. Similar findings were obtained in each of three separate experiments.

These data on H-9 cells prompted us to ask whether similar findings might be obtained in the case of in vitro-grown lymphocytes that we attempted to infect by HIV-1. It is necessary to point out that the protocol used to infect such cells involves an initial prestimulation step of peripheral blood lymphocytes (PBLs) by PHA (see Materials and Methods). However, CyA was not used to treat cells at this stage, as it probably would have interfered with cellular proliferation.22,23 Rather, treatment with CyA was only enacted at later times, ie, seven to 14 days after IL-2 had been added to the culture medium. At the dose of CyA used in these studies (ie, 0.1 μg/mL), no effect of drug on the IL-2-dependent growth of such peripheral blood-derived lymphocytes was observed.24

The data of Fig 2 show that lymphocytes that had been exposed to CyA (0.1 μg/mL) for 24 hours before addition of HIV-1 were refractory to viral infection. Addition of drug at two hours following exposure to HIV-1 was moderately inhibitory of viral antigen expression, in that positively fluorescent cells did not begin to appear in the cultures until 12 days after infection. In contrast, cultures not treated with CyA, or those that received CyA at 24 hours after viral infection, were positive for the viral antigens p17 and/or p24 by five days after infection. The percentages of infected lymphocytes in these studies ranged between 6% and 14%; these percentages are consistent with previous data obtained in this laboratory25 as well as with the work of other investigators.26,27

**Concentration Dependence of the CyA-Mediated Inhibitory Effect**

The data of Table 2 indicate that pretreatment for 24 hours of H-9 cells with CyA was able to mediate interference with HIV-1 infection in a concentration-dependent fashion. In this context, doses of CyA of 0.05 μg/mL and higher were able to interfere completely with infection, while lesser concentrations were largely ineffectual.

Similar results are illustrated in Fig 3, which depicts the

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### Table 1. Effect of 0.1 μg/mL CyA on Infectability of H-9 Cells by HIV-1

<table>
<thead>
<tr>
<th>Time of Addition of CyA (h)</th>
<th>Time of Removal of CyA (h)</th>
<th>Reverse Transcriptase Activity (cpm/mL)*</th>
<th>% Fluorescent Cells in Secondary Culture†</th>
</tr>
</thead>
<tbody>
<tr>
<td>-24</td>
<td>+2 h</td>
<td>3,149</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>—</td>
<td>76,823</td>
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<td>—</td>
<td>65,394</td>
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<tr>
<td>24</td>
<td>—</td>
<td>158,397</td>
<td>47</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>146,580</td>
<td>52</td>
</tr>
</tbody>
</table>

*Twenty-four-hour culture fluids were harvested at 12 days after initial infection by HIV-1 and processed for reverse transcriptase activity as described in Materials and Methods. Background activity was 2,934 cpm.

†Percentages of infected cells in previously uninfected cultures of H-9 cells were estimated by indirect immunofluorescence assay after five days, as described in Materials and Methods.
results of a study in which CyA was not present during the period of viral adsorption but was added two hours after initial exposure to HIV-1. In this instance, drug concentrations of 0.1 μg/mL or higher had an inhibitory effect with regard to expression of viral p17 and/or p24 antigen, while concentrations below 0.1 μg/mL were relatively without effect.

Effect of Removal of CyA From HIV-1-Exposed Cultures

We were curious to know whether those peripheral blood-derived lymphocytes in which CyA appeared to retard, but not eliminate, viral antigen expression would continue in this fashion, even if the drug were removed from the culture medium. Toward this end, cells that had been treated with CyA from two hours after exposure to HIV-1 were centrifuged after either five days or 20 days and resuspended in fresh CyA-free medium. These cells were then followed for expression of viral p17 and/or p24 by indirect immunofluorescence. Figure 4 shows that in both cases, the cultures quickly began to express such antigens in an efficient manner.

Effect of Chronic Exposure of PBLs to CyA on Infectability by HIV-1

The fact that pretreatment of PBLs with CyA appeared to render them resistant to infection by HIV-1 led us to ask whether cells obtained from patients on long-term CyA therapy for prevention of allograft rejection might share this property. This question was answered by obtaining blood from each of four individuals who had received kidney transplants at least 2 years previously and who had been on CyA therapy ever since, as well as from two control donors.

Table 2. Concentration-Dependent Effect of CyA in Preventing Infection of H-9 Cells by HIV-1

<table>
<thead>
<tr>
<th>Concentration of CyA (μg/mL)</th>
<th>Reverse transcriptase activity (cpm/mL)</th>
<th>% Fluorescent Cells in Secondary Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.001</td>
<td>845,238</td>
<td>45</td>
</tr>
<tr>
<td>0.01</td>
<td>926,462</td>
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</tr>
<tr>
<td>0.05</td>
<td>653,905</td>
<td>53</td>
</tr>
<tr>
<td>0.10</td>
<td>871</td>
<td>0</td>
</tr>
<tr>
<td>0.50</td>
<td>1,052</td>
<td>0</td>
</tr>
</tbody>
</table>

*CyA was included in tissue culture medium for 24 hours prior to infection by HIV-1 and was also present during the two hours of viral adsorption.

†Reverse transcriptase assays were carried out on culture fluids five days after infection.

‡Percentages of infected cells were determined five days after infection as described in Materials and Methods. Infectious culture fluids were harvested from primary cultures five days after infection, for the purpose of infecting secondary cultures.
Infection of the lymphocytes of these individuals by HIV-1 was performed according to the protocol described in Materials and Methods. Table 3 shows that the lymphocytes of each of these four donors became infected by HIV-1 and to the same extent as cells obtained from healthy control donors. Cells from these CyA-treated individuals could not be infected, however, if they had been maintained in tissue culture in the presence of drug prior to exposure to HIV-1. Thus, lymphocytes from patients on long-term CyA antirejection therapy behaved identically in this regard to cells from healthy donors.

Effect of CyA on Infection by HIV-1 of U-937 Cells and Monocytes/Macrophages

The fact that CyA could apparently be used successfully, in pretreatment protocols, to protect both H-9 cells and peripheral blood-derived lymphocytes from infection by HIV-1 led us to ask whether similar data might be obtained in the case of monocytes/macrophages. The results listed in Table 4 indicate that no such protective effect of CyA could be achieved with regard to either cells of the U-937 line of monocytes or primary cultures of human monocytes/macrophages.

DISCUSSION

The results of this report indicate that CyA may exert an inhibitory effect with regard to the ability of HIV-1 to infect and replicate in T lymphocytes. The evidence suggests that this inhibition is probably manifested in either of two ways. First, pretreatment of cells with CyA for 24 hours before incubation with HIV-1 seemed to completely prevent infection, as determined by production of infectious progeny virus, generation of reverse transcriptase activity, and expression of the viral antigens p17 and p24. These experiments were carried out using both a susceptible line of human T lymphocytes, H-9 cells, and freshly obtained human peripheral blood lymphocytes. These findings suggest that CyA can act to prevent infection of T cells by HIV-1, possibly by interfering with the ability of virus to bind to its specific receptor.

The presence of CyA, from the start of viral adsorption only, did not have the same restrictive effect (Table 1). This suggests that the action of CyA was probably induced and was not a direct antiviral effect of this drug.

In addition to the foregoing, it seems clear that CyA may act to restrict viral replication and viral antigen expression, if added to cells shortly after or during infection. It is important to note that the use of CyA in this regard was never completely inhibitory to the ability of the virus to cause infection. Rather, it appeared that CyA was able to exert a strong suppressive effect with regard to the number of cells that were able to express the viral antigens p17 and/or p24 at any given time. Removal of drug from the culture medium invariably led to the rapid expression of these viral markers, indicating that the cells in question had indeed become infected, but that the drug had apparently succeeded for a time in interfering with some aspect of viral replication.

In contrast, attempts to duplicate these findings with both the U-937 line of human monocytes and with primary cultures of human monocytes/macrophages were not successful. This may point to the possibility that such cells may become infected by HIV-1 through means other than their CD4 receptor. Investigations along these lines are now in progress in a number of laboratories.

These experiments were initiated because we hoped to provide some in vitro rationale for the potential use of CyA in the therapy of AIDS. While our data do suggest that CyA might be able to exert some prophylactic effect with regard to T cells that have never been exposed to HIV-1, it is also true that this drug was not able to interfere with viral replication in the case of preinfected cells.

It is worth mentioning that CyA has no known antiviral activity. Its known effects on the immune system are multifold and include interference with production of IL-2, and other lymphokines, decreased responsiveness to antigenic and mitogenic stimuli, and decreased responsiveness to intracellular calcium signaling.22,28-31 CyA has been reported in a study on AIDS patients to promote the proliferation of T4 cells while causing a decrease in numbers of T8 lymphocytes.32 The mechanism of CyA-mediated interference with infection of T cells by HIV-1 is not clear at this time, although it is likely that the effect of this drug is mediated at the level of the CD4 receptor. Evidence in favor of such a hypothesis is that the addition of CyA at times after viral adsorption was relatively noneffective. Indeed, CyA had no effect at all on H-9 cells that had been preinfected by HIV-1.

We should also point out that CyA had no effect in these studies on proliferation of either H-9 cells or of peripheral blood-derived lymphocytes nor on expression of CD4 antigen. While CyA can indeed interfere with primary proliferative responsiveness of mitogen-stimulated lymphocytes,22,23 such cells were not used in this study. Rather, we used as targets HIV-1–cultured lymphocytes that had been initiated by stimulation with PHA, but which were subsequently grown in the presence of exogenous IL-2. PHA was not included in these protocols at any stage of attempted infection by HIV-1. Thus, the documented inhibitory effects of CyA on PHA-driven lymphocyte mitogenesis are not an issue.

Table 3. Infection by HIV-1 of Peripheral Blood Lymphocytes From Patients on Long-Term CyA Therapy and From Controls

<table>
<thead>
<tr>
<th>Donor</th>
<th>% Fluorescent Cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CyA Present</td>
</tr>
<tr>
<td>Control</td>
<td>0*</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>CyA-treated</td>
<td>0</td>
</tr>
<tr>
<td>CyA-treated</td>
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<tr>
<td>CyA-treated</td>
<td>0</td>
</tr>
<tr>
<td>CyA-treated</td>
<td>0</td>
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</tbody>
</table>

*In the "CyA present" protocol, cells were preincubated with drug (0.1 μg/mL) for 24 hours prior to infection by HIV-1. Drug was also present during the two hours of viral adsorption; subsequent maintenance of cells was in drug-free medium. In the "CyA absent" protocol, infection was carried out as described in Materials and Methods. Positive cells were scored, five days after infection, by indirect immunofluorescence assay using a combination of monoclonal antibodies to viral p17 and p24.
here. Our studies on peripheral blood-derived lymphocytes were performed because of concern that results obtained with the H-9 leukemic cell line might not reflect those on normal cells. In fact, our data indicate that similar results were observed with both cell types.

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