The Use of Cyclosporine, FK506, and SDZ NIM811 to Prevent CD25 Quiescent Peripheral Blood Mononuclear Cells from Producing Human Immunodeficiency Virus

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It has been shown that the combined use of two pharmacologic agents can inhibit human immunodeficiency virus (HIV) production by peripheral blood mononuclear cells in vitro. One, an anti-CD25 immunotoxin (IT), kills activated T cells that produce virus; the other, the immunosuppressive drug cyclosporine, prevents the quiescent cells, which harbor HIV, from becoming activated. The present study compares the antiviral activities of two agents, SDZ NIM811 and FK506, to that of cyclosporine. In combination with the anti-CD25 IT, these drugs significantly suppressed virus production. In the absence of prior addition of the IT, the ability of the drugs to inhibit virus production was much lower, suggesting that they work effectively in latently infected cells. In the case of SDZ NIM811, the inhibition of virus production was accompanied by a modest inhibition of cell proliferation. In contrast, FK506 exerted strong antiproliferative activity. Cyclosporine was both moderately antiproliferative and a potent antiviral agent.

Recent studies have demonstrated the dynamic nature of human immunodeficiency virus (HIV) replication in vivo and have indicated that in persons with CD4 cell counts <500/ mm³, the majority of HIV detected in plasma is derived from newly infected, short-lived, activated CD4+ cells [1, 2]. However, the persistence of viremia even in the initial weeks of therapy with potent antiretroviral agents suggests the existence of a population of latently infected cells that become activated or chronically produce low levels of virus, or both. Indeed, several studies have demonstrated that quiescent cells outnumber actively replicating cells [3] and that these cells are a potentially important source of clinically relevant variants [1].

We previously described [4] a therapeutic regimen consisting of an immunotoxin (IT), which kills HIV-infected activated cells, and cyclosporine, which inhibits activation of latently infected (nonproducing) cells. Although neither pharmaceutical alone was effective in completely inhibiting virus production in cultures containing both activated and quiescent cells (which were later activated), in combination they completely suppressed production of HIV. Since normal cellular determinants were targeted, this approach should not result in the selection of viral mutants. In addition, the mechanism of action of these immunomodulators is entirely different from that of currently available antiretroviral agents, so cross-resistance would not be expected with these drugs.

In our initial work, cyclosporine was used to target quiescent, HIV-infected cells because of its well-known ability to inhibit T cell activation. However, it is now known that cyclosporine also has antiviral activity by virtue of its ability to block the interaction between viral Gag and cellular cyclophilin A [5-7]. At the dosages used in our previous studies, cyclosporine did not effectively suppress virus production unless the activated (CD25+) cells were eliminated [4].

The present study was designed to compare the antiviral and antiproliferative activity of cyclosporine with those of two other agents, SDZ NIM811 and FK506. FK506 is structurally unrelated to cyclosporine, but it also inhibits T cell activation by binding to calcineurin [8]. SDZ NIM811 is a nonimmunosuppressive analogue of cyclosporine that still has anti-HIV activity [9].

Materials and Methods

Immunotoxin and drugs. RFT5-dgA was prepared and purified as previously described [10] and was used at a concentration of 10 nmol/L throughout the study. Cyclosporine (Sandimmune-i.v., 50 mg/mL; Sandoz, East Hanover, NJ) and FK506 (Prograf, Tacrolimus injection, 5 mg/mL; Fujisawa, Deerfield, IL) were stored at 4°C. SDZ NIM811, a nonimmunosuppressive 4-substituted derivative of cyclosporine ([Melle-4]cyclosporine) was obtained from Sandoz Forschungsinstut (Vienna) [9]. It was stored at 1 mg/mL in ethanol at −80°C.

Virus and peripheral blood mononuclear cells (PBMC). Virus stocks and PBMC were prepared and infections induced as described [10, 11].

Elimination of CD25+ PBMC prior to infection with HIV. PBMC were cultured at 10⁴/mL for 3 days in flasks containing...
either complete culture medium (CM) [11] or CM plus IT. On the fourth day, cells were washed and infected with HIV. After infection, cells were washed again and cultured at 10^6/mL/well for 6 days in 24-well tissue culture plates coated with the anti-CD3 monoclonal antibody (MAb) 64.1 [4], in CM alone, or in CM plus drugs. Six days after infection, the concentration of viral p24 antigen in cell-free supernatants was determined using a commercially available ELISA kit (DuPont, Boston). Preliminary experiments demonstrated that the IC_{50} in both unfractionated and quiescent PBMC were 0.43 and 0.24 μg/mL for cyclosporine, 0.75 and 0.23 μg/mL for FK506, and 0.94 and 0.48 μg/mL for SDZ NIM811, respectively. At the same time, aliquots of cells (2 × 10^5) were pulsed with [3H]thyidine (1 μCi/well) for 6 h in 96-well plates to assess the effect of the drugs on cell proliferation. The different treatments were performed in triplicate wells, and all experiments were repeated at least three times.

**Combined treatment of HIV-infected PBMC in a coculture system.** A coculture system was used to evaluate further the combined effect of the anti-CD25 IT and the pharmacologic agent(s) on virus production and on transmission of HIV from infected to uninfected cells. Fresh PBMC were infected with HIV on day 1. On day 2, HIV-infected PBMC were incubated for 3 days in 24-well plates at 10^5 cells/mL, 0.6 μL/well with CM or CM plus IT. On day 5, the media were replaced with either CM or CM plus IT. On day 8 (after 6 days of treatment), the IT (or CM from untreated controls) was removed, and the cells were thoroughly washed and cultured overnight in CM. On day 9, the cells were centrifuged, and 10^6 phytohemagglutinin (PHA)-activated (5 μg of PHA/mL for 3 days) PBMC obtained from the same donor were added to each well. Cells in one plate did not receive PHA-activated cells (to assess the effects of IT and drugs on the initially infected PBMC). Half the wells in each group (including those containing cells that were not cocultured) also received cyclosporine, FK506, or SDZ NIM811. Three days later (day 12), levels of p24 in cell-free supernatants were determined. Each experiment was done at least three times, and all variables were tested in triplicate.

**Results**

**Effect of cyclosporine, FK506, and SDZ NIM811 on virus production and cell proliferation.** Treatment of fresh PBMC with 10 nmol/L RFT5-dgA prior to infection with HIV generates a population of quiescent CD25^- HIV-infected cells [4] in which production of viral p24 is suppressed to virtually undetectable levels. Stimulation of these cells with solid-phase anti-CD3 MAb 64.1 results in the production of high levels of virus [4]. To test the effect of different drugs on unfractionated and quiescent CD25^- HIV-infected PBMC, cells were infected with virus and were then cultured for 6 days in the presence of solid-phase anti-CD3. Figure 1 shows the effect of the three drugs on both production of virus and cell proliferation. As shown in the left panels, at 1 μg/mL, cyclosporine, FK506, and SDZ NIM811 reduced the amount of virus produced by unfractionated PBMC (CD25^+ and CD25^- cells) >90%, 82%, and 55%, respectively. The right panels show that 1 μg/mL cyclosporine, FK506, or SDZ NIM811 reduced p24 production by the CD25^-PBMC >95%, >95%, and 82%, respectively.

To determine to what extent these reductions in viral p24 production were cyclophilin-mediated versus a consequence of the inhibition of cell proliferation, we tested the ability of these three drugs to inhibit proliferation of HIV-infected unfractionated PBMC and quiescent CD25^- cells. At 1 μg/mL, FK506 reduced proliferation of both unfractionated PBMC and CD25^-PBMC by 80%. Cyclosporine reduced proliferation of unfractionated PBMC and CD25^-PBMC by 58% and 32%, respectively. SDZ NIM811 suppressed proliferation of both unfractionated PBMC and CD25^-cells by 20%. Taken together, our results suggest that the inhibition of both cell proliferation and Gag-cyclophilin interactions in the CD25^- cells leads to optimal anti-HIV activity and that cyclosporine is the most effective drug because it exerts both effects.

**The effect of cyclosporine, FK506, and SDZ NIM811 on virus production and transmission in an autologous coculture system.** To further increase the sensitivity of our virus detection assay, we used a coculture system in which PHA-activated PBMC obtained from the same donor were added to IT-treated or untreated HIV-infected cells. As shown in figure 2, on day 11, untreated cells (not cocultured) produced 21 ng/mL p24, while under the same conditions, IT-treated cells produced <1% of this amount. This confirms our previous results demonstrating that anti-CD25 IT can eliminate activated CD25^- cells that produce HIV. When the drugs were added on day 8 to control cells not cocultured with PHA-activated cells, p24 production was reduced by 68%–79%. In IT-treated CD25^- cells, production of p24 was reduced >99.7% (cyclosporine and FK506) and >99.95% (SDZ NIM811).

When HIV-infected cells were cocultured with PHA-activated cells, production of p24 was significantly increased. In the absence of any drug, up to 110 ng/mL p24 antigen was detected in the supernatants of CM-treated PBMC. Concentrations of p24 were reduced by 53%, 63%, and 68% in the presence of FK506, SDZ NIM811, and cyclosporine, respectively. Hence, these drugs do inhibit HIV production by activated cells, but only partially. Addition of cyclosporine or SDZ NIM811 to IT-treated cells resulted in a >99% reduction of p24 levels, and addition of FK506 reduced virus production by 97%.

These results demonstrate that all three drugs are highly effective at suppressing the production of HIV from CD25^- cells, even in the presence of PHA-activated PBMC.

**Discussion**

Three major findings emerge from this study: When used in combination with an anti-CD25 IT, cyclosporine, FK506, and SDZ NIM811 suppressed HIV replication and transmission to virtually undetectable levels. These drugs were highly effective in preventing quiescent HIV-infected cells from producing virus, even in the presence of a strong activating signal. The
Production of p24 Antigen

![Graph of p24 Antigen Production](image)

Cell Proliferation

![Graph of Cell Proliferation](image)

Figure 1. Effect of different drugs on HIV production and cell proliferation. Peripheral blood mononuclear cells were cultured in tissue culture flasks containing either complete medium (CM) or CM + immunotoxin (IT). On day 4, cells were washed and infected with HIV. After infection, cells were cultured for 6 days in 24-well tissue culture plates previously coated with anti-CD3 monoclonal antibody 64.1 (see Materials and Methods). At 6 days after infection, concentration of viral p24 antigen in cell-free supernatants was determined, and aliquots of cells were pulsed with [3H]thymidine to assess effect of tested drugs on cell proliferation. Effect of cyclosporine (○), FK506 (■), and SDZ NIM811 (▪) on viral p24 production (upper panels) and on [3H]thymidine incorporation (lower panels) expressed as % of untreated controls.

ability of the three drugs to suppress virus production in latently infected cells was achieved either by inhibition of cell proliferation alone (FK506), by blocking Gag-cyclophilin interactions (SDZ NIM811), or by both mechanisms (cyclosporine).

Although our results differ to some extent from those reported previously, in which these drugs reduced HIV replication very effectively [9, 12, 13], variations in experimental design probably explain the differences. Indeed, previous studies used cell lines or standard cultures of PBMC instead of quiescent CD25 cells. In addition, the concentrations of drugs used in other studies were higher than those used in our experiments.

This ability of the three drugs to inhibit virus production by quiescent HIV-infected cells was most likely achieved by one or two mechanisms. FK506 suppressed virus production by virtue of its ability to inhibit cell proliferation. This was not unexpected, since FK506 binds to a different cellular target, FKBP, and FKBP does not bind to Gag [6]. Cyclosporine suppressed virus production in quiescent cells both by inhibiting cell proliferation and by blocking Gag-cyclophilin interactions. On the other hand, SDZ NIM811, which was minimally immunosuppressive, reduced virus production efficiently because it inhibited Gag-cyclophilin interactions. Since SDZ NIM811 is both minimally immunosuppressive and highly effective, it may be the drug of choice clinically in conjunction with IT therapy.

The use of cyclosporine and other immunomodulators to treat HIV infection has been suggested previously on the basis of
Figure 2. Combined treatment of HIV-infected peripheral blood mononuclear cells (PBMC) in coculture system. Fresh PBMC were infected with HIV on day 1. On day 2, HIV-infected PBMC were divided into 2 aliquots and cultured in complete medium (CM) alone or CM + immunotoxin (IT) for 6 days. On day 9, 10^6 phytohemagglutinin (PHA)-activated PBMC from same donor were added to each well. One plate did not receive PHA-activated cells in order to assess effect of IT and drugs on infected PBMC alone. In addition, half of wells in each group (including those lacking cocultured cells) received cyclosporine (□), FK506 (◇), SDZ NIM811 (◆), or CM alone (▲). On day 12, cell-free supernatants were assayed for p24 content.

their ability to reduce viral replication and inhibit apoptosis. Indeed, cyclosporine has been administered to patients at different stages of HIV infection with variable results [14, 15], as would be predicted from the large number of activated, HIV-producing cells present. Since those studies did not include detailed measurements of virus loads or immune function, it is critical that careful assessment of virologic and immune parameters be included in future clinical studies using these agents.

In summary, results of the current study confirm and extend our previous observations that combining agents with different mechanisms of action to target both activated and quiescent HIV-infected cells should be considered in designing new therapeutic strategies for HIV disease.

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References