Recent findings show that human immunodeficiency virus (HIV-1) protease inhibitors designed to specifically inhibit the aspartic protease of HIV-1 nonetheless exert various effects on immune cell function in vitro and in vivo. Dendritic cells (DC), central players of the immune system, express several aspartic proteases that are important for DC function. In the present study, we demonstrate that all of the HIV-1 protease inhibitors tested affect DC maturation. In addition, saquinavir had a strong inhibitory effect on the T-cell stimulatory capacity of mature DC. In contrast, indinavir had only a slight effect on DC induced T-cell proliferation and allowed efficient transduction of DC with a replication-incompetent HIV-1 vector designed for DC-based immunotherapy. HIV-1 protease inhibitors that have little or no effect on DC function may be preferable for combination with immunotherapy for HIV/AIDS.

Highly active antiretroviral therapy, including reverse transcriptase inhibitors and at least one HIV protease inhibitor, has proven to be extremely effective in treating HIV infection (1). HIV-1 protease inhibitors were designed to be highly specific for the HIV-1 aspartic protease. However, inhibitory activity toward cellular aspartic proteases (2–4) and interactions with other host cell factors (5–7) have been described. This impacts on leukocyte activation (8), antigen presentation, and T-cell responses (9). Thus, HIV-1 protease inhibitors may directly modify the host’s immune response, in addition to their antiviral activity, providing one possible explanation for the observation, that in some patients treated with protease inhibitors, an increase in CD4+ T-cell counts was detected despite of unchanged HIV viremia (10–12).

Dendritic cells process and present foreign antigens to both CD4+ and CD8+ T-cells, thereby inducing primary and second-ary immune responses. Degradation of foreign antigens by intracellular proteases, including aspartic proteases, is essential for processing and presentation of antigen by antigen presenting cells to lymphocytes (13). Inhibition of this antigen presenting machinery alters the host’s immune response, as has been shown recently for HIV-1 protease inhibitors in an animal model (9). Recent estimates of the decay of latent virus in patients suggest that lifelong antiviral therapy will be required to control HIV infection (14). As control of viremia may also be essential for the success of a subsequent antiviral immunotherapy (15), the potential effects of protease inhibitors on immune cell function are significant. The present study aimed to investigate the effect of HIV-1 protease inhibitors on DC phenotype and function, and to test the feasibility of using DC for immune based therapy for HIV infection, in conjunction with antiretroviral therapy regimens.

EXPERIMENTAL PROCEDURES

Reagents—The following cell culture supplements were used: granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leukine; Immunex, Seattle, WA), interleukin 4 (IL-4) (R & D Systems, Minneapolis, MN) and lipopolysaccharide (LPS; Sigma). Tetanus toxoid from Clostridium tetani was purchased from Calbiochem. The HIV-1 protease inhibitors indinavir (Crixivan; Merck, West Point, PA), ritonavir (Norvir; Abbott, North Chicago, IL), saquinavir (Fortovase; Roche, Nutley, NJ), and nelfinavir (Viracept; Agouron, La Jolla, CA) were obtained from the University of California, San Diego pharmacy. The latter inhibitors were dissolved in dimethyl sulfoxide (Me2SO). As controls, cells were either left untreated or were treated with a comparable concentration of Me2SO but without HIV-1 protease inhibitor.

Generation of Monocyte-derived DC—DC were generated from buffy coats of healthy donors as described recently (16). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation in Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). Plastic-adherent PBMC were incubated for 7 days in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated human serum AB (Gemini, Calabasas, CA), 1% nonessential amino acids (Mediatech), GM-CSF (100 IU/ml), and IL-4 (1,000 units/ml). Mature DC were generated by incubating immature DC for 2 days in medium supplemented with 10 ng/ml of LPS.

Construction and Preparation of Retroviral Vectors—Construction and preparation of the vesicular stomatitis virus G protein (VSV-G) pseudotyped vector HIV-1ΔEαV containing the enhanced green fluorescent protein gene was performed as described previously (16).

Transduction of Monocyte-derived Dendritic Cells—Monocyte-derived DC were transduced with the vector HIV-1ΔEαV at multiplicity of infection (m.o.i.) of 5, in the presence of 4 μg/ml polybrene (Sigma), GM-CSF, and IL-4 for 16 h at 37 °C. DC were then washed and incubated for 4 days in GM-CSF- and IL-4-containing medium.

Immunofluorescence—Immunophenotyping of cells was accomplished by using phycoerythrin-conjugated anti-CD11c, anti-CD14, anti-CD80, anti-CD86, anti-HLA-DR, isotype control antibody (all from Becton Dickinson, Mountain View, CA), anti-CD40 (BIOSOURCE Int., Camarillo, CA), and anti-CD83 (Immunotech, Marseille, France). The analyses were carried out on a flow cytometer (Epics Elite, Coulter).

Viability Assays—The number of viable cells was determined by trypan blue exclusion assay (Sigma). Cells were observed under a Nikon UFX-I1A microscope (Tokyo, Japan).
TABLE I
Viability and yield of DC derived from PBMC treated with or without HIV-1 protease inhibitor

|               | Control | Indinavir | Ritonavir | Saquinavir | Nelfinavir |
|---------------|---------|-----------|-----------|------------|-----------|
| Viability    | 92 ± 2  | 96 ± 2    | 86 ± 13   | 86 ± 10    | 90 ± 6    |
| Yield (× 10^6) | 3.5     | 2.9       | 3.5       | 2.5        | 3.7       |
| Median       | 1.4–5.6 | 1.6–7.8   | 1.3–4.7   | 0.8–3.8    | 1.0–4.9   |

* Mean percentage ± S.D. from at least three experiments is shown.

** Number of immature DC generated from 10^8 PBMC after 7 days of culture; results of at least three experiments are shown.

Effect of HIV-1 Protease Inhibitors on Viability and Yield of Monocyte-derived DC—The HIV-1 protease inhibitors indinavir, ritonavir, saquinavir, and nelfinavir were added to monocyte-derived DC cultures at a total concentration of 15 μg/ml, a concentration equal to or higher than the peak plasma concentration of these HIV-1 protease inhibitors usually achieved during treatment. None of the HIV-1 protease inhibitors significantly affected viability or yield of the obtained immature DC (Table I).

Effect of HIV-1 Protease Inhibitors on Maturation of DC—The aspartic protease cathepsin D is up-regulated during monocyte maturation (20) and, together with other members of the aspartic protease family, plays an important role in antigen processing (21, 22). To investigate the effect of HIV-1 protease inhibitors on DC maturation, immature DC were stimulated with LPS. DC maturation was accompanied by an increase in expression of surface antigens involved in T-cell co-stimulation (Table II). All of the HIV-1 protease inhibitors tested led to a markedly reduced expression level of CD80 when compared with control (Table II). In addition, saquinavir significantly reduced the expression levels of CD40 and CD86, while indinavir, ritonavir, and nelfinavir reduced the expression of CD40 but not CD86 (Table II).

Effect of HIV-1 Protease Inhibitors on T-cell Stimulation by Immature DC—The majority of DC cultured for 7 days with GM-CSF and IL-4 stained moderately to strongly positive for HLA-DR, CD86, CD11c, CD40, and, at lower relative intensities, for CD80 and CD83, while CD14 was not detectable (Fig. 1). This profile is characteristic of functionally immature DC (18). A comparable surface antigen expression profile was detected after treatment of DC with 15 μg/ml indinavir (Fig. 1). Similar results were observed after treatment of DC with ritonavir, saquinavir, or nelfinavir (data not shown).

Effect of HIV-1 Protease Inhibitors on T-cell Proliferation—Pepstatin A, a natural inhibitor of aspartic proteases, has been shown to partially inhibit protein degradation in lysosomes (19) as well as antigen-specific proliferation (3). To determine whether HIV-1 protease inhibitors produced similar effects, the ability of DC to process and present the recall antigen tetanus toxoid to autologous T-cells in the presence of HIV protease inhibitors was examined. Untreated DC (control) stimulated tetanus antigen specific T-cell proliferation in a dose-dependent manner (Fig. 2, A and B). DC treated with indinavir at a concentration of 7.5 or 15 μg/ml induced a tetanus-specific T-cell response at a level comparable with control (Fig. 2A). Similar results were observed for DC treated with ritonavir, saquinavir, or nelfinavir (Fig. 2B).
Effect of HIV-1 Protease Inhibitors on DC Differentiation

| TABLE II  | Effect of HIV-1 protease inhibitors on DC maturation |
|-----------|--------------------------------------------------|
|           | Indinavir | Ritonavir | Saquinavir | Nelfinavir |
| CD40      | -14.5 ± 4  | -39.0 ± 13 | -24.7 ± 16 | -23.9 ± 7  |
|           |  0.004    |  0.0001   |  0.02     |  0.0002   |
| CD80      | -47.9 ± 13 | -51.4 ± 20 | -52.1 ± 22 | -57.3 ± 23 |
|           |  0.005    |  0.002    |  0.002    |  0.001    |
| CD86      | -17.0 ± 8  | -7.1 ± 15  | -21.5 ± 12 | -18.0 ± 24 |
|           |  0.4      |  0.4      |  0.009    |  0.2      |

* Immature DC were cultured in the presence or absence of 15 µg/ml HIV-1 protease inhibitor and stimulated for 2 days with LPS. The surface antigen profile was determined as described in the legend to Fig. 1. Mean percentage of change, compared with control, plus/minus S.D. of 11 experiments is shown. ** t test, significant if p < 0.05.

lower DC concentrations tested (Fig. 3). In contrast, nelfinavir did not affect the T-cell stimulatory capacity of DC at any DC/T-cell ratio tested (Fig. 3).

Effect of Indinavir on Single Cycle Infection of DC by a Replication-incompetent Viral Vector—Recently, several studies proposed the use of replication-incompetent vectors based on HIV-1 as immunotherapy for HIV/AIDS (16, 23–25). Co-cultivation of vector-transduced DC and T-cells from HIV-1-infected subjects as a mean to prime or boost antiviral immunity ex vivo leads to strong viral replication (data not shown) (26), which in turn impairs the immune function of these cells (27). Addition of an HIV-1 protease inhibitor to DC/T-cell co-cultures will suppress viral replication, thereby preventing virus induced immune dysfunction. Furthermore, a previous in vivo study suggests that the combination of antiretroviral therapy (in this case excluding HIV-1 protease inhibitors) and virus-specific immunotherapy is superior to the use of either therapy alone (15). Conflicting data exist regarding whether HIV protease inhibitors block early steps of viral infection (28–31). To assess the effect of indinavir on transduction of immature DC with a replication-incompetent HIV-1-based vector, we first performed drug susceptibility testing on CEM-GFP cells as has been described previously (17). In the presence of indinavir, viral replication was markedly reduced in HIV-1_LAI-infected CEM-GFP cells at all m.o.i. tested (Fig. 4A). In contrast, the transduction efficiency of immature DC with the immunotherapeutic vector HIV-1ΔENΔV3 (16) was comparable in the absence or presence of indinavir (Fig. 4B), thus suggesting that gene therapeutic modification of DC in the presence of indinavir is feasible.

DISCUSSION

In the present study the effect of HIV-1 protease inhibitors on DC differentiation and function was investigated. Matura-
T-cell stimulatory capacity of mature DC. Ritonavir, indinavir, and nelfinavir strongly inhibited the expression of CD80 and to a lesser extent CD40, while the expression of CD86 was unchanged. The ability of DC to stimulate T-cell proliferation was reduced by ritonavir at all DC/T-cell ratios tested. Indinavir had a slight effect on DC induced T-cell proliferation only at the lower DC concentrations tested, while at higher DC concentrations the T-cell stimulatory capacity of indinavir treated DC was comparable with control. Nelfinavir had no significant effect on DC-induced T-cell proliferation.

Whether long term treatment with combinations of one or several HIV-1 protease inhibitors may aggravate the effects observed in our present study is currently being investigated. Preliminary data suggest that DC cultivated from monocytes of HIV-positive individuals differ markedly in their expression level of several T-cell co-stimulatory molecules, including CD40, CD80, and CD86, which is associated with the viral load in the respective individuals (32). Since in the subjects studied, protease inhibitor treatment was linked to a lower viral load, it is difficult to distinguish whether the antiviral effectiveness of the respective drugs, the drugs themselves, or a combination of both may have influenced the activation status of DC.

The surface expression of CD80 and CD86 on DC upon stimulation with LPS is regulated by nuclear factor (NF)-κB activation (33). A group of adapter proteins, the tumor necrosis factor receptor-associated factors, have been shown to be involved in NF-κB activation (34–37). A recent study suggests that tumor necrosis factor receptor-associated factor-dependent NF-κB activation can be blocked by peptatin A, an inhibitor of aspartic proteases, but not indinavir or ritonavir (38). The latter finding may be due to the low concentration of the HIV-1 protease inhibitors used in the prior study (38). It remains to be determined whether HIV-1 protease inhibitors at higher concentrations interfere with tumor necrosis factor receptor-associated factor-dependent NF-κB activation or a different signal transduction pathway.

Blocking of certain DC-expressed surface molecules, such as CD40 and CD86, has been shown to reduce the T-cell stimulatory capacity of DC (39, 40). Accordingly, the reduced up-regulation of CD40 and CD86 on mature DC by saquinavir led to the most pronounced inhibition of DC induced T-cell proliferation, while inhibition of only CD40 and CD80 expression, but not CD86 expression, on mature DC by ritonavir, indinavir, and nelfinavir had little or no effect on T-cell stimulation by DC.

Recent studies demonstrate that ritonavir inhibits the chymotrypsin-like activity of the 20 S proteasome (7), leading to an impairment of antigen presentation and, ultimately, of cytotoxic T-lymphocyte responses in mice (9). This effect was exerted to a lesser extent by saquinavir, and not at all by neither indinavir nor nelfinavir, similar to the pattern observed in the present study. Thus, the effectiveness of adjunctive immunotherapies given to HIV-1-infected individuals receiving ritonavir or saquinavir as antiviral therapy may be compromised.

In conclusion, indinavir and nelfinavir, when compared with ritonavir and saquinavir, affect DC immunophenotype and their T-cell stimulatory capacity to a lesser extent and therefore may be a better choice for drug regimens used in combination with immunotherapy for HIV/AIDS.

Acknowledgments—We acknowledge the expert technical assistance of Maureen Ibanez. We are also thankful to the UCSD Center for AIDS Research for use of core facilities.

REFERENCES

1. Carpenter, C. C., Cooper, D. A., Fischl, M. A., Gatell, J. M., Gazzard, B. G., Hamner, S. M., Hirsch, M. S., Jacobsen, D. M., Katzenstein, D. A., Montaner, J. S., Richman, D. D., Saag, M. S., Schechter, M., Schooley, R. T., Thompson, M. A., Vella, S., Yin, P. G., and Volberding, P. A. (2000) J. Am. Med. Assoc. 283, 381–390
2. Gruber, A., Speth, C., Bukkens-Vogel, E., Zangerle, R., Rurgov-Zepelin, M., and Fatkenheuer, G. (1999) J. Acquir. Immune Defic. Syndr. Hum. Retroviruses 20, 203–209
3. Bode, H., Schmidt, W., Schulze, J. D., Fromm, M., Zippel, T., Wahn, M. A., Bendfeld, R., Riecken, E. O., and Ullrich, R. (1999) AIDS 13, 2595–2597
4. Schmidtke, G., Holzguth, H. G., Bogoy, M., Kairies, N., Groll, M., de Giuli, R., Emch, S., and Groettrup, M. (1999) J. Biol. Chem. 274, 35734–35740
5. Weichold, F. F., Bryant, J. L., Pati, S., Barents, O., Gallo, R. C., and Reitz, M. S. J. M. (1999) J. Hum. Virol. 2, 261–269
6. Evrard, B., Grootveld, M., Klenner, F., de Giuli, R., Booth, B. L. J., Curvolo, V., Bennecke, M., Jetteau, F., Zinkernagel, R. M., and Lotteau, V. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13120–13124
7. Perrin, L., and Teleni, A. (1998) Science 280, 1871–1873
8. Hewitt, E. W., Treumann, A., Morrice, N., Tatnell, P. J., Kay, J., and Watts, C. (2000) Arch. Biochem. Biophys. 385, 322–332
9. Rouzaut, A., Lopez-Moratalla, N., and de Miguel, C. (2000) Arch. Biochem. Biophys. 380, 153–163
10. Diment, S., and Stahl, P. (1985) J. Biol. Chem. 260, 1074–1075
11. Levitz, S. M. (1998) N. Engl. J. Med. 338, 5–14
12. Essaye, S. M., Kim, M., Gonzalez, C., Rigaud, M., Kaul, A., Chandwani, S., Hoover, W., Lawrence, R., Spiegel, H., Pollack, H., Krasinski, K., and Borkowsky, W. (1998) AIDS 12, 2523–2532
13. Kiese, R. J., and Chapman, H. A. (1999) Curr. Opin. Immunol. 11, 107–113
14. Muzio, M., Natoli, G., Saccani, S., Levrero, M., and Mantovani, A. (1998) J. Exp. Med. 187, 171–179
15. Black, J. L., Bieber, A., and Segall, H. (2000) J. Immunol. 165, 1350–1359
16. Saccani, S., Levrero, M., and Mantovani, A. (1998) J. Immunol. 161, 512–517
17. Het, Z., Venzon, D. P., Toussaint, M., Gervais, A., and Mendel, M. J. (1999) J. Biol. Chem. 274, 153–160
18. Nelfinavir had little or no effect on T-cell stimulation by DC.

Effect of HIV-1 Protease Inhibitors on DC Differentiation