Antiretroviral Therapy with Protease Inhibitors Has an Early, Immune Reconstitution–Independent Beneficial Effect on *Candida* Virulence and Oral Candidiasis in Human Immunodeficiency Virus–Infected Subjects

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Highly active antiretroviral therapies (HAARTs) that contain human immunodeficiency virus (HIV) protease inhibitors (PIs) or nonnucleoside reverse-transcriptase inhibitors (NNRTIs) were compared for their effect on secretory aspartyl proteinase (Sap), a virulence trait for mucosal candidiasis. In therapy-naive HIV-positive subjects, oral Sap was detected in 11, 6, 3, 0, and 0 of 15 subjects treated with PI-HAART and in 7, 7, 9, 6, and 5 of 15 subjects treated with NNRTI-HAART, on days 0, 14, 30, 90, and 180 of treatment, respectively. In another 30 subjects, Sap was detected in 0 and 7 of 15 subjects after 1 year of treatment with PI-HAART or NNRTI-HAART, respectively. The anti-Sap effect of PI-HAART was associated with clinical resolution of oral candidiasis but not with late and inconstant recovery of anticandidal cellular immunity. In all subjects, the 2 therapeutic regimens compared well in increasing $CD4^+$ cell count and abating viremia. Thus, PIs exert an early, immune reconstitution–independent effect on *Candida* virulence in the oral cavities of HIV-positive subjects.

Highly active antiretroviral therapy (HAART) that includes human immunodeficiency virus (HIV) protease inhibitors (PIs) has shown remarkable efficacy against virus replication and against progression to and mortality from AIDS [1–4]. Beneficial effects have been noticed, in particular, in the field of opportunistic infections (OIs) since early HAART introduction in the clinical care of patients with AIDS [3–5]. Repeated observations and current opinion attribute these clinical benefits mainly, if not exclusively, to immune reconstitution, as usually measured by the elevation of circulating $CD4^+$ cells and by the at least partial recovery of T cell responses against recall antigens [6–9]. However, several findings have documented that the clinical efficacy of HAART may not be paralleled by the reconstitution of specific immune responses against agents of OI [10, 11].

We have considered that HAART-induced early decline of oral candidiasis (OC), one of the most frequent OIs in HIV-infected subjects, might also depend on an additional direct effect of PIs against *Candida albicans,* the most common cause of OC in such patients [12]. This hypothesis was based, first, on clinical observations that resolution of refractory OC occurred in some PI-treated patients well before recovery of $CD4^+$ cell count and, second, on the anticandidal activity of some PIs [13]. This activity was demonstrated in vitro, as well as in an animal model of mucosal candidiasis, and was mediated by the inhibition of *C. albicans* secretory aspartyl proteinase (Sap), a family of virulence enzymes that belong to the same family as HIV protease [13–16]. The above findings raised the issue of whether the anticandidal activity of PI could be relevant to the clinical efficacy of PI-containing HAART regimens.

To address this issue, we compared subjects who received HAART with PI with other subjects who were receiving a PI-sparing HAART regimen with a nonnucleoside reverse-transcriptase inhibitor (NNRTI) [16] for OC incidence, oral *Candida* carriage, and Sap production in the oral cavity. In a first study, 30 antiretroviral therapy–naive subjects who began HAART that included PIs or NNRTIs were longitudinally assessed, at various time intervals, for a total follow-up of 180 days. In a second, cross-sectional study, 30 subjects who had been receiving either therapeutic regimen for $\geq$1 year were compared. In both studies, specific immune reconstitution of anti-*Candida* response also was assessed, to examine the extent of this recovery and its possible relationship with the effect of PI-HAART or NNRTI-HAART on oral Sap level and OC incidence.

Subjects and Methods

Study setting. The Catholic University Hospital is a 1700-bed tertiary care center located in Rome, Italy, with ~60,000 patient ad-

Received 21 June 2001; revised 18 September 2001; electronically published 3 January 2002.

Informed consent was obtained from patients about the scope and nature of the investigation. The clinical studies were conducted according the institutional rules of the Catholic University, Rome, Italy, after approval by the internal ethical committee.

Financial support: National AIDS Research Program (contract 50C/B to A.C. and R.C.).

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The Journal of Infectious Diseases 2002;185:188–95

0022-1899/2002/18502-0007$02.00

missions each year. There is a 60-bed unit for the admission of HIV-infected patients and a day hospital for their outpatient care.

Longitudinal study. Seventy-one HIV-infected subjects who were naive for antiretroviral therapy and who were admitted as outpatients from 1 July 1 through 31 December 1999 were considered. For the purpose of the present study, 15 subjects who began PI-HAART (group 1) and 15 who began NNRTI-HAART (group 2) were selected randomly. The time of the beginning of therapy has been designated as time 0 in the analysis, and all subjects were monitored for 180 days.

Cross-sectional study. One hundred and fourteen HIV-infected subjects who were admitted as out-patients or in-patients and who had been receiving HAART for $\geq$1 year without any change of therapy were considered. From this population, 15 HIV-infected subjects receiving HAART with a PI (group 3) and 15 receiving HAART with an NNRTI (group 4) were selected randomly.

Parameters evaluated. For each subject enrolled in the study, the following data were obtained (scheme A): age, sex, HIV risk behavior, active injection drug abuse, stage of HIV infection [17], previous manifestations of HIV infection, date of AIDS-defining illness, presence of OC and history of previous OC episodes, concurrent OIs, risk factors for OC antimicrobial therapy, type and use of prophylaxis for *Pneumocystis carinii* pneumonia and toxoplasmosis, use of other medications (antituberculous agents, antivirals, corticosteroids, and antacids), numbers of circulating $CD4^+$ T cells (cells/mm^3) and peripheral polymorphonuclear cells (PMNL; cells/mm^3), and HIV viremia, with a limit of detection of $<$50 HIV RNA copies/mL. The analyzed risk factors for OC included neutropenia (PMNL count $<$1000 cells/mm^3), alcoholism, cirrhosis, diabetes, neoplastic diseases, and chronic renal failure. For each patient, we also obtained blood samples (scheme B) for analyzing lymphoproliferative response to *C. albicans* antigen, tetanus toxoid, and phytohemagglutinin (PHA; see below). In the longitudinal study, all subjects underwent scheme A at 14 (except for determination of $CD4^+$ T cell counts and HIV viremia), 30, 90, and 180 days and scheme B at baseline and 30 and 180 days.

Laboratory methods. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood samples by density gradient centrifugation on Lymphoprep solution (Nycomed Pharma) and were resuspended at 10^6 cells/mL in RPMI medium (Gibco-BRL) supplemented with 5% human AB serum (Sigma Chemical), 2 mmol L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco-BRL). Triplicate PBMC cultures were stimulated with 10 μg/mL PHA (Sigma), 10 μg/mL tetanus toxoid (kindly supplied by Chiron-Biocine), or with different doses (10 and 50 μg/mL) of a purified antigenic extract from *C. albicans* (MP-F2) that contains immunodominant mannoprotein antigens of the fungus and can be used, without loss of sensitivity, in place of whole, inactivated *Candida* cells as antigenic stimulator of PBMC proliferation [18]. Lymphocyte proliferation was measured as [^{3}H]thymidine incorporation after 3 (PHA) or 7 (tetanus toxoid and *Candida* antigen) days of incubation under 5% CO_2 at 37°C, as reported elsewhere [12]. The stimulation index (SI) was calculated by dividing the radiolabel incorporation by stimulated PBMC cultures by the incorporation of unstimulated control cultures. A positive lymphoproliferative response was assumed when an SI $\geq$5 with incorporation values $>$1500 cpm was measured. Data published elsewhere [18] and our own unpublished data have indicated that $>$90% of healthy, HIV-negative adult subjects would have SI and count-per-minute values greater than or equal to the selection cutoff for positive response.

Oral *Candida* carriage and in vitro Sap production by *Candida* isolates were assessed as described elsewhere [19–21]. The diagnosis of OC, microbiologically documented on the first episode, was made routinely by the typical signs and symptoms. All OC were clinically categorized into 3 types: pseudomembranous, erythematous, and hyperplastic forms, as described in detail elsewhere [12].

The amount of salivar Sap was measured by ELISA [19, 20, 22]. In brief, 1 mL of saliva was centrifuged at 6500 *g* for 10 min, and the cell-free supernatant was treated with 0.5 mL of 50% thrichloracetic acid (TCA). Besides precipitating the protein, this treatment unbinds Sap from any substrate or antibodies, thus allowing for the measurement of both free and bound Sap. The precipitate in TCA was washed with ethanol, dissolved in 1% SDS, boiled for 3 min, diluted in 0.2 *M* carbonate buffer (pH 9.6), and applied (70 μL) to the microtest plates. A rabbit serum raised against a highly purified Sap preparation [19, 20] was used (at a 1:100 dilution) and, after 2 h of incubation at 37°C, a second antibody (phosphatase-conjugated anti–rabbit IgG4, 1:100 dilution; Sigma) was added, and the reaction was detected by the addition of nitrophenol phosphate (Sigma) as substrate. The detection limit of this reaction was 20 ng/mL [19, 20]. The specificity of the reaction was always confirmed by Western blot [20]. A subject was considered to be Sap positive when Sap was found in his or her saliva (detected by ELISA and confirmed by Western blot).

Statistical analysis. Quantitative variables were tested for normal distribution and were compared by means of Student's 2-tailed *t* test. Differences in group proportions were assessed by use of the χ^2 test or, for small numbers, Fisher's exact test. A repeated-measures analysis of variance test was used for comparison of paired samples. A 2-tailed test of significance at the $P < .05$ level was used to determine statistical significance. All statistical analysis was performed with the use of the software program Intercooled Stata (StataCorp).

Results

Longitudinal study: clinical findings. The baseline characteristics of all subjects enrolled in the longitudinal study are described in tables 1 and 2. The 2 groups were rather homogeneous with regard to demographic characteristics, HIV viremia, and number of peripheral $CD4^+$ cells at the baseline. The group taking PI-HAART had more episodes of OC and esophageal candidiasis before entering the study and had more OC episodes at the time of enrollment than the NNRTI-HAART group, although no statistical significance was reached in any comparison. All subjects began HAART with 2 reverse-transcriptase inhibitors (RTIs), including nelfinavir (12 subjects [80%]) or indinavir (3 subjects [20%]) in group 1 and nevirapine (12 subjects [80%]) or efavirenz (3 subjects [20%]) in group 2. As shown in table 2, HAART exerted a significant reduction of viremia and increase in peripheral $CD4^+$ cells throughout the follow-up period, with no statistically significant differences between the 2 therapeutic regimens. However, the number of OC episodes did rapidly decline during PI-HAART (from 7 at baseline to 3 and 1, respec-

Table 1. Baseline characteristics of 30 therapy-naive human immunodeficiency virus (HIV)–infected subjects before beginning highly active antiretroviral therapy with protease inhibitors (group 1) or nonnucleoside reverse-transcriptase inhibitors (group 2).

Characteristic	Group 1 ($n = 15$)	Group 2 ($n = 15$)	*P*	OR (95% CI)
Sex, men : women	9:6	8:7	.71	1.31 (0.24–7.06)
Age, mean years ± SD	36 ± 7	38 ± 8	.47	2.06 [a]
Stage of HIV infection[b]				
B	8 (53)	12 (80)	—	—
C	7 (47)	3 (20)	.12	3.50 (0.55–24.61)
Active drug abuse	1 (6)	1 (6)	1.00	—
Time from AIDS diagnosis, mean days ± SD	104 ± 63	275 ± 252	.11	170[a]
Relapsing oral candidiasis[c]	5 (33)	3 (20)	.41	2.00 (0.30–14.42)
Previous esophageal candidiasis	2 (13)	1 (6)	.54	0.46 (0.01–7.93)
Presence of oral candidiasis	7 (47)	3 (20)	.12	3.50 (0.55–24.61)
Candida albicans oral carriage	11 (73)	8 (53)	.44	2.40 (0.41–14.82)

NOTE. Data are no. (%) of subjects, except where noted. CI, confidence interval; OR, odds ratio.
[a] Differences between means.
[b] According to Centers for Disease Control and Prevention definitions [17].
[c] More than 3 episodes during the previous year.

tively, after 14 and 30 days of therapy). Oral *Candida* carriage also was progressively reduced in subjects receiving PI-HAART. In contrast, neither OC nor fungus carriage was significantly affected by 180 days of treatment with NNRTI-HAART (table 2).

Longitudinal study: immunologic findings. As shown in figure 1, only 3 of 15 subjects initiating PI-HAART were responsive to stimulation of their PBMC in vitro with *Candida* antigen, and none were responsive to tetanus toxoid, whereas the majority of subjects clearly responded to stimulation by the mitogen (PHA). The median SI of the 3 subjects who responded to MP-F2 was relatively low (8; range, 5–19). After 30 days of treatment, almost all subjects were responsive to PHA, yet only 3 more subjects (with respect to the baseline) showed positive proliferation to *Candida* antigen, and still none showed positive proliferation to tetanus toxoid. After 180 days of treatment, only 5 subjects had positive a SI to MP-F2 (2 of whom were already positive at baseline), and only 3 subjects were responsive to tetanus toxoid.

Table 2. Follow-up of 30 human immunodeficiency virus (HIV)–infected subjects receiving highly active antiretroviral therapy that included protease inhibitors (group 1) or nonnucleoside reverse-transcriptase inhibitors (group 2).

		No. of days				
Group, characteristic	Baseline	14	30	90	180	*P*
Group 1						
$CD4^+$ cell count, cells/mm^3 ±	139 ± 110		175 ± 77	272 ± 60	286 ± 96	
SD (range)	(14–341)	—	(65–300)	(135–403)	(114–536)	<.001[a]
HIV RNA level, copies/mL ±	141,760 ± 149,638		38,748 ± 46,488	9968 ± 19,799	1119 ± 2120	
SD (range)	(11,800–500,000)	—	(500–187,000)	(<50–56,700)	(<50–6900)	<.001[a]
No. with oral candidiasis	7	3	1	1	0	<.001[b]
No. with oral *Candida albicans* carriage	11	11	8	6	5	.005[b]
Group 2						
$CD4^+$ cell count, cells/mm^3 ±	202 ± 170		252 ± 177	375 ± 235	456 ± 393	
SD (range)	(13–530)	—	(20–601)	(26–991)	(29–1492)	<.001[a]
HIV RNA level, copies/mL ±	119,963 ± 180,973		61,976 ± 102,780	11,216 ± 26,792	1922 ± 7325	
SD (range)	(5300–500,000)	—	(<50–350,000)	(<50–98,000)	(<50–28,400)	.001[a]
No. with oral candidiasis	3	0	2	2	2	1.00[b]
No. with oral *C. albicans* carriage	8	8	11	6	4	.10[b]
P, group 1 vs. group 2[c]						
$CD4^+$ cell count	.23	—	.13	.11	.11	
HIV RNA level	.72	—	.43	.88	.68	
Oral candidiasis	.12	.22	1.00	1.00	.48	
C. albicans carriage	.44	.26	.26	1.00	.69	

NOTE. Odds ratio for time: baseline, 1.00; 14 days, 1.00; 30 days, 0.42; 90 days, 0.18; and 180 days, 0.18. A dash (—) indicates not done.
[a] Analysis of variance test for repeated measures.
[b] χ^2 for trend.
[c] Student's *t* test.

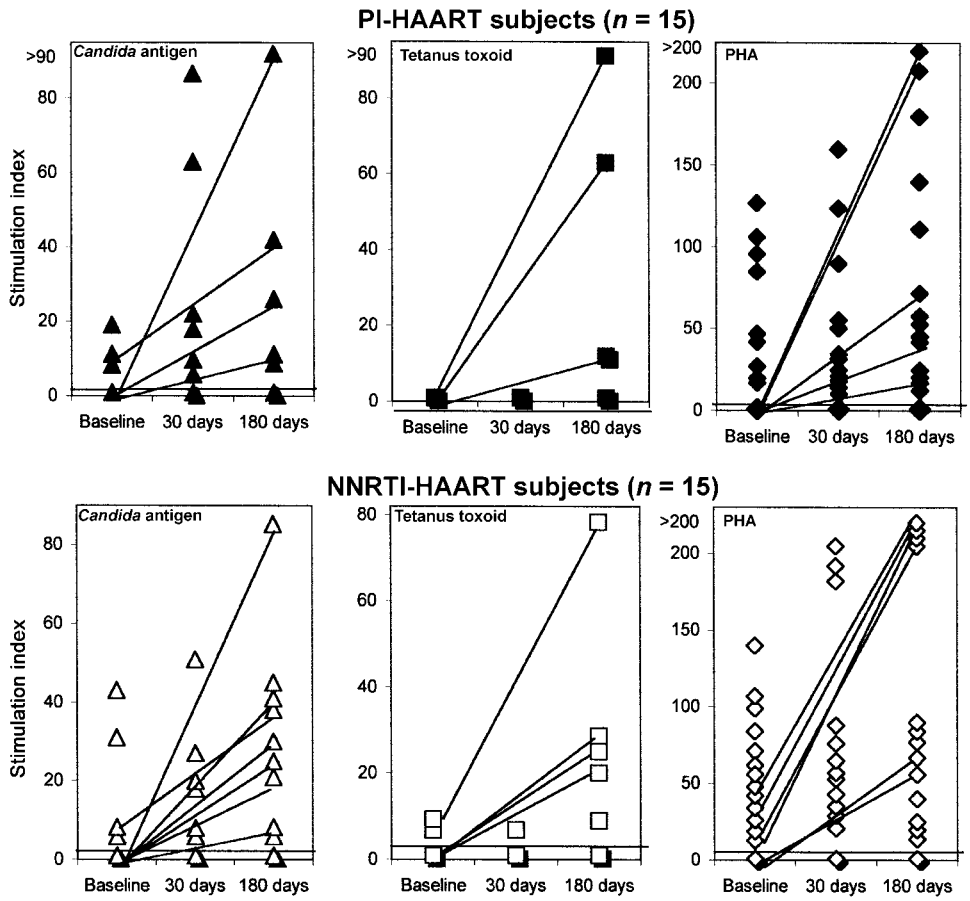

Figure 1. Proliferation of peripheral blood mononuclear cells of human immunodeficiency virus–positive subjects in response to *Candida* antigen, tetanus toxoid, and phytohemagglutinin (PHA) during highly active antiretroviral therapy (HAART) with protease inhibitors (PIs; *top*) or nonnucleoside reverse-transcriptase inhibitors (NNRTIs; *bottom*). Baseline is day 0 of treatment. The line over the abscissa indicates the cutoff determination of positive response (see Subjects and Methods for further details). The lines joining symbols indicate those subjects with significant increase in lymphoproliferation between baseline and 180 days.

Among the subjects who received NNRTI-HAART, the proportion of *Candida*-responsive subjects was substantially equivalent to those of PI-HAART subjects at the baseline. However, it clearly increased during treatment to reach positive lymphoproliferation to *Candida* antigen in the majority (9/15) of the subjects after 180 days. Five subjects also responded to the tetanus toxoid, and almost all responded to PHA. Overall, considering only the subjects who were unresponsive to *Candida* at baseline, 6 subjects showed functional recovery of anticandidal response in the NNRTI-HAART group, versus 3 in the PI-HAART group (*P,* not significant).

Salivar Sap. Figure 2 shows that the subjects of the 2 therapeutic groups differed markedly in the effect of therapy on the amount of salivar Sap. The percentage of Sap-positive, *Candida*-positive subjects and the mean ± SE Sap amount decreased substantially, from 100% to 55% and from 271 ± 46 to 152 ± 21 ng of Sap/mL of saliva, respectively, after only 14 days of PI-HAART administration. Along this trend, the enzyme was present in only 3 of 8 *Candida*-positive subjects after 30 days, to become undetectable in any subject after 90 and 180 days from treatment initiation, thus providing a highly significant ($P < .01$) decreasing trend. In no case was Sap found in the saliva without *C. albicans* isolation (this Sap-producing *Candida* species was isolated in >90% of subjects). Notably, however, the decreased amount or the lack of Sap detection was observed even in the presence of 6 and 5 isolations of the fungus on days 90 and 180, respectively (figure 2 and table 2). Moreover, these isolates produced as much Sap in vitro as did the isolates from salivar Sap–positive subjects (data not shown).

In the NNRTI-HAART group, neither the number of Sap-positive subjects among *Candida*-positive subjects nor the amount of the enzyme (despite some fluctuation; figure 2) was seen to decrease significantly over the duration of treatment, although the baseline mean value of salivar Sap was lower in this group of subjects than it was in PI-treated ones. Two patients, who were initially Sap-negative, converted to Sap positivity by

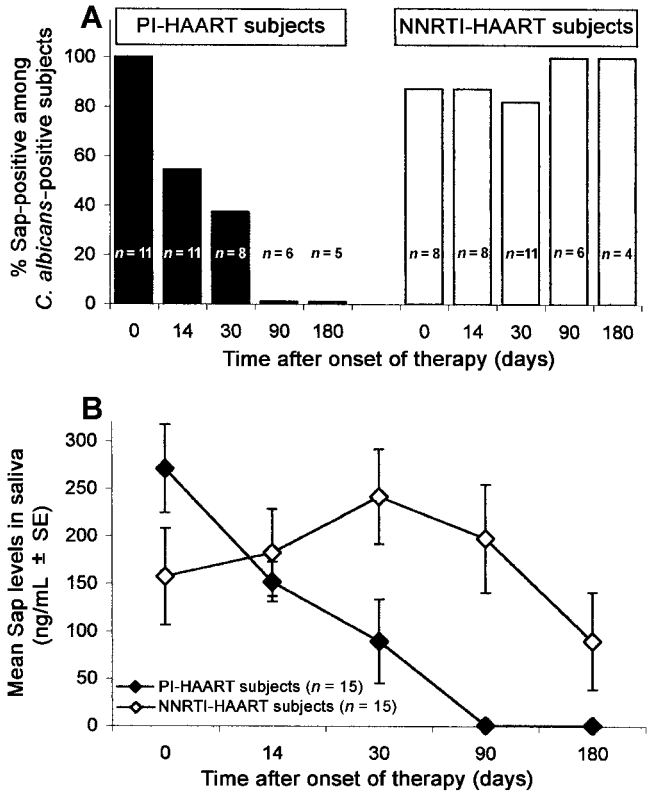

Figure 2. Percentage of subjects with secretory aspartyl proteinases (Sap; *A*) and Sap content *(B),* as measured by ELISA in the saliva of human immunodeficiency virus–positive subjects. *A,* Percentage of Sap-positive subjects among *Candida albicans*–positive subjects; *B,* Sap level in the saliva of all subjects of the 2 groups. See Subjects and Methods for further details. HAART, highly active antiretroviral therapy; NNRTI, nonnucleoside reverse-transcriptase inhibitor; PI, protease inhibitor.

14 or 30 days of treatment. All 6 and 4 *Candida*-positive subjects of this therapeutic regimen still had Sap in their oral cavities on days 90 and 180, respectively, of observation (figure 2).

Cross-sectional study. The 2 groups (15 subjects each) did not differ significantly regarding demographic characteristics, time from the beginning of therapy, HIV viremia, and number of peripheral $CD4^+$ cells. All subjects underwent HAART with 2 RTIs, including indinavir (11 cases [73%]) or ritonavir (4 cases [27%]) in group 3 and nevirapine in group 4. Table 3 shows the relevant microbiological and immunologic data of both groups of patients.

Concerning lymphoproliferation to *Candida* antigen, the 2 groups did not differ significantly either in the proportion of subjects who responded to therapy (11 and 7 of 15 for PI-HAART and NNRTI-HAART, respectively) or in the mean ± SD SI (34 ± 38 vs. 32 ± 34, respectively). Eleven and 8 subjects receiving PI-HAART and NNRTI-HAART were seen to harbor *Candida* species in their oral cavities, respectively (again, >90% of the isolates were *C. albicans*). No subject suffered from OC. Nonetheless, the 2 groups differed markedly in the proportion of subjects with measurable Sap in the saliva (0 and 7 in the PI-HAART and NNRTI-HAART groups, respectively; $P < .001$). The mean ± SD amount of salivar Sap in the NNRTI-HAART patients was 162 ± 104 ng/mL, which is comparable to that found in therapy-naive subjects at the initiation of the treatment (see above). All *C. albicans* isolates from either group of patients were comparable Sap producers in vitro (data not shown).

Discussion

As for other OIs, OC of HIV-infected subjects proved to be exquisitely sensitive to regimens that used cocktails of antiretroviral agents (HAART) with an HIV PI [2, 5]. It is usually assumed that the efficacy of these therapeutic regimens against OI depends on the recovery from the profound lymphopenia that is characteristic of OI-affected subjects and is associated with a degree of reconstitution of specific cell-mediated immunity. This may be helped by desequestration of anergic suppressor $CD4^+CD25^+$ T cells previously entrapped at the focus of infection [23]. However, there are anecdotal reports and circumstantial observations that the decrease of OC incidence has occurred, in several subjects, too early to be fully accounted for by the immune reconstitution of anticandidal cell-mediated immunity [12].

The data reported in the present article strongly suggest that reconstitution of systemic anti-*Candida* T cell response, whatever its source and mechanisms, does not consistently—that is, with reference to the whole group of treated patients—occur during the first 180 days of PI-HAART. A more substantial immune recovery of anticandidal response was observed in the subjects in the NNRTI-HAART group, a therapeutic regimen that, however, appears to be less efficacious than the PI-HAART against OC and oral fungal carriage within the first period of treatment (tables 1 and 2). The low frequency of immune reconstitution in our PI-treated subjects of the longitudinal study was not due to some technical fault, as demonstrated by the more efficient and extensive lymphoproliferative response to the *Candida* antigen by the chronically treated patients, coupled with the substantial $CD4^+$ cell count increase, nor was it attributable to some nonspecific immunosuppression of our subjects, as shown by their high responsiveness to the polyclonal stimulant and the decrease of potentially immunoinhibitory viremia load. Notably, it has been reported recently that HIV-positive subjects with OC had lymphoproliferative responses to *Candida* antigens that were substantially similar to those of HIV-negative subjects, with no specific effect of HAART on those responses [24]. Finally, the antigenic preparation of *C. albicans* used here (MP-F2) has been used elsewhere to rather precisely monitor the characteristic fall in antigen recognition during the progression of HIV infection and immunosuppression [18].

We hypothesized elsewhere that the early clinical benefit of PI treatment against OC could be due, in part, to the PI capacity of inhibiting production and activity of Sap, a major virulence fac-

Table 3. Baseline characteristics of 30 human immunodeficiency virus (HIV)–infected subjects treated for >1 year with highly active antiretroviral therapy (HAART) with protease inhibitors (group 3) or nonnucleoside reverse-transcriptase inhibitors (group 4).

Characteristic	Group 3 ($n = 15$)	Group 4 ($n = 15$)	P	OR (95% CI)
Sex, men:women	9:6	6:9	.27	2.25 (0.42–12.75)
Age, mean years ± SD	40 ± 8	40 ± 10	.98	NC
Stage C of HIV infection[a]	11 (73)	7 (47)	.13	3.14 (0.54–19.59)
Time from beginning of HAART, mean months ± SD	16 ± 4	15 ± 6	.58	NC
Candida albicans oral carriage	11 (73)	8 (53)	.25	2.41 (0.41–14.82)
C. albicans isolation	9 (60)	7 (47)	.34	2.06 (0.37–12.09)
$CD4^+$ cell count, mean cells/mm^3 ± SD	322 ± 214	402 ± 211	.31	NC
HIV RNA level, mean copies/mL ± SD	3973 ± 12,061	504 ± 1362	.28	NC
Subjects with Sap	0	7 (47)	.006	NC
Sap concentration, mean μg/mL ± SD	<20[b]	162 ± 104	—	—
Mean SI	34 ± 38	32 ± 34	.92	−1.71
Positive lymphoproliferative response	11 (73)	7 (47)	.13	3.14 (0.54–19.59)

NOTE. Data are no. (%) of subjects, except where noted. CI, confidence interval; NC, not calculable; OR, odds ratio, Sap, secretory aspartyl proteinase; SI, stimulation index.

[a] According to Centers for Disease Control and Prevention definitions [17].

[b] All subjects.

tor in mucosal candidosis [12, 13]. The PI concentrations needed to inhibit Sap in vitro fall in the range of those achievable in the mucosal fluids of subjects who receive HAART [21–23, 25–27]. Moreover, we have detected enzymatically active Sap in the oral cavity of HIV-infected patients [19]. Finally, it has been shown repeatedly that HIV-infected patients harbor strains of *C. albicans* with very high potential for Sap production [19, 20, 28], a property that some authors have ascribed to Sap induction by HIV envelope proteins [29]. However, the in vivo relevance of the anti-Sap activity of PI could not be assessed previously in the absence of a suitable control. Thus, we sought evidence of the anti-Sap effect in subjects receiving PI-HAART and of its relationship with resolution of OC and recovery of anticandidal cellular immunity by comparing them with subjects receiving NNRTI-HAART instead of PI-HAART.

To this aim, we enrolled 60 HIV-positive subjects for 2 randomized, comparative studies. The first study was a longitudinal follow-up of therapy-naive subjects who were assessed at intervals during a 180-day period of therapy. The second study was cross-sectional and involved 2 groups of 15 subjects each who had been treated with HAART combining either PIs or NNRTIs with other RTIs of HIV replication for >1 year. The length of chronic treatment and any other demographic characteristics did not differ significantly between the 2 groups of subjects.

In the cross-sectional investigation, all 7 subjects in the NNRTI-treated group from whom *C. albicans* was isolated were found to have Sap in their saliva, to a level that did not significantly differ from the Sap level in the saliva of *C. albicans*–positive, therapy-naive subjects at baseline. In clear contrast, 0 of 9 subjects of the PI-treated group with positive *C. albicans* isolation had any Sap detectable in the saliva. This was clearly due to some in vivo inhibition of Sap production, because the numbers of *C. albicans* (Sap producer) strains isolated from both groups were comparable, and all isolates, whatever the source, showed the same Sap production capacity in vitro.

The results of the present study of therapy-naive subjects who were followed-up for 180 days not only confirmed the specific anti-Sap effect of PI treatment (and that this effect was not due to eradication of the fungus from the oral cavity) but also showed that inhibition of Sap production in vivo occurred quite early after therapy administration. In fact, of the 11 PI-treated subjects with Sap in their saliva at the baseline, 5 had no Sap after 14 days, and 8 had no Sap after 30 days of treatment, despite still being colonized by *Candida* in their oral cavities (11 and 8 subjects, respectively). After 90 and 180 days, no subject had salivar Sap, although some of them still harbored *C. albicans* in their oral cavities. Because all these isolates were Sap producers in vitro, it follows that the early effect of PI was due to inhibition of Sap production activity in vivo rather than to the elimination of the fungus from the oral cavity or selection of non-Sap producer strains of the fungus. In contrast, all 7 Sap-positive NNRTI-HAART–receiving subjects at the baseline were still positive, with baseline-comparable mean salivar Sap amount, after 30 days, and 6 and 4 of them even after 90 and 180 days, respectively, of treatment. Moreover, 2 initially Sap-negative subjects converted to Sap positivity by 30 days of treatment. Thus, NNRTI-HAART, in clear contrast to PI-HAART, had no direct effect on Sap production by *C. albicans* in the oral cavity, which is in keeping with the absence of activity in vitro (data not shown).

Importantly, PI-HAART and NNRTI-HAART did not differ substantially in any other respect, particularly with respect to

the increase of $CD4^+$ cell count and the viremia decrease. In particular, the subjects receiving NNRTI-HAART showed a trend for a more substantial recovery of immune response to *Candida,* compared with the the subjects receiving PI-HAART, particularly notable in the longitudinal study. From these data, it appears to be quite unlikely that immune recovery, as measured at the peripheral level, is the critical factor explaining either the *early* decrease of OC episodes or the abatement of fungus virulence in HIV-positive subjects who receive PI. However, longer treatment with a more sustained specific immune reconstitution, coupled with marked elevation of $CD4^+$ T cells, might well control *Candida* disease, even in the presence of oral colonization and Sap production, as documented here for patients taking NNRTI-HAART in the cross-sectional investigation.

We are fully aware that the number of subjects investigated in each of the 2 studies is relatively low—thus, caution is required in the extension of our results to every population of subjects receiving PI-HAART. However, the cumulative evidence gained from both the serial trend measurements in the longitudinal study and the cross-sectional investigations rather strongly suggests 2 conclusions: (1) PI-containing HAART regimens have the distinctive advantage of inhibiting the production and activity of a major virulence factor of *C. albicans,* particularly involved in mucosal (oral and vaginal) candidiasis [15], and (2) this effect may be therapeutically relevant in the early phase of therapy, when immune reconstitution is still inconsistent. In principle, the control of *Candida* virulence might also favor immune system reconstitution itself by decreasing a harmful antigenic load [13, 30]. A similar effect of PI-HAART might well be of relevance in other OIs caused by microorganisms with aspartic proteinases playing a role in the mechanisms of mucosal or systemic pathogenicity [31].

Acknowledgments

We thank Daniela Adriani, Anna Maria Marella, and Giuseppina Mandarino, for their dedicated assistance.

References

1. Deeks SG, Smith M, Holodniy I, Kahn JO. HIV-1 protease inhibitors: a review for clinicians. JAMA **1997**; 277:145–55.
2. Palella FJ, Delaney KM, Moorman AC, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. New Engl J Med **1998**; 338:853–60.
3. Sepkowitz KA. Effect of HAART on natural history of AIDS-related opportunistic disorders. Lancet **1998**; 351:228–30.
4. Brodt HR, Kamps BS, Gute P, et al. Change incidence of AIDS-defining illness in the era of antiretroviral therapy. AIDS **1997**; 11:1731–38.
5. Zingman BS. Resolution of refractory AIDS-related mucosal candidiasis after initiation of didanosine plus saquinavir. N Engl J Med **1996**; 334: 1674–5.
6. Autran B, Carcelain G, Li TS, et al. Positive effects of combined antiretroviral therapy on CD4 T cell homeostasis and function in advanced HIV disease. Science **1997**; 277:112–6.
7. Autran B, Carcelain G, Li TS, et al. Restoration of the immune system with anti-retroviral therapy. Immunol Lett **1999**; 66:207–11.
8. Pakker NG, Roos MT, van Leeuwen R, et al. Pattern of T-cell repopulation, virus load reduction, and restoration of T-cell function in HIV-infected persons during therapy with different antiretroviral agents. J Acquir Immune Defic Syndr Hum Retrovirol **1997**; 16:318–26.
9. Angel JB, Kuman A, Parato K, et al. Improvement in cell-mediated immune functions during potent anti–human immunodeficiency virus therapy with ritonavir plus saquinavir. J Infect Dis **1998**; 177:898–904.
10. Mezzaroma I, Carlesimo M, Pinter E, et al. Long-term evaluation of T-cell subjects and T-cell function after HAART in advanced stage HIV-1 disease. AIDS **1999**; 13:1187–93.
11. Hsieh S, Hung C, Pan S, et al. Restoration of cellular immunity against tuberculosis in patients with HIV-1 and tuberculosis with effective antiretroviral therapy. J Acquir Immune Defic Syndr **2000**; 25:212–20.
12. Cauda R, Tacconelli E, Tumbarello M, et al. Role of protease inhibitors in preventing recurrent oral candidosis in patients with HIV infection: a prospective case-control study. J Acquir Immune Defic Syndr **1999**; 21: 20–5.
13. Cassone A, De Bernardis F, Torosantucci A, Tacconelli E, Tumbarello M, Cauda R. In vitro and vivo anticandidal activity of human immunodeficiency virus protease inhibitors. J Infect Dis **1999**; 180:448–53.
14. Hube B. *Candida albicans* secreted aspartyl proteinase. Curr Top Med Mycol **1996**; 7:55–69.
15. De Bernardis F, Arancia S, Morelli L, et al. Evidence that members of the secretory aspartyl proteinases gene family, in particular *SAP2,* are virulence factors for *Candida* vaginitis. J Infect Dis **1999**; 179:201–8.
16. Conway B. Initial therapy with protease inhibitor–sparing regimens: evaluation of nevirapine and delavirdine. Clin Infect Dis **2000**; 30(Suppl 2): S130–4.
17. Centers for Disease Control and Prevention. 1993 Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR Morb Mortal Wkly Rep **1992**; 41(RR-17):1–19.
18. Quinti I, Palma C, Guerra EC, et al. Proliferative and cytotoxic responses to mannoproteins of *Candida albicans* by peripheral blood lymphocytes of HIV-infected subjects. Clin Exp Immunol **1991**; 85:485–92.
19. De Bernardis F, Chiani P, Ciccozzi M, et al. Elevated aspartic proteinase secretion and experimental pathogenicity of *Candida albicans* isolates from oral cavities of subjects infected with human immunodeficiency virus. Infect Immun **1996**; 64:466–71.
20. De Bernardis F, Mondello F, Scaravelli G. High aspartyl proteinase production and vaginitis in human immunodeficiency virus–infected women. J Clin Microbiol **1999**; 37:1376–80.
21. Angiolella L, De Bernardis F, Bromuro C, et al. The effect of antimycotics on secretory acid proteinase of *Candida albicans.* J Chemother **1990**; 2: 55–61.
22. Cassone A, De Bernardis F, Mondello F, Ceddia T, Agatensi L. Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. J Infect Dis **1987**; 156:777–83.
23. Bucy RP, Hockett RD, Derdeyin CA, et al. Initial increase in blood $CD4^+$ lymphocytes after HIV antiretroviral therapy reflects redistribution from lymphoid tissues. J Clin Invest **1999**; 103:1391–8.
24. Leigh JE, Barousse M, Swoboda R, et al. *Candida*-specific systemic cell–mediated immune reactivities in human immunodeficiency virus-positive persons with mucosal candidiasis. J Infect Dis **2001**; 183:277–85.
25. Acosta EP, Kakuda TN, Brundage RC, Anderson PL, Fletcher CV. Pharmacodynamics of human immunodeficiency virus type 1 protease inhibitors. Clin Infect Dis **2000**; 30(Suppl 2):S151–9.
26. Hughens PWH, Burger DM, Hoetelmans RMW, et al. Saliva as a possible specimen for monitoring compliance and plasma levels in patients treated with indinavir [abstract 32330]. In: Program and abstracts of the 12th

World AIDS Conference (Geneva). Stockholm: International AIDS Society, **1998**:586–87.

27. Car A, Cooper DA. HIV protease inhibitors. AIDS **1996**;10(Suppl A): 151–7.
28. Ollert MW, Wende C, Gorlich M, et al. Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus–positive patients. J Clin Microbiol **1995**;33:2543–9.
29. Gruber A, Lukasser-Volg E, Borg–von Zepelin M, et al. Human immunodeficiency virus type 1 gp160 and gp41 binding to *Candida albicans* selectively enhances candidal virulence in vitro. J Infect Dis **1998**;177: 1057–63.
30. Mencacci A, Spaccapelo R, Del Sero G, et al. $CD4^+$ T-helper cell responses in mice with low-level *Candida albicans* infection. Infect Immun **1996**; 64:4907–14.
31. Atzori C, Angeli E, Mainini A, et al. In vitro activity of human immunodeficiency virus protease inhibitors against *Pneumocystis carinii.* J Infect Dis **2000**; 181:1629–34.