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Improvement in immune parameters and human immunodeficiency virus-1 viral response in individuals treated with 16α-bromoepiandrosterone (HE2000)

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ABSTRACT

A randomised, double-blind, placebo-controlled study examined the safety, tolerance, immunological effect and anti-human immunodeficiency virus (HIV) activity of sub-cutaneously administered HE2000 (16α-bromoepiandrosterone) as monotherapy in treatment-naïve patients with HIV-1. Twenty-four patients received five sequential daily doses of 50 or 100 mg of HE2000 or placebo every 6 weeks for up to three courses, and were followed thereafter for 3 months. HE2000 was safe, with transient injection site reactions being the main side-effect. Peripheral blood samples, collected serially, were analysed for changes in immune cell phenotypes. Significant increases were observed in the numbers of circulating dendritic cells, early activated ($CD69^+CD25^-$) CD8 T-cells and T-NK cells after administration of 50-mg doses of HE2000 ($p < 0.05$). Gene expression in peripheral blood mononuclear cells was analysed by real-time RT-PCR. Before treatment, HIV-1-infected patients had significantly elevated transcripts for a number of inflammatory mediators ($p < 0.012$). After 50 mg or 100 mg HE2000, but not after placebo, there were significant sustained decreases in IL-1β, TNF-α, IL-6 and Cox-2 transcripts ($p < 0.05$). There were no significant differences in CD4 cell numbers, although patients receiving 50-mg doses demonstrated a significant decrease in viral load (− 0.6 log; $p < 0.01$). Anti-HIV-1 T-cell responses were analysed serially using GAG-peptides to stimulate cytoplasmic IFN-γ responses. After three courses, the 50-mg dose group demonstrated a significant increase in CD8 T-cell response against two distinct GAG peptide pools ($p < 0.03$). These findings suggest that immune-based therapies may be able to impact viral load by decreasing inflammation and/or stimulating CD8 T-cells.

Keywords AIDS, 16α-bromoepiandrosterone, HE2000, human immunodeficiency virus, immune stimulation, therapy

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INTRODUCTION

Infection with human immunodeficiency virus (HIV) degrades the immune system, rendering the host susceptible to opportunistic infections and death. It is an expanding chronic disease that currently threatens the lives of more than 40 million people worldwide. 16α-Bromoepiandrosterone (HE2000) is an immune modulator that has the potential to stimulate host immune defences against a pathogen that has already infected the host. In a preliminary report of a non-human primate model of simian HIV infection (Frincke *et al.*, International Society for Antiviral Research, Jerusalem, 1999), it was reported that HE2000 increased survival time. Furthermore, HE2000 administered to cats infected with feline immunodeficiency virus lowered their viral set point when compared with administration of a placebo [1].

HE2000 was first studied in patients in an open-label, safety, tolerance, pharmacokinetic and anti-HIV activity study conducted in South Africa in treatment-naïve, HIV-infected patients (Hollis Eden Pharmaceuticals, San Diego, CA, USA; data on file). The data indicated increases in circulating dendritic cell numbers and other cells important to host protection. Based on those observations, the present study was

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designed as a randomised, double-blind, placebo-controlled trial to explore further the potential of HE2000 to improve the immune status of patients with HIV-1 infection.

MATERIALS AND METHODS

Study design

Study HE2000-009, conducted in Belville and Durban, South Africa, between November 2000 and January 2002, was a randomised, double-blind, placebo-controlled study of the safety, tolerance, immunological effect and anti-HIV activity of sub-cutaneously administered HE2000 in HIV-infected, treatment-naïve patients. The inclusion of a vehicle placebo control and the double-blind randomised design allowed an unbiased estimation of the contribution of HE2000 to the immune modulation effects. HE2000 was supplied as a solution containing 100 mg HE2000 in 1 mL of a vehicle formulation consisting of PEG200, propylene glycol, benzyl benzoate and benzyl alcohol (Hollis Eden Pharmaceuticals). The placebo consisted of the vehicle only, without the active pharmaceutical ingredient HE2000. Patient criteria included female or male patients, aged >18 years, with a CD4 cell count ≥200 cells/mm^3, a plasma HIV RNA level between 5×10^3 and 1×10^6 copies/mL, as measured by the bDNA assay (Bayer, Tarrytown, NY, USA), and a Karnofsky score of at least 70. Patients did not take anti-retroviral medication during the treatment or follow-up phases of the study.

Patients were assigned randomly to one of two dose levels of HE2000 (50 or 100 mg) or to a placebo in a 3:1 ratio. Placebo groups received the equivalent volume of vehicle as the HE2000 groups (0.5 mL for 50 mg; 1 mL for 100 mg). In this study, a treatment course consisted of 5 consecutive days of once-daily sub-cutaneous injections of HE2000 or placebo equivalent, followed by a 37-day observation period. Subjects received up to three 5-day treatment courses over a 19-week period. All study participants were provided with a nutritional supplement of multivitamins with minerals (Centrum™; Wyeth, Madison, NJ, USA) throughout the study. The Medicines Control Council, the regulatory body of the Republic of South Africa, and the local ethics committees approved the protocol and the written consent form. The study was conducted in accordance with the International Conference on Harmonization, Good Clinical Practice Guidelines, National Good Clinical Practice Guidelines and the Declaration of Helsinki principles.

Flow cytometry

Whole blood was incubated with sets of four monoclonal antibodies (Becton Dickinson, San Jose, CA, USA) to measure cell sub-sets using a FACSCalibur (Becton Dickinson). The four-colour panels consisted of APC, PerCP, FITC and PE reagents, respectively, in the following combinations: T-cell activation (CD3/CD8/CD69/CD25, CD3/CD4/CD69/CD25) [2]; NK populations (CD3/CD8/CD16/CD56); T-cytotoxic cells (CD3/CD8/CCR3/CCR5); and dendritic cells (CD11c/HLA-DR/lineage markers CD3, CD16, CD14, CD19, CD56/CD123). List-mode data (25 000–50 000 events) were analysed using FCS Express (De Novo Software, Thornhill, Ontario, Canada). $CD3^+$ cell sub-sets were identified by serial gating of (i) nucleated cells, (ii) lymphocyte/lymphoblastoid cells, and (iii) $CD3^+$ cells, followed by gating of the sub-set of interest. The absolute frequency per μL of a cell sub-set was estimated by the following equation:

$$f(S) = p(G) \times f(G)$$

where G denotes a set of gated cells, S denotes a cell sub-set contained in the gate, f stands for a frequency (in cells/μL) in a set, and p(G) is the proportion of gated cells in the sub-set. For $CD4^+$ or $CD8^+$ cell sub-sets, p(G) was determined from a $CD4^+$ or $CD8^+$ gated region, and f(G), the absolute $CD4^+$ or $CD8^+$ cell frequency, was determined using the FACSCount test. For dendritic cells, p(G) was determined from a nucleated cell region that was defined using a forward vs. orthogonal scatter plot, and f(G), the absolute white blood cell (WBC) count, was determined using an automated cell counter (Advia 120; Bayer, Tarrytown, NY, USA).

Flow phenotypes were analysed before and serially throughout the study after HE2000 treatment or placebo. Results were calculated as percentage change from baseline (pre-treatment) frequency. Treatment effects were investigated for dendritic cells ($Lineage^-HLA^-DR^+CD11c^+/CD123^+$) [3], early activated CD8 T-cells ($CD3^+CD8^+CD69^+CD25^-$) [4,5], T-NK cells ($CD3^-CD8^+CD16^-CD56^+$) [6,7], and T cytotoxic 1 cells ($CD3^+CD8^+CCR3^-CCR5^+$) [8,9].

Inflammatory mediator transcripts

Samples from seven healthy South African donors were analysed concurrently at two time-points (2 weeks apart), and from HIV-1-infected patients before and serially throughout the study after treatment with HE2000 or placebo. Peripheral blood was collected into CPT-Vacutainers (Becton Dickinson) and peripheral blood mononuclear cells (PBMCs) were isolated according to the manufacturer's protocol. Two million PBMCs were cultured in 1 mL of RPMI 1640 containing fetal bovine serum 10% v/v for 3 h at 37°C to establish baseline transcription levels. After lysis in 300 μL MagnaPure lysis buffer (Roche, Branchburg, NJ, USA), the samples were frozen at −70°C. After thawing, the lysates were mixed and transferred into the MagnaPure sample cartridge, and mRNA was isolated with the MagnaPure-LC apparatus (Roche) using the mRNA standard protocol for cells. The elution volume was set to 50 μL. Two aliquots of 8.2 μL RNA were independently reverse-transcribed using AMV-reverse transcriptase and oligo-(dT) as primer (First Strand cDNA synthesis kit; Roche) according to the manufacturer's recommendations. After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 μL and stored at −20°C until PCR analysis.

Target sequences were amplified using LightCycler Primer Sets (designed by T. Giese; Search-LC, Heidelberg, Germany) with the LightCycler FastStart DNA Sybr Green I Kit (Roche) according to the manufacturer's protocol. RNA input was normalised by the average expression of two housekeeping genes, β-actin and cyclophilin B, in healthy donors. As this factor remained constant, all samples were deemed comparable.

The copy number was calculated from a standard curve, obtained by regressing known input concentrations of a plasmid vs. the PCR cycle number at which the detected fluorescence intensity reached a specific value. Results from

two independent analyses for each sample and parameters were averaged and presented as adjusted transcripts/μL cDNA.

Cell-mediated immunity

Frozen PBMCs were thawed and stimulated separately with two pools of HIV GAG peptides (GAG pool 1 and GAG pool 2; C. Brander, Massachusetts General Hospital, Boston, MA, USA), and with anti-CD49d and anti-CD28. Cells were permeabilised and stained with CD4, CD8, CD69 and IFN-γ antibodies, and were then analysed for cytoplasmic IFN-γ production by multiparameter flow cytometry.

CD4 and viral load determinations

CD4 counts were assessed using a FACSCount (Becton Dickinson), and viral loads were measured using the bDNA assay. Quality assurance procedures were adhered to for bDNA, and internal and external controls were used to validate each batch of samples. Average $\log_{10}$ changes in viral loads were calculated as the average for each dose group of the change from $\log_{10}$ baseline values for individual patients at each visit.

Statistical methods

The study was designed to explore the safety of HE2000 and to understand its effects on immunological biomarkers. Thus, it was not powered to formally detect efficacy in any parameter. Demographical and background characteristics of the treatment groups at baseline were compared by exact Wilcoxon and exact chi-square tests. For any given patient response y_t at time t, the percentage change from baseline was defined as $100*(y_t - y_0)/y_0$, where y_0 denotes the patient baseline response. Hence, a negative value indicates a decrease from baseline. Percentage changes were tested for their absolute significance with the exact Wilcoxon signed rank test and pairwise by treatments with the exact Wilcoxon Mann–Whitney test. All comparisons were adjusted for multiplicity by means of the step-down Bonferroni method. Each individual GAG response was preserved in the analysis. Baseline values entered the model as covariates. The time-series structure of the study design extended from 21 days before baseline to day 133, plus three monthly follow-up visits. Resulting time-sequences were submitted to generalised linear mixed models [10]. These models allow for heterogeneous variances and non-normal responses. Analyses were performed with the original data and via Savage scores for skewed distributions. Overall significant results were followed by pairwise comparisons adjusted for multiplicity. Statistical analyses were performed with the use of SAS software (SAS Institute, Cary, NC, USA) and StatXact software (Cytel Software Corp., Cambridge, MA, USA) to calculate exact p values.

RESULTS

Baseline characteristics and disposition of patients

The disposition of patients for analysis is presented in Table 1. All patients in the HE2000 50-mg group completed the study. One patient in the placebo group did not complete the study because of a protocol violation. Three patients in the HE2000 100-mg group were discontinued from the study: one became pregnant, one was lost to follow-up, and one withdrew consent. No patients died during this study or discontinued treatment during the trial because of treatment-related adverse events. The baseline characteristics of the patients are listed in Table 2. Randomisation resulted in balanced distribution concerning age, gender, race or ethnic group, viral load and CD4 count, considering the small sample size.

Table 1. Disposition of patients according to treatment

	HE2000			
Group	**50 mg**	**100 mg**	**Placebo**	**Total**
Enrolled (24)[a]	9 (37.5)	9 (37.5)	6 (25.0)	24
Completed (20)	9 (37.5)	6 (25.0)	5 (20.8)	20
Discontinued (4)	0 (0)	3 (12.5)	1 (4.2)	4
Reason				
Protocol violation	0 (0)	0 (0)	1 (4.2)	1
Consent withdrawn	0 (0)	1 (4.2)	0 (0)	1
Lost to follow-up	0 (0)	1 (4.2)	0 (0)	1
Terminated by investigator	0 (0)	1 (4.2)	0 (0)	1

[a]Sample size and percentage relative to row total.

Table 2. Baseline characteristics of patients included in the study

	HE2000		
Characteristic	**50 mg**	**100 mg**	**Placebo**
n [a]	9	9	6
Age, years	35 (28,38)[b]	31 (28,37)[h]	44.5 (41,50)
Gender, F/M[c]	6/3	7/2	2/4
Race[d], *n* (%)			
African	7 (29.2)	5 (20.8)	5 (20.8)
Caucasian	1 (4.2)	1 (4.2)	0 (0)
Other	1 (4.2)	3 (12.5)	1 (4.2)
Viral load[e]	38 (14,58) [b]	25 (14,81)	36 (14,66)
CD4 count[f]	482 (354 568)[b]	382 (267 438)	287 (247 482)[g]

[a]Sample size.
[b]Median (inter-quartile range).
[c]Female/male.
[d]Race or ethnic group.
[e]10^3 copies/mL.
[f]$CD4^+$ cells/μL.
[g]Based on $n = 5$ (one patient without baseline value).
[h]100 mg group vs. placebo, $p < 0.04$.

Safety

All 24 patients were considered in the safety analysis. The most commonly reported treatment-related adverse events were injection site reactions. Local reactions, including pain, erythaema, induration, burning, swelling and pruritis,

occurred in 8/9, 9/9 and 4/6 patients in the 50-mg, 100-mg and placebo groups, respectively. These reactions were described as transient and mild-to-moderate in severity.

Changes in immune phenotypes

Changes in the numbers of cell subsets were analysed for dendritic cells, early activated CD8 T-cells, T-NK cells and T cytotoxic 1 (Tc1) cells. The changes in immune phenotypes from baseline values are shown in Table 3. In the placebo group there was a significant decrease in T-NK cells ($p < 0.05$). Patients in the 50-mg dose group showed significant increases in dendritic cells ($p < 0.05$) and activated CD8 T-cells ($p < 0.01$). Patients in the 100-mg dose group had a significant increase in Tc1 cells ($p < 0.05$). Pairwise comparisons demonstrated significant differences ($p < 0.05$) between the 50-mg and placebo groups for dendritic cells, activated CD8 T-cells and T-NK cells.

Changes in inflammatory mediator transcripts

HIV-1-infected patients displayed elevated levels of a number of inflammation-related transcripts in their circulating mononuclear cells before treatment compared with healthy volunteers (Table 4). Each pairwise comparison was significant after adjustment for multiple testing ($p < 0.012$).

Serial analyses during the study showed no significant change in the levels of transcripts for four inflammatory mediators (IL-1β, TNF-α, IL-6 and Cox-2) in the placebo group (Table 5), whereas both the 50-mg and 100-mg dose groups showed significant decreases ($p < 0.05$ and $p < 0.01$, respectively) in transcripts for all four mediators. Decreases were observed in the treated groups after the first course of treatment, and these persisted throughout the study. The differences between each HE2000 group and the placebo group were significant for IL-1β, TNF-α and Cox-2 ($p < 0.05$), and there were trends for IL-6 ($p < 0.07$ for both HE2000 groups).

Table 3. Percentage change from baseline in cell subsets

	%[b] vs. own baseline			%[c] vs. placebo	
Cell subset[a]	50 mg *n* = 9	100 mg *n* = 9	Placebo *n* = 6	50 mg	100 mg
Dendritic	13.6*	0.0	− 7.0	20.6*	7.0
Act. CD8	24.6**	3.1	− 3.5	28.1*	6.6
T-NK	12.2	0.2	− 18.6*	30.8*	18.8
Tc1	14.0	34.6*	1.5	12.5	33.1

[a]Dendritic cells: lineage$^-$HLA$^-$DR$^+$CD11c$^+$/CD123$^+$ Act: CD8: CD3$^+$, CD8$^+$, CD69$^+$, CD25$^-$; T-NK cells: CD3$^-$CD8$^+$CD16$^-$CD56$^+$ Tc1 cells: CD3$^+$, CD8$^+$, CCR3$^-$, CCR5$^+$.
[b]Comparison of average percentage change from baseline.
[c]Pairwise comparisons of active treatment minus placebo.
*$p < 0.05$; **$p < 0.01$.

Table 4. Inflammatory mediator transcripts in healthy donors and human immunodeficiency virus (HIV)-1 patients pre-treatment

	Median transcript levels	
Gene	Healthy donors *n* = 7	HIV patients* *n* = 22
IL-1β	1973	19 662
TNF-α	99	1133
MIP-1α	616	15 732
IL-6	36	1543
IL-8	5425	56 909
IL-10	6	15
Cox-2	160	1255
M-CSF	14	211
RANTES	52	191
GM-CSF	1	105
MCP-1	1008	7224
IFN-γ	7	41

*$p < 0.012$ in all cases.

Table 5. Selected inflammatory mediator transcript changes after treatment

	Median percentage change from baseline				
	Treatment[a]			Treatment comparisons[b]	
Gene	50 mg (*n* = 7)	100 mg (*n* = 9)	Placebo (*n* = 6)	50 mg vs. Placebo	100 mg vs. placebo
IL-1b	− 65*	− 66**	− 19	− 46*	− 47*
TNF-α	− 82*	− 79**	0.3	− 82*	− 79*
IL-6	− 83*	− 90**	0.9	− 84#	− 91#
Cox-2	− 60*	− 58**	− 17	− 43*	− 41*

[a]p values refer to the significance relative to 0% change.
[b]p values adjusted for multiplicity.
*$p < 0.05$. **$p < 0.01$. #$p < 0.07$.

Changes in circulating CD4 cells and viral loads

There were no significant changes in the numbers of circulating CD4 and CD8 T-lymphocytes between groups. Fig. 1 shows the average $\log_{10}$ changes in viral loads over time. At the end of the study, on average, the 50-mg dose group showed a −0.66 log change in viral load, the 100-mg group showed a −0.45 log change, and the placebo group showed a −0.19 log change from baseline values. A statistically significant downward linear component was detected in the viral load of the 50-mg dose group relative

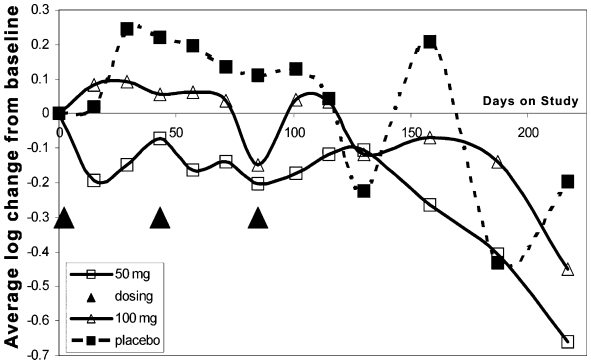

Fig. 1. Average $\log_{10}$ change in viral load for each dose group.

the group receiving placebo ($p < 0.01$). The estimated regression coefficients were −0.00294 ($\log_{10}$/day) for the 50-mg group ($p < 0.001$), and −0.00084 ($\log_{10}$/day) for the placebo group ($p < 0.33$).

Cellular immunity to HIV

There were no significant differences in HIV GAG peptide cytoplasmic IFN-γ responses in CD4 T-cells between treated and placebo groups. Similarly, there were no significant differences between groups during the dosing period (up to day 89) (data not shown). Because of a potential time-lag to improve the immune response in HIV-1 infected patients, and because the viral load change in the 50-mg dose group only became apparent after dosing (days 102–219), cellular immune responses were investigated during this period. Cytoplasmic cytokine analyses were performed on patients with baseline samples, and were available for seven patients in the 100-mg group, four patients in the 50-mg group, and four patients in the placebo group. Fig. 2 shows an increase in CD8 T-cell response to both GAG pool 1 and GAG pool 2, respectively, during this period, reaching 0.7% of the CD8 T-cells with GAG pool 1 and 1.5% with GAG pool 2 for the 50-mg group. Similar responses were not seen in the 100-mg dose group or the placebo group. Comparisons among the groups indicated that the changes observed were significant for the 50-mg dose group as opposed to both the placebo (p 0.005 for GAG pool 1; p 0.03 for GAG pool 2) and the 100-mg dose group (p 0.02 for GAG pool 1; p 0.001 for GAG pool 2) (Table 6).

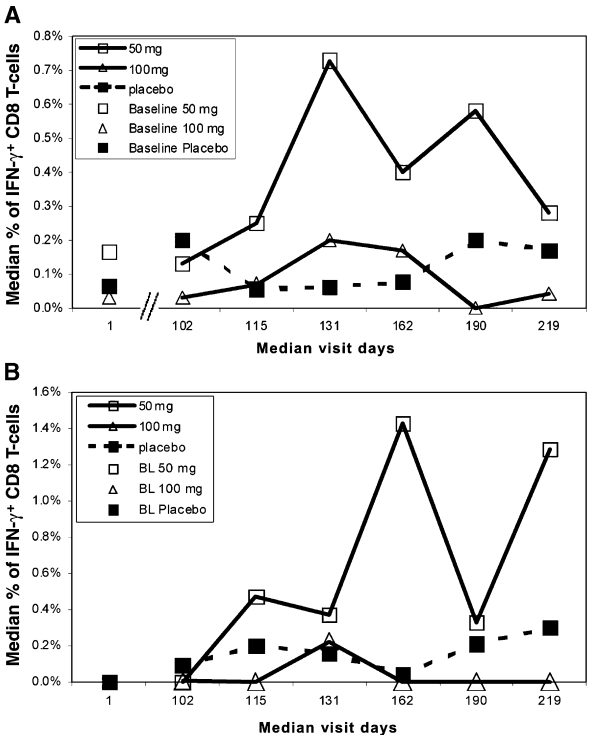

Fig. 2. Cytoplasmic CD8 T-cell IFN-γ responses to (**A**) GAG peptide pool 1 and (**B**) GAG peptide pool 2. Baseline values are indicated at day 1.

Table 6. CD8 T-cell cytoplasmic IFN-γ responses to peptides

GAG peptides	Comparison	Difference[a] (SEM)	p[b]	Adjusted p[c]
Pool 1	100 mg vs. 50 mg	− 24% (8%)	0.008	0.02
	100 mg vs. placebo	10% (9%)	0.35	1.0
	50 mg vs. placebo	32% (9%)	0.002	0.005
Pool 2	100 mg vs. 50 mg	− 34% (11%)	0.0004	0.001
	100 mg vs. placebo	2% (13%)	0.4	1.0
	50 mg vs. placebo	37% (15%)	0.004	0.03

[a]Difference in treatment means.
[b]Raw p values for the significance of the difference.
[c]p values adjusted for multiplicity by the Bonferroni procedure. SEM; standard error of the mean.

DISCUSSION

HE2000, at both doses studied, significantly decreased IL1-β, TNF-α, IL-6 and Cox-2 transcripts associated with a chronic inflammatory state. This decrease in the chronic inflammatory state associated with HIV-1 infection may lead to an improvement in innate and adaptive immunity. Patients infected with HIV-1 produce elevated inflammatory mediators [11–13]. One of the challenges of immune therapy for HIV-1 infection is to decrease the non-specific chronic inflammatory

process, while improving specific immune responses. Administration of anti-inflammatory agents to HIV-1 patients can decrease specific immunity [14–16], while administration of IL-2 (to improve immunity) leads to increases in pro-inflammatory mediators, prompting immunosuppressive therapy [14].

Evidence is also presented for improvement in innate and adaptive immune components after treatment with HE2000. Dendritic cells and activated CD8 T-cells increased after treatment with 50 mg of HE2000. T-NK cells decreased over time in the placebo group, but not in the HE2000 groups. T-cytotoxic cells (Tc1) increased in number with 100 mg of HE2000. Circulating dendritic cell numbers are decreased in HIV-1 patients [17–19]. $CD8^+CD69^+$ T-cells, associated with in-vitro responses from control and HIV-infected patients [20,21], and with early in-vivo responses to simian immunodeficiency virus infection [22–24], were also decreased in HIV patients [25]. Decreased CD56 expression on CD8 T-NK cells may be associated with decreased T-cell cytotoxicity in individuals infected with HIV-1 [26] or hepatitis C virus [27]. These cells are implicated in the production of soluble factors that inhibit HIV-1 replication [28] as well as IFN-γ [29]. The improvement in innate and adaptive immunity might be expected to result in a decrease in viral load, and an enhanced ability to prevent opportunistic infections.

HIV-1 GAG peptide-specific CD8 IFN-γ responses were analysed in order to investigate the specificity of the observed stimulation of adaptive immunity. An increase in specific responses in the 50-mg dose group was demonstrated, concomitant with a significant decrease (− 0.6 log) in viral load. This prompts speculation that the increases observed in circulating dendritic cells and early activated CD8 T-cells might have contributed to the generation of HIV-1-specific CD8 responses that might, in turn, contribute to the observed decrease in viral load. The possible contribution of T-NK cells to these observations is unknown. All patients were supplied with daily doses of multivitamins to control for nutritional effects on immunity [30–33], and this supplementation is expected to contribute to the minimal effects observed in the placebo group when compared with an untreated population. The decrease in viral load was not accompanied by a concomitant increase in circulating CD4 T-cells. It is possible that HE2000 treatment also results in CD4 cells remaining in the peripheral sites combating infection.

The reasons for the differences observed between the 50-mg and 100-mg dose groups are unclear. This was a pilot study, with three of the nine patients in the 100-mg dose group discontinuing the trial. Daynes *et al.* [34] have shown a biphasic IL-2 response by lymphocytes from the action of dehydroepiandrosterone in mice [34] and in human cells [35]. The diminished response with higher doses of HE2000 may be explained by these observations.

The improvement in innate and adaptive immunity may extend beyond early HIV-1 infection. It has also been demonstrated that HE2000 has activity in a murine model of tuberculosis [36], restoring Th1 responses and accelerating chemotherapy-induced bacterial clearance. Furthermore, HE2000 can lead to malarial parasite clearance in a rat model [37]. The results from the present study were obtained in the absence of anti-retroviral drugs. With further clinical development, this compound might prove useful in combination with anti-retroviral drugs for the treatment of HIV-infected patients.

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