

Immunological Basis for IgE Hyper-Production in Enfuvirtide-Treated HIV- Positive Patients

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We previously reported that enfuvirtide (ENF) treatment is accompanied by a selective increase of serum IgE. We asked whether ENF had intrinsic capability to direct B-lymphocytes to switch to IgE and/or if it could drive CD4 T cells to a Th2 phenotype. ENF was added *in vitro*: (a) to B-lymphocytes stimulated with IgE-switch inducing stimuli; (b) to peripheral blood mononuclear cells. Total IgE production by B cells and IL4 and IFN- γ production by CD4 T lymphocytes were evaluated, respectively. ENF had no measurable effect on the IgE production by B-lymphocytes. In contrast, it sharply increased the IL4 to IFN- γ (a correlate of the Th2 phenotype) when added *in vitro* to T cells from healthy donors or from single ENF-treated patients. The hyper-IgE production in ENF-treated patients is associated with the *in vitro* induction of a type-2 phenotype in CD4 T cells.

KEY WORDS: Enfuvirtide (ENF); Immunological response; IgE, Th1; Th2; IFN- γ ; IL-4; B-lymphocytes; T-lymphocytes; Ig switch.

INTRODUCTION

Enfuvirtide (ENF) is the first anti-HIV drug belonging to the entry inhibitor category that has entered clinical use (1, 2). Its activity is based on the inhibition of HIV-1 replication via binding to a gp41 region of the viral envelope,

which is involved in the fusion to host cell membranes (3–5). Large phase III clinical trials have shown that ENF plus an optimized background of other antiretroviral drugs leads to superior clinical benefit than the optimized background alone in terms of gain of CD4 cell counts and reduction in HIV viraemia (6, 7). ENF is currently administered with a twice-daily 90 mg dose by the subcutaneous route. Induration and nodules, pain/discomfort, erythema, and pruritus (6–8) are observed in virtually all treated patients at injection site, and collectively indicated as injection site reactions. Injection site reactions are generally mild and tend to diminish over time (9, 10). Histopathological studies on cutaneous biopsy specimens have described an inflammatory response consistent with a localized hypersensitivity reaction, reminiscent of a granuloma annulare and categorized as interstitial granulomatous drug reaction (11). Clinically, systemic hypersensitivity reactions have been reported in less than 3% of patients (6, 7). Eosinophilia was also reported to occur more frequently in subjects taking ENF (9, 10). We previously described a significant and selective increase of total IgE in ENF-treated patients monitored for up to 24 weeks. In that study, patients had been recruited to the EAP (Early Access Program)(protocol MV 16721), where ENF was introduced as part of a “salvage” combination in subjects with a CD4 count below 100 cells/ μ L (12). The serum IgE increase was observed both in those with normal as well as in those with altered IgE levels at baseline, although it was faster in the latter, and it was associated neither to hypereosinophilia nor to cases of systemic hypersensitivity (12).

Here, we studied whether the ENF peptide was capable to directly drive *in vitro* B-lymphocytes to switch to IgE production and/or whether it could act through a Th2 skewing mechanism.

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METHODS

Study Population

Patients who were studied were part of a previously described cohort (12) who received ENF by the subcutaneous route at 90 mg twice-daily for at least 4 weeks in addition to their backbone of antiretroviral drugs. The EAP study was approved by the San Raffaele Hospital Ethical Committee and a signed informed consent was obtained from each participant. For B-cell purification, three allergic individuals were selected among laboratory workers. They had a clinical history of seasonal rhinoconjunctivitis and positive *in vitro* assay for grass pollen specific IgE. For T-cell lines establishment with the ENF peptide, six blood donors were included as controls, who were recruited from the laboratory personnel. A negative clinical history for allergic diseases and a negative skin prick test to common aeroallergens was used to define those blood donors as nonallergic. All subjects were informed on the nature of the study and agreed to sign an informed consent.

B-Lymphocyte Purification

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from allergic donors by density-gradient centrifugation on Ficoll Hypaque (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) (13). Cells were then washed and resuspended in RPMI-1640 containing 10% heat-inactivated FCS (Hyclone Laboratories, Logan, Utah, USA), 2 mM L-glutamine, 50 μ g/mL streptomycin, and 100 U/mL penicillin (Life Technologies Inc., Rockville, Maryland, USA) (complete medium). Purified B-cell populations were isolated from PBMC by positive sorting with Dynabeads conjugated to CD19 using the Dynal T-cell negative isolation kit according to the manufacturer's instructions (Dynal Inc., Lake Success, New York, USA). FACS analysis of the resultant purified cells showed that they were more than 97% CD19 positive B-lymphocytes.

In Vitro IgE Production

B-lymphocytes (2×10^5 cells/mL) were cultured in complete medium with or without IL-4 (100 U/mL; DNAX Research Institute, Palo Alto, California, USA), plus anti-CD40 monoclonal antibody (mAb) 626.1 (5 μ g/mL; a kind gift of Dr. D. Vercelli, Arizona Respiratory Center, Tucson, AZ, USA) and, in some cases, plus ENF peptide (50 μ g/mL). After 14 days, supernatants were harvested and assessed for their IgE content. Every 3–4 days, half the culture supernatant was replaced by a

new complete medium with or without IL-4 as described previously (14).

IgE Measurement

Levels of total IgE in culture supernatants were measured using ELISA. Microtiter plates (Maxisorb; Nunc, Rochester, New York, USA) were coated overnight at 4°C with 50 μ L/well of anti-human IgE mAbs 7.12 and 4.15 (a kind gift of Dr. D. Vercelli) at 2 μ g/mL each in 0.1 M NaHCO₃ buffer pH 9.0. After washing with PBS containing 0.05% Tween 20, wells were blocked for 4 h at room temperature with 2% dry milk in PBS containing 0.01% sodium azide. Culture supernatants or purified IgE standards (Amersham Pharmacia Biotech) were added 50 μ L/well to washed plates and kept in a humidified chamber overnight at 4°C. Plates were washed and 50 μ L of 1:1,000 diluted horseradish peroxidase-labeled (HRP-labeled) rabbit anti-human IgE (DAKO Corp., Carpinteria, California, USA) was added for 4–6 h at room temperature. After washing, the plate was developed with 100 μ L of *o*-phenylenediamine dihydrochloride (OPD)(Sigma Chemical Co., Milan, Italy), and the reaction was stopped by adding 100 μ L of 10% H₂SO₄ and read at 492 nm using an ELISA reader (LP 400; Sanofi Diagnostique Pasteur, Marnes-la-Coquette, France). The sensitivity of the assay was 0.3 ng/mL. Levels of specific IgE were measured in ELISA plates coated (overnight at 4°C) with ENF peptide (50 μ g/mL in 0.1 M NaHCO₃ buffer pH 9.0). All the other steps remained the same as in the total IgE assay. IgE antibodies specific to most sensitizing allergens in the Southern European area were measured with a routine qualitative laboratory assay (Phadiatop) from Pharmacia (Uppsala, Sweden) which is commonly used in routine clinical practice as a preliminary screening for IgE-mediated allergies and provide a “yes” or “no” type of answer (15).

IgE Absorption on Solid-Phase ENF

Absorption of IgE on different antigens (ENF, grass allergen, Fc fragment of human IgG, tetanus toxoid [TT]) was performed as previously described (16, 17). The Fc fragment of human IgG was purchased from Southern Biotechnology (Birmingham, AL, USA), the grass allergen extract was a lyophilized commercial preparation of *Phleum pratense* (ALK Abellò Italy, courtesy of dr Silvia Pecora) with a content in group V allergen of 10 μ g/mL. Tetanus toxoid was obtained from Connaught Labs, Willowdale, Ontario, Canada. Antigen coating was performed overnight at 4°C at 5 μ g/mL for all antigens, except ENF [50 μ g/mL]). Total IgE were measured in sera before and after repeated, sequential passages on

solid-phase coated antigens. As negative control, absorption on 2% dry-milk (blocking buffer) coated wells was made. A serum from a patient with a single major sensitization to grass pollen, 1385 ng/mL total IgE and a high titer of grass-specific IgE (class IV according to the Pharmacia RAST classification) was also included as a control for the efficiency of absorption.

ENF-Responsive T-Cell Lines

Proliferation and cytokine profile of ENF-stimulated T-cell lines derived from normal healthy donors and from ENF-treated patients were established as follows. PBMC were cultured in 24-well plates at a cell concentration of 2×10^6 /mL in complete medium containing ENF peptide at 50 or 10 μ g/mL concentration. As controls, an irrelevant 21-mer peptide was used, whose sequence corresponded to amino acids 86–106 of human chemokine receptor 5 (18). At day 7, cells were re-stimulated with autologous, 2500-rad irradiated PBMC (which had been previously frozen) at a 1 to 1 ratio with effector cells in the presence of the antigen (ENF or the control peptide). At day 14, intracellular staining and proliferation assays were performed to compare the efficiency of this experimental system in the establishment of ENF and control peptide specific T-cell lines and their respective cytokine profiles.

Intracellular Staining

Cells were harvested and re-stimulated for 4 h with phorbol 12-myristate 13-acetate (PMA, 5 ng/mL) (Sigma) in the presence of a protein transport inhibitor (brefeldin A, 10 μ g/mL) (Sigma). Cells were then reacted with mAbs to membrane bound antigens (Leu3A to human CD4) then fixed and reacted with single cytokine specific monoclonals in permeabilizing buffer (PBS buffer containing 0.05% saponine, Sigma). The mAbs specific to IL-4 or IFN- γ were covalently linked to phycoerythrin and fluorescein isothiocyanate (FITC), respectively. All reagents for intracellular staining experiments were from Pharmingen, where not otherwise indicated. Readings were performed with a FACScan cytofluorimeter, equipped with Cellquest software (Becton Dickinson). Cells were preliminary gated for physical parameters in order to exclude cellular debris ("lymphogate"). The percent of CD4 cells positive for each given cytokine was determined as compared to control cell preparations, which were reacted with fluorochrome- and isotype-matched monoclonal antibodies of irrelevant specificity (Pharmingen).

Proliferation Assays

T lymphocytes were cultured with or without the antigen they were originally stimulated with, in the presence of autologous antigen presenting cells, for 2 days. Phytohemagglutinin (PHA, 5 μ g/mL) (Sigma) was included as a positive control. Tritiated thymidine (3H-TdR, Dupont, Cologno, Milano, Italy) was added in the last 18-h of culture. Cells were harvested with a FilterMate Universal Harvester (Perkin Elmer Life Sciences, Boston, Ma, USA) and 3H-TdR incorporation, expressed as counts per min, was measured with a beta-counter (Topcount NXT Microplate Scintillation and Luminescence Counter, Perkin Elmer Life Sciences).

Statistical Analysis

The Wilcoxon's matched-pairs signed-ranks test was used to compare the ratio of the percentage of IL-4 to IFN- γ positive CD4 T lymphocytes from healthy donors following *in vitro* stimulation with different stimuli *versus* the same ratio before stimulation (baseline). A *p* value < 0,05 was considered significant.

RESULTS

Switch Induction on B-Lymphocytes

Purified B-lymphocytes were used for an *in vitro* assay of IgE-switch induction. Lymphocytes were isolated from three allergic donors in order to optimize the yield of the experiment. *In vitro* addition of the ENF peptide at 10 or 50 μ g/mL did not increase the IgE production induced by simultaneous stimulation with monoclonal antibody to CD40 plus recombinant IL-4 or with either stimulus only. In Fig. 1 a representative experiment out of three is shown. The total IgE production following anti-CD40 plus IL-4 stimulation (positive control) was above 10 ng/mL in the three donors who were tested.

IgE Absorption on Solid-Phase ENF

No relevant reduction were observed in total IgE content following up to six passages of absorption on solid-phase ENF of sera from five ENF-treated patients who had total serum IgE levels ranging from 804 to 2011 ng/mL (Fig. 2, left panel). A limited reduction (within 20% of the original value) was observed, which did not differ neither in the extent nor in the passage-dependent pattern from that obtained with absorption on irrelevant antigens, such as tetanus toxoid, the Fc fragment of human IgG, or the blocking reagent (not shown). In contrast, grass pollen absorption dramatically reduced total IgE levels

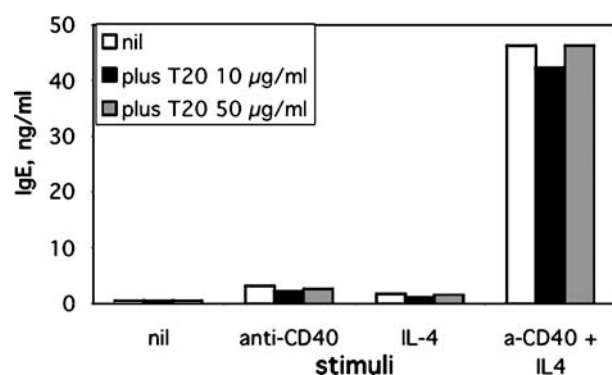


Fig. 1. *In vitro* induction of IgE secretion (in ng/mL, on the y-axis) by purified B-lymphocytes from one healthy donor following physiological stimuli (IL-4, antibody to CD40, or both, on the x-axis) in the presence or absence of the ENF peptide at the two different concentrations indicated in the legend. "Nil" indicates IgE production in the absence of any stimuli or upon stimulation with ENF only, at the two different concentrations indicated in the legend.

in the serum from the grass allergic patient (Fig. 2, right panel).

Th2 Polarization of CD4 Lymphocytes from ENF-Treated Patients

In vitro stimulation of PBMC from three ENF-treated patients whose blood was taken at baseline and after 24 weeks of ENF therapy was performed. Secondary T-cell lines were derived and the IL-4/IFN- γ cytokine profile was studied. An increase in the proportion of IL-4, but not IFN- γ positive CD4 T cells was observed in these three patients in the second as compared to the first blood sample. A representative experiment is shown in

Table I. Th2 Polarization of CD4 T Lymphocytes from Three ENF-Treated Patients

Patient	Time	IL-4 (%)	IFN- γ (%)	IgE (ng/mL)	Phadiatop readings	Phadiatop (A.U./L)
RR21	$T = 0$	0.10	0.10	21	2092	4.45
	$T = 24$ weeks	2.11	0.30	370	2275	4.85
PGP22	$T = 0$	0.15	0.10	31	28	<0.35
	$T = 24$ weeks	2.15	0.14	496	28	<0.35
LE04	$T = 0$	0.15	0.11	838	136	<0.35
	$T = 24$ weeks	0.95	0.12	2131	168	<0.35

Note. The percent of CD4 positive T cells stained with mAb to IL-4 and IFN- γ is indicated. These evaluations were made in three patients (identified in the first column from left) before the beginning of ENF therapy ($T = 0$) and 24 weeks later ($T = 24$ weeks). Total serum IgE (ng/mL) and the results of the Phadiatop evaluation of specific IgE to most common sensitizing allergens are also indicated. Phadiatop is a qualitative test, yielding either a positive or a negative result (cutoff: 0.35 AU/mL). The absolute values (readings) of the reading of each sample are also reported.

Fig. 3. Single results are listed in Table I. Total IgE levels and the results of an assay measuring IgE specific for most sensitizing allergens (Phadiatop) at baseline and after 24 weeks of ENF therapy are also included in Table I.

Th2 Polarization of CD4 Lymphocytes in Culture with ENF

In vitro stimulation of PBMC with the ENF peptide was also performed in six untreated, nonallergic control individuals and secondary T-cell lines were derived. A remarkable increase in the ratio of CD4 T lymphocytes secreting IL-4/IFN- γ was observed when comparing T cells

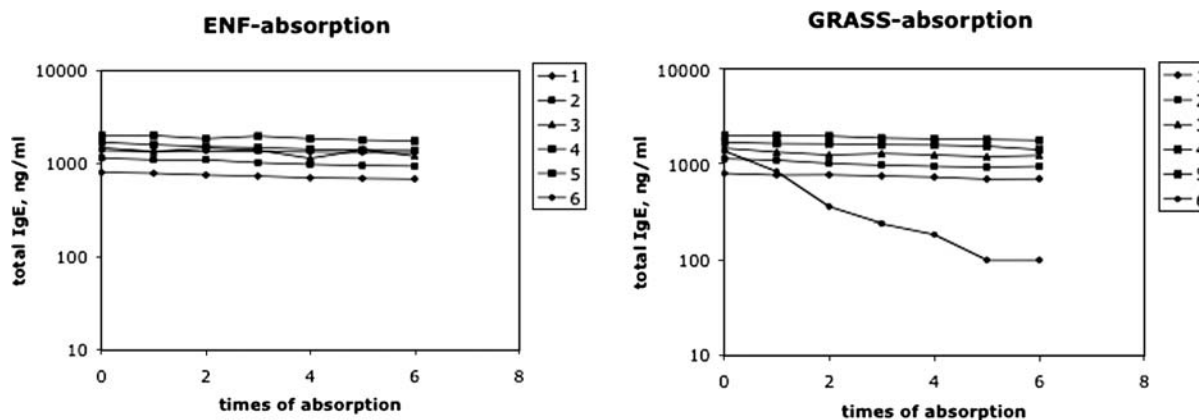


Fig. 2. Absorption of total IgE (ng/mL on the y-axis) following sequential passages (number on the x-axis) on plastic-bound ENF (left panel) or grass (right panel). Six sera were tested, of which five (number one through five, as indicated in the legend) were obtained from HIV-patients after 24-weeks of ENF-treatment and one (number six in the legend) from a grass-allergic individual.

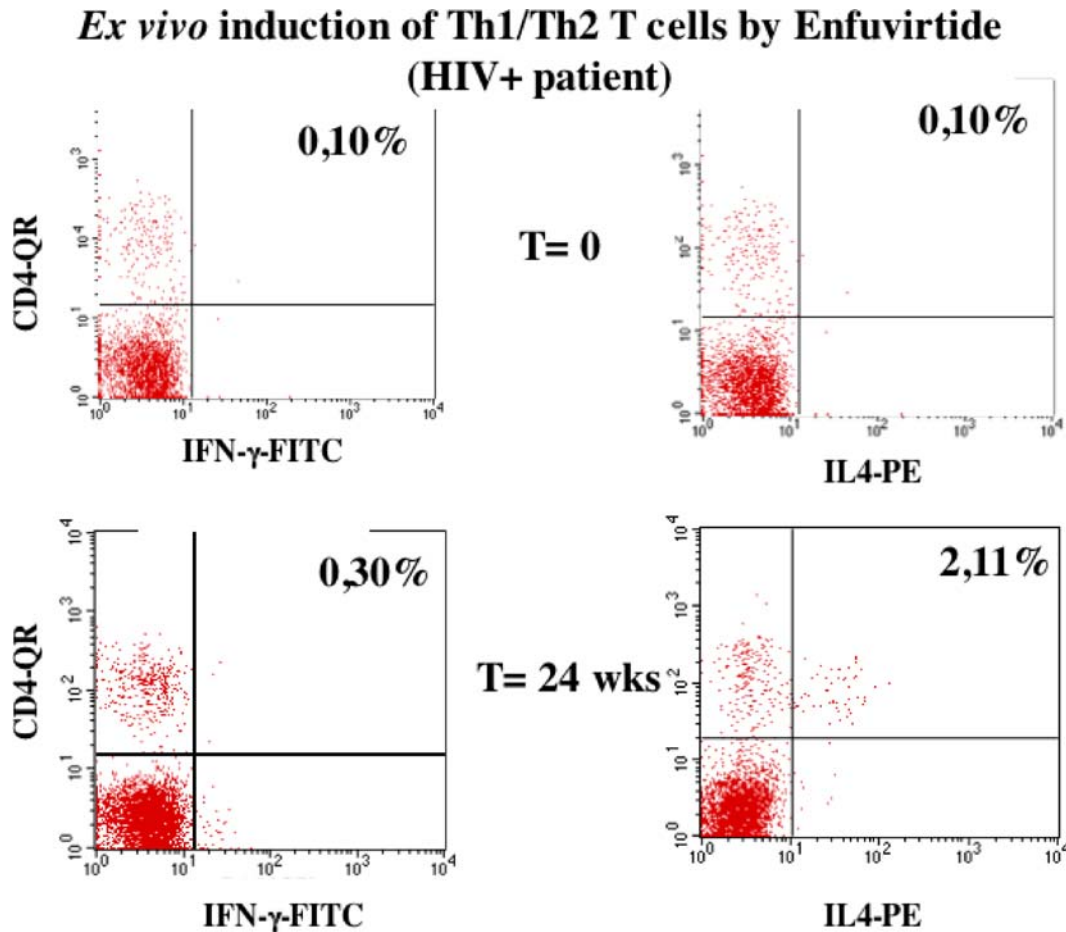


Fig. 3. Dot-plot cytofluorimetric analysis of IFN- γ (left panels) and IL-4 (right panels) expression by CD4 T lymphocytes from one ENF-treated patient stimulated *in vitro* with ENF. Cells were first gated for physical parameters in order to exclude cellular debris. The test was performed on samples taken before beginning ENF treatment (top panels) and 24 weeks later (lower panels), respectively. Samples were simultaneously stained with a quantum-red conjugated monoclonal antibody specific to the membrane CD4 (on the y-axis), with a fluorescein-conjugated monoclonal antibody to intracellular IFN- γ and with a phycoerythrin-conjugated monoclonal antibody to intracellular IL-4 (on the x-axis). Ten thousand events were acquired. The percentage of CD4 T cells expressing either IFN- γ or IL-4 is indicated in the top right quadrant of each panel and used as a measure of Th1/Th2 functional polarization.

at baseline with secondary T-cell lines. A representative reading of one ENF-T-cell line is pictured in Fig. 4. Both the absolute proportion of IL-4-producing T cells and the ratio of IL-4 to IFN- γ positive CD4 T cells were significantly augmented in ENF- as compared to control-peptide-stimulated lymphocytes ($p = 0.0312$ for both) (Table II). Notably, the proliferative response of secondary T-cell lines toward the ENF peptide was similar to that elicited by the control peptide (not shown), suggesting that the observed difference in cytokine polarization did not correlate with a different intrinsic immunogenicity of the compared peptides.

DISCUSSION

The main finding of the present study is that ENF treatment is inducing in HIV-1 positive patients a systemic immunological disequilibrium, which is not interfering with the efficient suppression of the viral load. This phenomenon is characterized by a production of increased IgE levels, is not dependent on a direct effect on B-lymphocytes but is likely driven by the induction of Th2 cells. The clinical consequences of this side effect are limited to poorly symptomatic reactions at the injection site, likely due to the fact that these IgE are not ENF-specific.

In vitro induction of Th1/Th2 T cells by Enfuvirtide (healthy donor)

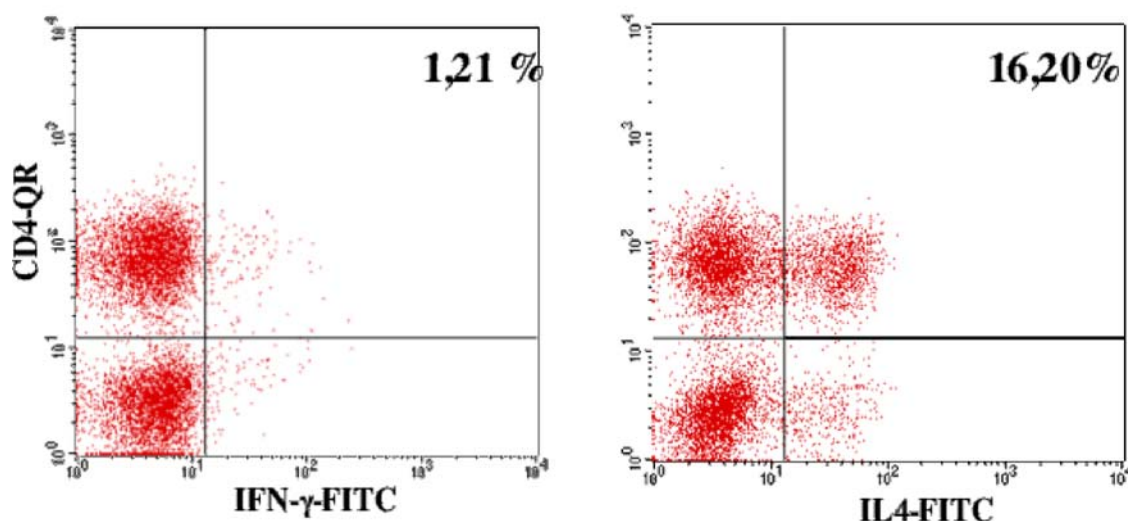


Fig. 4. Dot-plot cytofluorimetric analysis of IFN- γ (left panel) and IL-4 (right panel) expression by CD4 T lymphocytes from one healthy donor, stimulated *in vitro* with ENF. Cells were first gated for physical parameters in order to exclude cellular debris. Samples were simultaneously stained with a quantum-red conjugated monoclonal antibody specific to the membrane CD4 (on the y-axis), with a fluorescein-conjugated monoclonal antibody to intracellular IFN- γ and with a phycoerythrin-conjugated monoclonal antibody to intracellular IL-4 (on the x-axis). The percentage of CD4 T cells expressing either IFN- γ or IL-4 is indicated in the top right quadrant of each panel and used as a measure of Th1/Th2 functional polarization.

Class switching occurs through class switch DNA recombination and usually requires engagement of CD40 on B cells by CD40 ligand on Ag-activated CD4(+) T cells (19). We asked whether ENF could directly activate B lymphocytes by a T-cell independent mechanism, as was observed with some Epstein–Barr virus membrane proteins (20). We could induce *in vitro* IgE production by B lymphocytes by activating them *via* CD4 and IL-4

Table II. IL-4 and IFN- γ Production by CD4 T Cells

	IL-4	IFN- γ	IL-4/ IFN- γ ratio
Baseline	0.22 (0.20–0.47)	0.94 (0.60–1.18)	0.42 (0.28–0.44)
ENF	25.32 (7.89–38.93)*	5.18 (3.05–6.33)	4.16 (1.67–6.73)*
Control	2.50 (1.95–4.01) (ns)	5.27 (3.91–6.49) (ns)	0.48 (0.44–0.63) (ns)

Note. Median values (and interquartile ranges) of the percentages of IL-4 and IFN- γ CD4 T cells at baseline and following *in vitro* stimulation of PBMC from six healthy donors with ENF or with a control peptide, as indicated. The median values (and interquartile ranges) of the IL-4 to IFN- γ ratios for each experimental setting are also indicated. ns nonsignificantly different *versus* baseline.

* $p = 0.0312$ *versus* baseline.

(13, 14), but none of these stimuli could be substituted neither amplified by the exogenous addition of ENF. In principle, in the absence of T cells IgE switch could still be driven by membrane signals and cytokines of mast cells or basophil origin (21, 22), which could have been recruited by ENF. Although the model we used did not directly address this possibility, infiltration of these cell types was not observed at ENF injection site (11), a fact that is discouraging this hypothesis. Further studies are needed to explore this issue.

Notably, the accessibility from severely immunocompromized HIV patients of T cells, which were suitable for studies implying a measurable response to *in vitro* stimulation, was quite limited. Indeed, due to the initially low CD4 T cell counts, in most cases we obtained viable cell lines only from samples collected after 24 weeks of T20 treatment, which did not allow comparison with the pretreatment situation. This problem narrowed the sample we analyzed down to three patients, which brings to consider these data as anecdotal evidence. However, in these three patients an increase in IL-4 production by CD4 T-lymphocytes was observed. Moreover, *in vitro* addition of ENF was capable to induce a statistically significant, selective increase of IL-4 but not IFN- γ , secretion by T cell

lines in a group of healthy donors. Taken together these *ex vivo* and *in vitro* data support the notion that ENF has an intrinsic property to induce, either directly or *via* antigen presenting cells (which were included in the experimental setting we used) a Th2 phenotype of naive T cells, which may in turn drive B cells to switch to IgE production.

Since the allergic status of patients included in the present study may have potentially affected the results we observed, the presence of IgE to major sensitizing allergens in the area where patients were living was assessed. In the entire cohort, the proportion of ENF-treated patients who had an increase in total IgE was similar in individuals with and without specific sensitizations (not shown). In particular, among the three ENF-treated patients who had a measurable increase in Th2 ENF-specific T cells, two were negative and one positive for allergen-specific IgE. Notably, no change of allergen-specific IgE levels was associated with the dramatic increase in total IgE correlated with ENF treatment. These results are in agreement with the observation that, in this same cohort, total IgE levels increased during ENF treatment independently on baseline IgE levels (12).

The polyclonality of serum IgE induced by ENF treatment was not directly assessed. We performed a standard agar gel electrophoresis, but we could not compare the serum pattern with that of a human monoclonal IgE, likely due to the insufficient sensitivity of the assay (not shown). However, solid-phase ENF could not adsorb IgE from sera of ENF-treated patients. Moreover, also the absence of systemic reactions in patients with increased IgE levels during subcutaneous ENF therapy and the negative results of specific immunosorbent assays for the detection of reagenic antibodies to ENF (not shown) suggest that the IgE hyper-production was polyclonal.

The ENF peptide, which corresponds to the carboxy-terminal ectodomain sequence of gp41 of the HIV-1 LAI strain, was found to bind to the low-affinity formyl-peptide receptor, to induce calcium influx, and to selectively suppress IL-12 production from human monocytes (23–25). Production of IL-12 by dendritic cells was instead unaffected despite functional expression of the formyl-peptide receptor (23). In our experimental system with T-cell lines, monocytes were maintained in order to provide the antigen presenting cells necessary to support the selective amplification of ENF-specific T cells. In principle, these monocytes were therefore in culture conditions compatible with driving T-cells toward the Th2-phenotype through the above-described mechanism. Thus, ENF treatment may result in the suppression of IL-12 production, which could have immunologic consequences. This may be particularly important because the concentrations of ENF required to suppress IL-12 production *in vitro* are at or

below the levels of ENF required in humans to suppress viral burdens *in vivo* (26).

According to a popular model, HIV progression could be associated with a switch from a Th1 to a Th2 phenotype (27, 28), based on the preferential replication of the virus in Th2 (and Th0) cells (29). Indeed, although the elevation of IgE can undoubtedly correlate with disease progression (30, 31) and IL-12 was found to restore *in vitro* anti-HIV-immunity (32), data were also reported on the HIV-dependent variation of cytokine production at the cellular levels, which were in contrast to the switch theory (33). Moreover, although the induction of a Th2 immune skewing by ENF could impair the efficacy of the anti-HIV immune response, it must be pointed out that the above reported observations date back to the pre-HAART era. In the clinical setting a more complex scenario can currently be envisaged from the balance between the effect of the highly counteracted HIV-replication on the viral side and the restoration of an efficacious antiviral immunity on the host side. In this perspective, it will be important to include the drug-induced total IgE increase as a prognostic variable in the long-term follow-up of patients who are treated with ENF.

Thus, in summary, the critical issue about ENF immunological and antiretroviral activity is the net balance between the putative IL-12 inhibiting action of ENF and the inhibition of the formation of the gp41 hairpin structures which mediate viral-cell membrane fusion (25, 34–36).

CONCLUSIONS

Our results support the notion that ENF is bringing clinical benefits, which by far overcome the associated immunological dysregulation (6, 7, 12). However, these data also suggest that immunological alterations associated with ENF treatment need to be carefully monitored over time, since growing numbers of patients are only now entering long-term treatment and increasingly larger numbers of individuals are treated, including patients with not extremely depressed CD4 counts.

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