Clinical and immunological effects of a 6 week immunotherapy cycle with murabutide in HIV-1 patients with unsuccessful long-term antiretroviral treatment

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In an effort to evaluate the potential of non-specific immunotherapy in restoring global immunity, we have examined the clinical tolerance and biological effects of a 6 week administration of the immunomodulator, murabutide, in chronically infected HIV-1 patients. Forty-two subjects, presenting weak immune reconstitution and ineffective virus suppression following long-term highly active antiretroviral therapy (HAART), were randomized to receive, or not, murabutide 7 mg/day on five consecutive days/week. Clinical and immunological parameters were monitored before and after the immunotherapy period. Administration of murabutide was generally well tolerated, although some grade III adverse events, reversible on treatment cessation, were observed. Interestingly, in comparison with pre-inclusion levels, at 1 week after the immunotherapy cycle, only murabutide recipients presented a significant increase in CD4 cells, platelet counts, and in the percentage of patients with undetectable viral loads (<50 copies/mL). Statistical significance between the two groups was only evident with the latter parameter. Some of these clinical changes were maintained even up to 12 weeks after murabutide administration, and were accompanied by an increased ability to mount cellular responses to active immunization with a recall antigen, and by a significant increase in the percentage of patients presenting positive lymphoproliferative responses to the viral antigen gp160. These results warrant further evaluation of extended periods or cycles of murabutide immunotherapy as adjunct to HAART.

Keywords: HIV, immunotherapy, murabutide, lymphoproliferation, chemokines

Introduction

Although highly active antiretroviral therapy (HAART) can provide sustained control of HIV-1 replication, it allows for only partial reconstitution of the immune system.1,2 In addition, in patients successfully treated with HAART, abnormalities of lymphoid tissue and ongoing viral replication persist.3,4 These findings, together with the reported limitations in the use of HAART including drug-associated toxicities and development of drug-resistant strains,5–7 necessitate additional therapeutic strategies to restore global immunity and long-lasting virus control. Besides the battery of new drugs currently under development and efforts to target virus entry or integration, immunotherapy has been heavily pursued in the management of HIV disease. Different forms of immunotherapy have been proposed, including cytokines, growth factors and virus-specific therapeutic vaccines (reviewed in references 8 and 9). Most of these approaches have been aimed at correcting defective elements of adaptive immunity and in recovering virus-specific responses. However, HIV-1 infection also has a dramatic impact on innate immunity. This is exemplified by the ability
of the virus to infect, persist and to cause functional defects in natural killer (NK) cells, in monocytes/macrophages and in dendritic cells.\textsuperscript{10-13} The use of HAART has been reported to improve limited functions of certain subpopulations of NK cells and antigen-presenting cells (APCs); however, profound impairment in the numbers and function of other subpopulations was found to persist, despite long-term control of viral replication.\textsuperscript{9,10,14-17} Moreover, cells of the innate immune system act as HAART-resistant virus reservoirs, contribute to virus dissemination and are believed to be the origin of defective HIV-specific lymphocyte responses in infected subjects.\textsuperscript{10-12,18} Taken together, these findings strongly argue for the necessity to correct innate immune dysfunctions, using non-specific immunotherapeutic approaches, in order to restore global immunity and more efficacious long-term control of HIV-1.

Murabutide is a synthetic immunomodulator capable of regulating the function of innate immune cells and of enhancing the host’s non-specific resistance against bacterial and viral infections.\textsuperscript{19-21} This synthetic glycopeptide is also reported to synergize with therapeutic cytokines in driving T helper 1 responses and in potentiating cytokine-induced antiviral and antitumour immunity.\textsuperscript{22-24} In contrast to many other exogenous immunomodulators, murabutide is apyrogenic, anti-inflammatory and well tolerated in humans.\textsuperscript{19,23,25-27} In addition, murabutide was found to activate HIV-1-infected APCs and CD4 cells, rendering them non-permissive to viral replication.\textsuperscript{28,29} However, the HIV-suppressive activity of murabutide is not linked to a direct effect on the virus, and correlates with regulated expression of cellular factors necessary for the completion of the virus life cycle. Recently, the clinical tolerance of single and five repeated administrations of murabutide in HIV-1 patients on HAART has been demonstrated.\textsuperscript{30} These studies also revealed the capacity of the immunomodulator to induce surrogate markers of antiviral immunity, including β-chemokine release and improved lymphoproliferative responses (LPRs) to viral antigens.

In an effort to evaluate the benefit of non-specific immunotherapy in HIV disease, we have conducted a randomized Phase I/II study in 42 HIV-1 patients presenting weak immune reconstitution and incomplete virus suppression after a mean duration of over 2 years on HAART. In this study, called Restimur, we have addressed the safety of a 6 week cycle of immunotherapy with murabutide, and monitored the changes in viral and immune parameters up to 12 weeks after the immunotherapy period. We have also analysed the ability of patients, from the control and the immunotherapy groups, to mount antibody and cell-mediated responses following active immunization with tetanus toxoid vaccine. The results obtained provide support for the use of non-specific immunotherapy, as adjunct to HAART, within the strategies aimed at restoring global immunity and long-term virus control in HIV disease.

### Patients and methods

The clinical study was approved by the local ethics committee in Lille, France, and complied with the Hong Kong amendment of the declaration of Helsinki (1989). All patients received detailed information on the tested drug and on the protocol, and signed a written consent form prior to participating in the study. Patients were recruited at the AIDS reference centre of Northern France, located in Tourcoing Hospital. Following acceptance in this study, patients were randomly assigned to enter either the immunotherapy or the control arm. This latter group did not receive placebo, but was followed identically to the immunotherapy group.

#### Study design and subject description

The trial was a randomized, comparative, open-labelled, 22 week Phase I/II pilot study in chronically infected HIV-1 patients. A sample-size calculation was performed, on the assumption that a 35% difference between the two groups could be achieved in the number of patients attaining ≥30% increase in CD4 counts, after the immunotherapy period. A unilateral test of significance, with 5% risk level and 80% power level, indicated the need to enrol 22 patients per group. During the study, a total of 42 patients were included and randomization led to the assignment of 22 patients to the control and 20 patients to the immunotherapy arm. Subjects were required to be older than 18 years, to have received stable HAART for >6 months, to have presented plasma viral loads (pVL) <5000 copies/mL on two successive, recent tests and to have demonstrated incomplete immune restoration. This was defined either by a <50% HAART-induced rise in CD4 cells, and with the counts remaining <500/mm\(^3\), or by CD4 counts <350/mm\(^3\) following at least 1 year of stable HAART. Exclusion criteria were pregnancy, acute infection at the time of inclusion, anti-inflammatory or antipyretic therapy in the days before inclusion and immunotherapy or vaccination either before or during the study period. After a pre-inclusion period of 2–4 weeks, patients received, or not, immunotherapy that consisted of a single subcutaneous administration of murabutide 7 mg/day, on five consecutive days/week and for a period of 6 weeks. For the first injection, patients were admitted to hospital for a period of 24 h. Subsequent injections were performed at the patient’s residence by a registered nurse. The baseline characteristics of patients were identical between the two groups (Table 1) and no significant differences could be noted in any of the measured parameters. However, it should be noted that the mean CD4 cell counts of the control group, either before any treatment or prior to initiation of HAART, were <200 cells/mm\(^3\), slightly lower than that in the immunotherapy group. This difference, which was not of statistical significance, may have been an effect of...
randomization. All patients continued to receive HAART throughout the study, and the treatment consisted of either two reverse transcriptase inhibitors and one protease inhibitor (17 patients in the control and 16 patients in the immunotherapy group), or two reverse transcriptase and two protease inhibitors.

**Treatment with murabutide**

Lyophilized murabutide (N-acetyl muramyl-L-alanyl-D-glutamine-O-n-butylerster; batch VA-001-06) dosed at 10 mg per vial was provided by ISTAC Biotechnology (Lille, France) and prepared as described elsewhere. Murabutide was solubilized extemporaneously in apyrogenic saline and administered by subcutaneous route in the deltoid area.

**Clinical and biological evaluations**

All patients were subjected to clinical and biological surveys 2–4 weeks before inclusion and 1, 6 and 12 weeks after the end of the immunotherapy cycle. The group randomized to receive murabutide was also requested to visit the hospital, 2 weeks after the start of treatment, for an additional clinical evaluation. Adverse events (AEs) were evaluated by the investigator and by the subject himself, and graded according to the common toxicity criteria, as previously described. Routine clinical and laboratory examinations, as well as pVL, were performed on each visit, as reported elsewhere. All pVL determinations were performed in the same laboratory (Lille University Hospital) using the Amplicor Monitor HIV-1 kit (Hoffman-La Roche, Basel, Switzerland), which has a sensitivity of detection of 50 copies/mL. For calculations of means and medians, all samples with pVL below the detection limit were assigned arbitrarily a value of 49 copies/mL.

**Flow cytometry analysis**

Blood samples (100 μL) collected on EDTA were drawn at each visit and incubated at room temperature for 20 min with 20 μL of fluorochrome-labelled monoclonal antibodies, directed against human leucocyte surface antigens, or their matched isotype controls. The following monoclonal antibodies were used: CD3-fluorescein isothiocyanate (FITC), CD4-phycoerythrin-cyanin 5.1 (RPE-Cy5), CD4-PE, CD14-FTTC, CD86-PE and HLA-DR-PE (Immunotech, Marseille, France); CCR5-PE and CXCR4-PE (Pharmingen, Rungis, France); the Lymphocyte Immuno Kit (Becton Dickinson, Rungis, France) to detect, by three-colour fluorescence on CD4 and CD8 cells, the co-expression of the activation markers CD38 and CD28, and the naïve T-cell markers CD62L and CD45RA. The stained samples were then treated at room temperature for 10 min with 2 mL of 1× FACS Lysing Solution (Becton Dickinson, Le Pont de Claix, France) to eliminate erythrocytes. The white blood cells were collected after a short centrifugation step, washed with PBS containing 5% BSA (Sigma, Saint Quentin Fallavier, France), and fixed with 1% paraformaldehyde in PBS before analysis on a FACSCalibur flow cytometer using the program CellQuest (Becton Dickinson). Using forward- and side-scatter characteristics, lymphocyte and monocyte gates were initially defined. Analysis within the monocyte population was carried out on CD14 cells, whereas lymphocytes were identified by the expression of either CD3/CD4 or CD3/CD8 surface antigens.

**Detection of phytohaemagglutinin (PHA)-induced cytokines**

Blood samples collected on heparin were obtained from patients at different time periods and peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Les Ulis, France). For the detection of PHA-induced cytokines, PBMCs were seeded at 1 × 10^6 cells per well of a 24-well plate and cultured in RPMI 1 mL (Life Technologies, Courbevoie, France) containing 10% fetal calf serum, at 37°C in a 5% CO₂ in atmosphere. Cultures were either left unstimulated or stimulated with PHA 5 μg/mL for 48 h. Supernatants were then collected and frozen at −20°C until tested. Duplicate wells were set up for each culture condition, and pooled supernatants from two wells were titrated for the

### Table 1. Baseline characteristics of patients included in the control and immunotherapy groups

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>Control group (n = 22)</th>
<th>Immunotherapy group (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode of infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>homosexual (%)</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>heterosexual (%)</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>other (%)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>21:1</td>
<td>16:4</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44 ± 9</td>
<td>43 ± 11</td>
</tr>
<tr>
<td>CDC category (number of patients; A/B/C)</td>
<td>3/12/7</td>
<td>5/10/5</td>
</tr>
<tr>
<td>Duration (months) of HIV seropositivity</td>
<td>84 ± 48</td>
<td>84 ± 36</td>
</tr>
<tr>
<td>previous ARV therapy</td>
<td>52 ± 29</td>
<td>35 ± 24</td>
</tr>
<tr>
<td>HAART</td>
<td>39 ± 10</td>
<td>26 ± 11</td>
</tr>
<tr>
<td>CD4 lymphocyte counts/mm³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>before any treatment</td>
<td>158 ± 11</td>
<td>214 ± 160</td>
</tr>
<tr>
<td>before HAART</td>
<td>181 ± 141</td>
<td>221 ± 157</td>
</tr>
</tbody>
</table>

CDC, USA Centers for Disease Control and Prevention; ARV, antiretroviral; HAART, highly active antiretroviral therapy.

Where relevant, numbers given are mean ± S.D.
levels of interleukin (IL)-2, IL-10, IL-13, interferon (IFN)-γ and regulated on activation, normal T cells expressed and secreted (RANTES), using commercially available ELISA kits (R&D Systems, Abingdon, UK).

Detection of serum cytokine levels
Serum samples were obtained prior to murabutide administration and at two time points after immunotherapy (weeks 1 and 12). Levels of IFN-γ, granulocyte-colony-stimulating factor (G-CSF), macrophage (M)-CSF, IL-1 receptor antagonist, IL-7, macrophage inflammatory protein (MIP)-1β and RANTES were determined using ELISA kits, following the manufacturer’s instructions (R&D Systems).

Lymphoproliferative assays
LPRs were evaluated according to a previously published standard protocol. The following antigens were used: soluble extracts derived from ultrasonically disrupted *Mycobacterium tuberculosis* (tuberculin, 5 µg/mL) kindly provided by J. L. Stanford, University College London Medical School, soluble extract from *Candida albicans* (20 µg/mL, Greer Laboratories, Lenoir, NC, USA), cytomegalovirus antigen (1:20 dilution, BioWhittaker, Walkersville, MD, USA), and recombinant HIV-1 p24, gp160 (Protein Sciences Corp., Meriden, CT, USA) and Nef proteins tested at 3, 5 and 5 µg/mL, respectively. The proliferative response to each antigen was assessed in triplicate and the stimulation index (SI) was calculated as the mean cpm for cells with antigen divided by the mean cpm for cells without antigen. To analyse the percentage of responders to a given antigen, patients presenting an SI >5 were considered as responders.

Responses to vaccination with tetanus toxoid
At the period corresponding to 1 week after the end of murabutide administration, 16 out of 20 and 18 out of 22 patients from the immunotherapy and control groups, respectively, received one shot of tetanus toxoid vaccine 40 IU/0.5 mL (Pasteur Vaccins, Marne-la-Coquette, France). All patients verified that they had not received a tetanus vaccination for at least 3 years. Antibody levels and LPRs to tetanus antigen were evaluated at two time points before (pre-inclusion and 1 week after murabutide immunotherapy) and at two time points after tetanus vaccination (corresponding to weeks 6 and 12 following the end of murabutide immunotherapy). Titration of IgG antitoxin antibodies was performed at the Laboratoire de Biologie Spécialisée, Institut Pasteur, Lille, France, using a commercially available tetanus ELISA diagnostic kit (Genzyme Virotech, Rüsselsheim, Germany) and LPRs were evaluated, following stimulation of PBMCs, with tetanus toxoid antigen 10 µg/mL (Statens Serum Institute, Copenhagen, Denmark).

Statistical analysis
To analyse the evolution of parameters within each group over time, the data points obtained at different testing periods were compared using covariance analysis on repeated measure events (Friedman block treatment test). Significance between paired values was evaluated by the non-parametric Wilcoxon matched-pairs test. Comparison of data between the two groups at one specific time point was carried out using the Mann–Whitney U-test or Fisher’s exact test. P values of <0.05 were considered statistically significant.

Results
Clinical and biological tolerance
Forty-two patients were enrolled in the trial between November 1999 and June 2000, and all completed the study. In the immunotherapy group, 16 patients (80%) were administered the full 6 week course of murabutide and four of them (20%) stopped treatment because of serious AEs, after 2 (one patient), 4 (two patients) or 5 (one patient) weeks of therapy. The clinical tolerance was judged as satisfactory, with 15% of the patients presenting no AEs and no patient experiencing grade IV toxicity. Eight patients (40%) presented mild grade I AEs, similar in nature to the previously described flu-like syndrome. More serious AEs, of grades II and III, were documented in five and four patients, respectively, and are presented in Table 2. All the observed AEs reversed spontaneously within a few days of treatment cessation, except for grade II pericarditis, with mild effusion, which necessitated the administration of colchicine for 6 weeks. Echocardiography revealed that the effusion had disappeared following 4 weeks of colchicine treatment. In addition, the thoracic oppression reported by some patients could not be linked to a cardiopulmonary aetiology, as determined by radiography, ECG and echocardiography. However, the biological tolerance was highly acceptable and 11 AEs were recorded (nine of grade I, one of grade II and one of grade III), with only two of them judged as possibly related to murabutide administration. These two AEs, an elevation in serum amylases (grade II) or lipases (grade III), detected in two patients, returned to baseline values at the end of immunotherapy. Moreover, 25% of the patients reported a feeling of improved wellbeing similar to that described by patients receiving 1 week’s murabutide immunotherapy.30

Cell counts and pVL
Before, and at different time periods after immunotherapy with murabutide, total and differential blood cell counts revealed no significant changes, either over time or between the two groups of patients, in red blood cells, total leucocytes,
lymphocytes, granulocytes or monocytes. However, by intra-group analysis, significant variations were observed in the number of CD4, but not CD8 lymphocytes, and in platelet counts among patients receiving immunotherapy (Table 3). The mean (±S.E.M.) ∆ change in CD4 cell counts at 1, 6 and 12 weeks following immunotherapy was, respectively, 37 ± 17, 47 ± 14 and 62 ± 22 cells/mm³. The corresponding values in the control group were –7 ± 18, 15 ± 17 and 10 ± 20. A significant difference in the ∆ change in CD4 cell counts was observed between the two groups in the first week following immunotherapy (P < 0.05, Mann–Whitney U-test). Similarly, analysis of the ratio of CD4/CD8 cells revealed a significant increase in the longitudinal intra-group analysis among the immunotherapy arm (P < 0.05, Friedman block treatment test) with no significant change (P > 0.05) detectable in the control group. In addition, comparison of the CD4/CD8 ratio between the two groups revealed a significantly higher ratio in murabutide recipients observed at 1 and 6 weeks after treatment (P < 0.05, Mann–Whitney U-test), which was not linked to a decrease in CD8 cells. Furthermore, analysis of the changes in platelet counts also revealed significant variations in the immunotherapy group, but not in control patients, and this was attributed to the significant increase observed 1 week after the end of immunotherapy (P < 0.05 versus pre-treatment values, Wilcoxon matched-pairs test). A similar and significant increase in platelet counts was also

Table 2. AEs observed in the immunotherapy group during the 6-week period of murabutide administration

<table>
<thead>
<tr>
<th>AEs</th>
<th>Number of AEs observed (grade II/III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myalgia/arthritis</td>
<td>7/4</td>
</tr>
<tr>
<td>Thoracic oppression</td>
<td>3/1</td>
</tr>
<tr>
<td>Asthenia</td>
<td>2/1</td>
</tr>
<tr>
<td>Anorexia</td>
<td>2/0</td>
</tr>
<tr>
<td>Headache</td>
<td>2/0</td>
</tr>
<tr>
<td>Lumbalgia</td>
<td>2/0</td>
</tr>
<tr>
<td>Decreased muscle strength</td>
<td>0/1</td>
</tr>
<tr>
<td>Dyspnoea at effort</td>
<td>0/1</td>
</tr>
<tr>
<td>Fever</td>
<td>1/0</td>
</tr>
<tr>
<td>Insomnia</td>
<td>1/0</td>
</tr>
<tr>
<td>Intercostal pain</td>
<td>1/0</td>
</tr>
<tr>
<td>Pericarditis with mild effusion</td>
<td>1/0</td>
</tr>
<tr>
<td>Pain at injection site</td>
<td>1/0</td>
</tr>
<tr>
<td>Irritability</td>
<td>1/0</td>
</tr>
<tr>
<td>Depression</td>
<td>1/0</td>
</tr>
<tr>
<td>Total</td>
<td>25/8</td>
</tr>
</tbody>
</table>

*AEs judged as probably or possibly caused by murabutide therapy are reported.

Among the 20 patients tested, five presented with grade II and four with grade III AEs.

Table 3. Cell counts and pVL evaluated before and after a 6-week period of immunotherapy with murabutide

<table>
<thead>
<tr>
<th>Period tested relative to immunotherapy</th>
<th>Control group (n=22)</th>
<th>Immunotherapy group (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral loads (copies/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 counts (cells/mm³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8 counts (cells/mm³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet counts (×10³/mm³)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Values expressed as mean ± S.E.M.
- Median values.
- Statistically significant differences from baseline values before immunotherapy (P < 0.05, by Wilcoxon matched-pairs test).

**AEs**

- **Myalgia/arthritis**
- **Thoracic oppression**
- **Asthenia**
- **Anorexia**
- **Headache**
- **Lumbalgia**
- **Decreased muscle strength**
- **Dyspnoea at effort**
- **Fever**
- **Insomnia**
- **Intercostal pain**
- **Pericarditis with mild effusion**
- **Pain at injection site**
- **Irritability**
- **Depression**

**Total**

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<tr>
<td>Total</td>
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</table>
detectable in the immunotherapy group, 2 weeks from the start of treatment (mean ± s.e.m., 237 ± 16 × 10^8 cells/mm^3). This suggested that the murabutide-induced increase in platelet counts was restricted to the period of administration and, unlike the effect on CD4 cells, was not long lasting. On the other hand, inter-group analysis of pVL revealed no significant differences at any time point tested. However, intra-group analysis of the changes with time revealed a trend for lower pVL only in the immunotherapy group (Table 3), and which almost attained statistical significance (P = 0.05). This effect was evident by the drop in median pVL to below the detection limit at all time points after immunotherapy. Furthermore, the percentage of patients with undetectable pVL in the control and immunotherapy groups were, respectively, 4.5 and 10 at pre-inclusion (P = 0.4634 by Fisher’s exact test), 27 and 65 (P = 0.0155) at week 1, 29 and 55 (P = 0.1085) at week 6 and 36 and 60 (P = 0.1106) at week 12 after the immunotherapy period. This indicated a trend for higher control of pVL in patients receiving the immunomodulator over a period of 6 weeks.

Changes in cell surface receptor expression

A comprehensive analysis of the cell-surface expression, on CD4 and CD8 lymphocytes, of virus co-receptors, activation markers (CD38/HLA-DR), apoptosis-related and co-stimulation molecules (CD95/CD28), and naive T-cell markers (CD62L/CD45 RA) did not reveal any inter- or intra-group differences at any time point tested (data not shown). The analysis was performed both on the percentages of positive cells, as well as on the mean fluorescence intensity. Similarly, no detectable changes in the level of expression of HLA-DR, CD86, CCR5 or CXCR4 could be noted in the CD14+ monocyte population (data not shown). These findings suggest that, in the patient populations tested, immunotherapy with murabutide does not induce significant phenotypical changes that are detectable after the treatment period.

PHA-induced cytokines

We next evaluated the capacity of PBMCs to release cytokines following stimulation with PHA, at the following three time points: pre-inclusion, and 1 week and 12 weeks after immunotherapy. The net induced levels of IL-2, IL-10, IL-13 and IFN-γ did not significantly vary with time, with treatment, or between groups. Similarly, when the Δ change in the induced levels was calculated for the period corresponding to week 1 and week 12 after immunotherapy, no significant differences could be observed between the two groups, although a trend for higher release of IL-2, IL-13 and IFN-γ was noted in the immunotherapy group (data not shown). In contrast, the PHA-induced levels of RANTES (in pg/mL) were found to be significantly higher (P < 0.05 by Wilcoxon matched-pairs test) among murabutide recipients, both at week 1 (3937 ± 521) and week 12 (4216 ± 581) after immunotherapy, in comparison with the observed levels at pre-inclusion (3373 ± 519). No such increase from baseline levels (3970 ± 510) could be noted among control patients tested at identical time points. Moreover, the Δ change in the induced RANTES levels, after 1 (631 ± 328) and 12 (844 ± 448) weeks of the immunotherapy cycle with murabutide, were significantly higher (P < 0.05 by Mann–Whitney U-test) than the corresponding values of the control group (–414 ± 345 at week 1 and –317 ± 922 at week 12).

Serum cytokine levels

A panel of seven serum cytokines and chemokines were analysed before and at two time points after immunotherapy with murabutide. Levels of IFN-γ and G-CSF were not detectable in sera from any patient at any time point tested. However, although serum IL-1 receptor antagonist, IL-7, M-CSF and MIP-1β were detectable before and after immunotherapy, no significant changes were observed either with time, with treatment, or between the two groups (data not shown). Interestingly, serum RANTES levels before the immunotherapy period were found to be significantly higher (P < 0.05) in control patients than the immunotherapy group (Figure 1a), a difference that may well be an outcome of randomization. However, the evolution of RANTES levels with time in the control group was that of a continuous drop, attaining statistical significance (P < 0.05, Wilcoxon matched-pairs test) at the period corresponding to 12 weeks after immunotherapy. In contrast, serum RANTES levels tended to increase in the immunotherapy group, at the two time points tested after murabutide administration, although this increase did not attain statistical significance (Figure 1a). Nevertheless, analysis of the Δ change in serum RANTES levels at week 1 and week 12 after the immunotherapy period revealed a significant difference between the two groups (P < 0.05, Mann–Whitney U-test) at the latter time point (Figure 1b).

LPRs

The lymphoproliferative assays, performed against three different recall antigens and at four different time points, showed no significant changes with time, with treatment, or between the two groups of patients (data not shown). This was the case whether the size of responses (SI) or the percentage of responders (SI > 5) against each antigen were evaluated. For instance, the percentage of responders to tuberculin, at the different time points examined, ranged between 32 and 36% in controls and 35 and 55% in the immunotherapy group, whereas the percentage of responders to Candida ranged between 77 and 100% and between 65 and 95% in the control and immunotherapy groups, respectively. Evaluation of the LPR to HIV-1 antigens, p24 and gp160, also revealed no
significant modification in the size of the responses. The mean SI (± S.E.M.) against p24 antigen for the control and immunotherapy groups, respectively, were 6 ± 2 and 7 ± 2 at pre-inclusion, and 4 ± 1 and 11 ± 5 at the end of the study. Similarly, the size of the responses to gp160 at the end of the observation period was 2 ± 1 for controls and 5 ± 2 for the group receiving immunotherapy. Interestingly, a trend towards higher responses to viral antigens was noted in the immunotherapy group, but the changes did not attain statistical values. On the other hand, whereas the percentage of responders to p24 did not vary significantly within or between groups, the percentage of responders to gp160 significantly increased with time among patients receiving immunotherapy (Figure 2). At pre-inclusion, none of the patients allocated to the immunotherapy arm responded to gp160, whereas 30% of these patients showed positive responses (mean SI > 5) after 12 weeks of immunotherapy. In contrast, the level of responders among control patients remained identical (9%) at all time points tested (Figure 2).

**Responses to vaccination with tetanus toxoid**

Finally, we evaluated the capacity of patients, who did or did not receive immunotherapy with murabutide, to respond to active immunization with tetanus vaccine. Thus, at the period corresponding to 1 week after murabutide administration, 18 and 16 patients, respectively, from the control and immunotherapy groups, were immunized with tetanus vaccine. The IgG antitoxin antibody titres, evaluated 5 and 11 weeks after vaccination, were found to be significantly higher in both groups of patients, as compared with the titres detected prior to vaccination (Figure 3). However, antibody responses to the vaccine were similar and not significantly different between the control and the immunotherapy groups. On the other hand,
the LPRs to tetanus antigen, evaluated either as SI or as percentage of responders, did not change significantly after vaccination among patients in the control group (Figure 3). In contrast, patients receiving murabutide immunotherapy presented improved proliferative responses to tetanus antigen, and the size of the responses at 12 weeks post-vaccination was significantly higher than that observed in the control group ($P < 0.05$, Mann–Whitney $U$-test). Moreover, the percentage of responders (SI > 5) in the immunotherapy group was found to increase significantly, at the two time points evaluated after vaccination, in comparison with the observed values at pre-inclusion ($P < 0.05$, Fisher’s exact test).

It is of interest that patients receiving the immunotherapy cycle represented a moderate, but not significant, increase in the percentage of responders to tetanus antigen, when examined just prior to tetanus vaccination at week 1 after the immunotherapy period (Figure 3). This could be explained by a limited improvement in the ability to respond to certain recall antigens following murabutide administration.

Discussion

Following long-term HAART, immunological and virological responses seem to vary among HIV-1-infected subjects, and in nearly 20% of patients CD4 cells do not recover despite successful virus suppression. This lack of recovery of CD4 cells has been attributed, at least in part, to lower thymic function. Furthermore, in patients presenting favourable immune reconstitution, immunological measures generally do not normalize to levels seen in persons without HIV infection. Thus, several strategies have been developed to enhance immune reconstitution in HIV-infected subjects and, more specifically, to restore HIV-specific immunity. However, the success of immunotherapy will depend on whether APC numbers and function are also restored. In this context, granulocyte–macrophage (GM)-CSF has been used to stimulate the function of APCs and to improve host defences against opportunistic infections. Although some contradictory data on the benefit of GM-CSF administration to HIV patients have been reported, it is evident that GM-CSF given over a 6 month period may result in increased CD4 cell counts and decreased virological breakthrough. However, no significant effect on the incidence of opportunistic infections has been found with GM-CSF adjunct therapy, and no evidence for functional recovery of innate and adaptive immunity has yet been reported.

In an effort to enhance innate immune functions with the aim of driving a global immune recovery, we have assessed the clinical tolerance, and the changes in immune parameters following a 6 week administration of the immunomodulator murabutide. HIV-1 patients included in this study reflected those who did not present an adequate immune reconstitution and a highly suppressed pVL following long-term HAART. The safety of murabutide was satisfactory and no grade IV toxicity was registered during the study. Few serious grade III AEs were noted in 20% of the treated patients. However, none
of the observed AEs were life-threatening and only four of the 20 patients in the immunotherapy group did not complete the full 6 week course of administration. Nevertheless, analysis of the changes in clinical and biological parameters was carried out in all 20 patients. In future studies, it would be helpful to evaluate whether a lower number of injections per week or a shorter duration of the immunotherapy cycle, would achieve similar biological effects and, at the same time, reduce the incidence and severity of AEs.

The most striking effect of the 6 week immunotherapy cycle with murabutide was the increase in CD4 cell counts, which was evident up to 12 weeks after the end of the injection period. The increase in CD4 counts paralleled the increase in the percentage of CD4 cells and in the CD4/CD8 ratio. The latter was not related to any detectable drop in CD8 cells among patients receiving murabutide. Although no significant differences in CD4 counts could be established between the two groups, at any time point tested, 4.5% and 35% of patients, respectively, in the control and the immunotherapy groups presented ≥30% increases in CD4 counts at two of the three observation periods. Moreover, at the time point responding to week 1 after immunotherapy, the Δ change in CD4 cells was significantly higher among murabutide recipients than control patients. In addition, immunotherapy with murabutide was found, by intra-group longitudinal analysis, to induce a significant increase in CD4 cell counts, at all time points tested after immunotherapy. This was definitely not the case in the randomized control group where the CD4 cell counts remained constant throughout the study duration. Although the baseline characteristics between the two groups were not significantly different, it is relevant to note that the immunotherapy group had slightly higher CD4 counts and a shorter duration of treatment. However, such differences are unlikely to explain the observed effects after immunotherapy and could well be attributed to the randomized nature of the trial. The mechanism of the murabutide-induced rise in CD4 cells has not been elucidated in this study; nevertheless, it could not be associated with the induction of detectable serum levels of IL-2, or with an increase in naïve lymphocyte populations. At this stage, it is difficult to propose a solid explanation for the immunotherapy-related increase in CD4 cells, and future studies would need to consolidate and address the mechanism of this very interesting and clinically relevant phenomenon. However, among all the other cell populations analysed, a significant rise could only be noted in platelet counts during and at the end of murabutide administration. This effect is different in nature from that observed on CD4 cell counts, since it was not maintained 6 and 12 weeks after immunotherapy, and may be explained by the capacity of the immunomodulator to induce the release of cytokines, including IL-6, with potential implications in megakaryopoiesis. In any case, the observed effect of murabutide on platelet counts may be of particular relevance in patients presenting HAART-induced platelet toxicity, and could constitute the basis for more detailed and targeted clinical studies.

The effect of immunotherapy on pVL was modest and did not attain statistical significance when comparisons were made between the two groups of patients. Nevertheless, subjects receiving immunotherapy did present a nearly significant drop (P = 0.05) in pVL by longitudinal intra-group analysis. In addition, at the end of the immunotherapy period, a significantly higher number of murabutide recipients had pVL below the detection limit. Thus, it is clearly evident that 6 weeks of immunotherapy with murabutide results in a trend for a stronger control of viral replication and in a considerable percentage of patients (40%) presenting pVL below the detection limit, at all three time points examined after immunotherapy. This effect may be explained by the previously reported HIV-suppressive activity of the immunomodulator, and could be equally linked with a profile of cell activation that renders infected cells non-permissive for viral replication. In this respect, it is worthwhile to point out that stimulation of HIV-infected cells by murabutide has been known to regulate the expression of selected cellular factors that could potentially contribute to virus control.

Results of the immunological assays performed before and after murabutide administration suggest that non-specific immunotherapy has the potential to bring about improved immune restoration. Although the profile of PHA-induced cytokines did not change considerably with treatment, the capacity of PBMCs to release the β-chemokine RANTES increased significantly in the immunotherapy group. Furthermore, despite a significant difference, at baseline, in serum RANTES levels between the two groups, patients receiving immunotherapy, in contrast to the control group, were found to present increased levels of circulating RANTES. These findings may be of relevance to the potential benefit of non-specific immunotherapy since RANTES is known to potentiate antigen-specific responses, and to suppress HIV-1 replication as well as activation-induced cell death in PBMCs of infected individuals. Moreover, higher RANTES production levels from ex vivo activated CD4 lymphocytes has been reported to correlate with lower pVL and with slower rates of CD4 lymphocyte decline. Interestingly, among four different β-chemokines tested, only serum levels of RANTES were reported to be consistently increased following five repeated administrations of murabutide in HIV-1 patients. Thus, the capacity of murabutide to favour a selective and continuous elevation in RANTES levels may partly explain its observed effects on CD4 lymphocytes and on pVL. On the other hand, the 6 week immunotherapy with murabutide did not have a major impact on the patients’ LPR to recall antigens, and only a weak or marginal improvement could be observed. This may be explained, among several factors, by the strong heterogeneity within each group in the responsiveness to a specific recall antigen, by the relatively long
duration of antiretroviral therapy that had already resulted in recovered responses to certain antigens (Candida), and/or by the employment of an insufficient period of immunotherapy; longer duration would have been required to potentiate the depressed responses. Nevertheless, although the approach used did not modify the size of LPR or the percentage of responders to HIV-1 p24 antigen, a gradual increase with time in the percentage of responders to gp160 was noted in the immunotherapy group. In fact, nearly one-third of the patients responded to gp160, at 12 weeks after the immunotherapy period, whereas none of the 20 patients in the same group presented positive responses when tested prior to immunotherapy. These findings are in agreement with previous reports indicating, on the one hand, the inefficacy of long-term HAART in chronically infected patients to induce recovery of LPR to viral antigens and, on the other, the capacity of five consecutive daily administrations of murabutide to induce a trend towards enhanced responsiveness to viral proteins when measured a few weeks after treatment.

HIV-1 infection has been reported to result in depressed humoral and cell-mediated immune responses to immunization with recall antigens. However, after treatment with HAART, an improved ability to respond to active immunization has been described, and this was correlated with HAART-induced increases in CD4 cells and with a decrease in pVL. Our findings, on the ability of patients under HAART to generate significant antibody responses following immunization with tetanus toxoid, are in agreement with the earlier reports. However, the anti-tetanus antibody titres, detected 5 and 11 weeks after vaccination, were identical between the groups that had or had not received murabutide immunotherapy. On the other hand, using lymphoproliferative assays against tetanus antigen as a measure of cell-mediated immunity against the vaccine, prior immunotherapy with murabutide was found to result in a capacity to mount higher LPRs, as well as in a higher percentage of patients capable of responding to the recall antigen. This discrepancy in the effects of immunotherapy on humoral and cellular responses to tetanus vaccination is currently unclear, although prompting explanations. First, the immunomodulator has been known to exert an adjuvant effect and enhance antibody responses only if administered simultaneously with the immunizing antigen (reported in 26 and unpublished observation). Second, the proteins used to measure LPRs and antibody titres were different. This raises the possibility that the antigen employed in measuring LPRs could contain a wider spectrum of antigenic determinants, thereby rendering the evaluation of cellular responses more comprehensive. Finally, it is possible that, under the conditions used, the effects of non-specific immunotherapy with murabutide primarily target effector mechanisms implicated in cell-mediated but not in humoral immunity. Additional studies would be needed to address these issues.

Results from this pilot study substantiate the findings from previous reports and indicate an interesting potential for non-specific immunotherapy in HIV-1 infection. However, key issues still need to be addressed soon in large, double-blinded, placebo-controlled trials. These concern the optimal frequency of injections per week, the duration of the immunotherapy cycle, the benefit of repeated cycles, and the potential of combined immunotherapies using murabutide with IL-2, IFN-α or GM-CSF. In addition, it would be interesting to evaluate immunotherapy with murabutide during drug holidays in order to improve the window of opportunity provided by supervised structured treatment interruptions. This strategy may be particularly relevant under conditions where treatment interruptions alone could not yield tangible clinical benefits.

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References


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