Sequence Conservation and Antibody Cross-Recognition of Clade B Human Immunodeficiency Virus (HIV) Type 1 Tat Protein in HIV-1–Infected Italians, Ugandans, and South Africans

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We determined immune cross-recognition and the degree of Tat conservation in patients infected by local human immunodeficiency virus (HIV) type 1 strains. The data indicated a similar prevalence of total and epitope-specific anti–Tat IgG in 578 serum samples from HIV-infected Italian (n = 302), Ugandan (n = 139), and South African (n = 137) subjects, using the same B clade Tat protein that is being used in vaccine trials. In particular, anti-Tat antibodies were detected in 13.2%, 10.8%, and 13.9% of HIV-1–infected individuals from Italy, Uganda, and South Africa, respectively. Sequence analysis results indicated a high similarity of Tat from the different circulating viruses with BH-10 Tat, particularly in the 1–58 amino acid region, which contains most of the immunogenic epitopes. These data indicate an effective cross-recognition of a B-clade laboratory strain–derived Tat protein vaccine by individuals infected with different local viruses, owing to the high similarity of Tat epitopes.

The development of a vaccine against human immunodeficiency virus (HIV) or AIDS that is capable of inducing sterilizing immunity has been hampered by the extreme cross-clade variability of the HIV envelope (Env) protein and by the incapacity of Env-based vaccine prototypes to elicit high and stable antibody titers of cross-clade neutralizing antibodies [1, 2]. This has led to the concept that the containment of virus replication and the subsequent control of disease progression is, at present, a more achievable goal of HIV/AIDS vaccine development [3–14]. This, in fact, would result in a reduced and virtually absent virus transmission to healthy individuals and in a reduced need for antiretroviral drugs by infected patients, which are key issues in developing countries.

For this goal, HIV regulatory proteins appear to be optimal targets. In particular, recent studies have demonstrated that the HIV-1 Tat protein is an attractive vaccine candidate. In fact, (1) Tat is expressed very early on virus entry, even prior to virus integration [15], (2) it plays a key role in the virus life cycle and infectivity [16–26], and (3) the anti-Tat immune response correlates with nonprogression of disease in infected individuals [27–31] (V.F., S.B., B.E., unpublished data). Recent studies have also indicated that native, bio-
logically active (but not oxidized) Tat targets dendritic cells (DCs), is very efficiently taken up by them, and induces their maturation and antigen-presenting function, which drives Th1-type immune responses. These responses are known to be key in immune function against viruses and are required for a vaccine prototype to be capable of controlling viral replication. In fact, preclinical studies done in cynomolgus monkeys have demonstrated that immunization with either the biologically active Tat protein or tat DNA is safe and protects against challenge with the highly pathogenic simian-human immunodeficiency virus 89.6P and that the protection (i.e., containment of infection and the blocking of disease onset) correlates with Th1-type responses.

On the basis of these results, phase 1 clinical vaccine trials with a biologically active Tat protein derived from the IIIB strain of HIV-1 (the BH-10 clone) in both seronegative and seropositive individuals are starting in Italy, and phase 2 studies are being prepared in Italy, Uganda, and South Africa. However, key issues that must be addressed for candidate antigens, preliminary to any vaccine trial, are the evaluation of the immune cross-recognition of the candidate antigen (clade B laboratory strain–derived Tat) by individuals infected with local HIV-1 subtypes in distinct geographical areas (i.e., Italy, Uganda, and South Africa) and the assessment of the degree of similarity of the Tat protein from viruses circulating in the populations where trials will be conducted with the candidate Tat vaccine.

To this end, we present a cross-sectional analysis of total and epitope-specific antibodies against the B clade laboratory strain–derived Tat and sequence analysis of Tat from local viruses done in samples from Italian, Ugandan, and South African HIV-infected individuals. Our results indicate cross-recognition of the Tat vaccine and a high sequence similarity of critical Tat regions (1–58 aa), independent of the circulating virus in the 3 countries.

PATIENTS AND METHODS

Study population. Cryopreserved serum samples and cells from Italian HIV-1–infected patients (n = 302) at different stages of disease were obtained from 6 clinical centers from Rome, Milan, Palermo, Turin, and Bari. HIV-1–positive patients were 66.5% male, with a median age of 33 years (range, 16–62 years). Age- and sex-matched HIV-1–negative individuals (n = 132) were selected from among blood donors and healthy individuals.

African samples included serum and cells from HIV-1–infected patients from Uganda (n = 139; St. Mary’s "Lacor" Hospital, Gulu) and South Africa (n = 137; HIV/AIDS Vaccine Division of the Perinatal HIV Research Unit, University of Witwatersrand, Johannesburg, and Medical Research Council, Durban). HIV-1–negative individuals (according to both HIV-1 and HIV-2 serological test results) were recruited from among hospitalized patients in each clinical center (74 from Uganda and 34 from South Africa). Informed consent was obtained from all patients, and the human experimentation guidelines of local institutional ethical committees were followed in each country.

HIV-1 Tat protein expression and purification. The Tat protein (1–86 aa) from the BH-10 clone of the IIIB strain of HIV-1 (clade B) was expressed in Escherichia coli, purified, and stored as described elsewhere. The purified Tat protein was fully monomeric and had full biological activity, as assessed by virus transactivation assays and by DC uptake studies. This protein has the same specifications of the Tat protein that is under good manufacturing practice (GMP) development for use in phase 1 clinical trials. Tat sticks easily to surfaces and, because of the presence of 7 cystein residues, it is particularly vulnerable to oxidative stresses that modify its structure, inducing protein multimerization and aggregates with loss of biological activity. Therefore, the lyophilized protein was resuspended just before use in a degassed buffer that contained 0.1% bovine serum albumin (BSA) and handled as described elsewhere.

Measurement of serum antibodies against the Tat protein. For the detection of anti-Tat antibodies, 2 different ELISAs were developed to obtain high levels of sensitivity (ELISA 1) and specificity (ELISA 2). For ELISA 1, we used a secondary antibody conjugated to a peroxidase-labeled dextran polymer (EnVision System; Dako) that carries a high number (up to 100) of enzyme molecules for each specific site of binding to the primary antibody. In addition, less-stringent criteria for the cutoff determination than those applied to ELISA 2 were chosen (see below). For ELISA 2, we used a secondary antibody with no amplification of the signal, and more restrictive criteria for cutoff calculations were applied.

All solutions for the ELISA tests were freshly prepared and kept at 4°C until use. Procedures and cutoff calculation for ELISA 1 were done as follows: 96-well microplates (Nunc-Immuno Plate MaxiSorp Surface; Nunc) were coated with 100 ng of Tat/well in 200 μL of 0.05 mol/L carbonate buffer (pH 9.6) and incubated overnight at 4°C. Wells were then saturated with PBS that contained 1% BSA and 0.05% Tween 20 (Sigma) for 90 min at 37°C and then washed 5 times with PBS that contained Tween 20 (0.05%) on an automatic plate washer (Sorin Biomedica). One hundred microliters of the patient serum samples, diluted at 1:100 (for IgG detection) or at 1:25 (for IgM detection) in PBS that contained 1% BSA and 0.05% Tween 20, were added to the wells and incubated at 37°C for 90 min. To correct for any unspecific binding, each sample was always assessed in duplicate against Tat or against the buffer in which Tat was resuspended (control). In addition, in all experiments, an anti-Tat rabbit polyclonal antiserum (diluted to 1:1000) was included as the positive control, and 5 known anti-Tat negative
human serum samples from healthy, HIV-seronegative individuals were included (in duplicate) in each plate as negative controls. After incubation, plates were washed 5 times with PBS that contained 0.1% Tween 20 and were subsequently incubated for 90 min at 37°C with 100 µL of rabbit anti-human IgG (1:570; Dako) or rabbit anti-human IgM (1:700; Dako) diluted in PBS that contained 1% BSA and 0.05% Tween 20, with the exception of the wells containing the anti-Tat polyclonal rabbit antiserum, which were incubated only with buffer. After washing, wells were incubated with 100 µL of the peroxidase-labeled dextran polymer conjugated to goat anti-rabbit immunoglobulins (1:500; Dako EnVision System) diluted with 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl (pH 7.6) that contained 0.1% Tween 20, and 5% skim milk for 1 h at room temperature. The plates were then washed and incubated with 100 µL of a solution of o-phenylenediamine dihydrochloride (4 tablets in 12 mL of distilled H₂O plus 5 µL of 30% H₂O₂; Dako) for 10 min at room temperature in the dark. One hundred microliters of a 0.5 mol/L H₂SO₄ solution were finally added to stop the reaction. Absorbance in optical density was measured at 490 nm using a microplate reader (Sirio SEAC; Calenzano). Cutoff values were established for each plate and in each run and were calculated as 3 SD above the mean of each of the values of the optical density at 405 nm (absolute and Δ values) of either 89 serum samples from Italian HIV-negative blood donors or 34 serum samples from South African HIV-negative individuals. Using the same calculation procedure, cutoff values for Ugandan serum samples were calculated on samples from 123 HIV-negative individuals and were found to be higher than those for Italian and South African serum samples (0.7 and 0.4 for the optical density at 405 nm absolute and Δ values, respectively).

**Epitope mapping of Tat-specific antibodies.** Epitope mapping of anti-Tat IgG-positive serum samples was done using ELISA 2. Purified synthetic peptides (Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy) were designed to partially overlap (aa 1–20, 21–40, 36–50, 46–60, 56–70, 65–80, and 73–86) the BH-10 clone–derived linear sequence of the Tat protein (aa 1–86). All peptides were used at 250 ng/well in 200 µL of PBS with Ca²⁺ and Mg²⁺, using the procedures described above. Cutoff values were determined for each peptide as described above.

**Phylogenetic analysis of HIV-1 subtypes from Italy, Uganda, and South Africa.** Phylogenetic analysis of HIV-1 subtypes was done on the p6/p7 gag-coding sequence by using the neighbor-joining program of the Genetics Computer Group package (Accelrys). DNA extracted from 0.5 × 10⁶ peripheral blood mononuclear cells with the QIAamp DNA Blood Minikit (QIAGEN) was amplified by 1-round polymerase chain reaction (PCR) using the BJ1 (5'-TAGAGAAATGTAGACAGCATG-3') and BJ2 (5'-CTAATACTGTATCATCTGCTCTGT-3') primer pair (positions 1817–1838 and 2418–2442, respectively, in the HXB2 sequence). The reaction mixture (40 µL) consisted of 10 mmol/L PCR Buffer II (Perkin-Elmer), 2 mmol/L MgCl₂, 200 µmol/L each dNTP, 200 mmol/L/L primers BJ1 and BJ2, and 1.3 U of Taq polymerase (AmpliTaq Gold DNA polymerase 5000; Perkin-Elmer). Approximately 0.35 µg of DNA was added to the mixture. An initial denaturation step at 95°C for 9 min was followed by 35 amplification cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 1 min) and by a final cycle at 72°C for 5 min. Amplified products were then sequenced with the Ready Reaction BIG-Dye Terminator Cycle Sequencing Kit (Perkin Elmer) using primers BJ1 and BJ2 and a Cycle Sequencer ABI PRISM 3700 (Applied Biosystems), according to the manufacturer's instructions.

**Sequencing of the tat gene.** For the amplification of the Tat-coding region, a nested PCR strategy was applied for each of the 2 exons. Specifically, amplification of the first exon (366 bp) was done with the UGS (5'-ATGGGGATACTGGGAAGGAGT-3') and UG6 (5'-CCACGTCTCTGCTCTTTCT-3') primer pair (positions 5707–5728 and 6202–6222, respectively, in the HXB2 sequence) in the first PCR round, followed by a second...
amplification round with the UG7 (3'-CAGAATTGGGTGCTCA-ACATACC-5′) and UG8 (3'-TACTATGGCCACACACACTAT-TGC-5′) primer pair (positions 5775–5796 and 7374–7398, respectively, in the HXB2 sequence). For the second Tat-encoding exon (299–307 bp), the UG1 (3'-TTGAATGGAGCAAGTG-GCA-5′) and UG2 (3'-GATTCACAGATATTGAGG-5′) primer pair (positions 8205–8225 and 8600–8619, respectively, in the HXB2 sequence) was used in the first PCR round, and the UG3 (3'-TAGAGGAGGCTTAATAGG-5′) and UG4 (3'-CTGGGTCCAGAAGTCTCCACA-5′) primer pair (positions 8287–8305 and 8567–8587, respectively, in the HXB2 sequence) was used in the second PCR round. In both rounds, the reaction mixture (100 μL) contained 10 mmol/L PCR Buffer II (Perkin-Elmer), 2 mmol/L MgCl2, 200 μmol/L each dNTP, 200 mmol/L primers, and 2 U of TaqGold (Perkin-Elmer). In the first round, ~0.1 μg of DNA was added to the mixture and thermocycled with an initial denaturation step at 95°C for 9 min, followed by 35 amplification cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 1 min) and 1 cycle at 72°C for 10 min. Ten microliters of each amplified product in the first round were then used for the second PCR round. To avoid cross-contamination, pre- and post-PCR manipulations were done in separate rooms. Amplified DNA fragments were purified using the QIAquick PCR Purification kit (QIAGEN), according to the manufacturer’s instructions. Sequencing of both DNA strands of the amplified products were carried out as described above, using UG7 and UG8 for the first tat exon and UG3 and UG4 for the second exon.

**Tat sequence analysis.** The predicted Tat protein sequence from field isolates was compared with that from the BH-10 clone (clade B), which is a fully functional 86-aa–long Tat with a stop codon after the first 14 aa residues of the second exon [6, 19, 21, 24, 32]. Comparison of the Tat sequences among field isolates, as well as against BH-10 Tat, was done considering only the first 86 aas, even though most of the fields isolates had a longer, second exon-encoded sequence.

The degree of similarity (percentage similarity) of the Tat protein sequence was evaluated by a distance calculation that used Kimura 2-parameter distances [34] as $100 - n\%$, where $n$ is the calculated distance. The percentage of similarity was evaluated against the Tat BH-10 sequence, and 2 groups were identified, the first one including sequences with $\geq 75\%$ of conservation and the second one including sequences with $<75\%$ of conservation. GeneBank accession numbers for these sequences are AY193980–AY193999 (Italian sequences), AY194000–AY194026 (South African sequences), and AY194027–AY194053 (Ugandan sequences).

**Statistical analysis.** Exploratory analysis was done using box plots to compare the distribution of the optical density values from the ELISA 2 for anti-Tat antibodies with the level of similarity of the Tat protein with Tat BH-10 (major, $\geq 75\%$; minor, $<75\%$). Differences were assessed using the Mann-Whitney U test.

**RESULTS**

Total anti-Tat antibodies were found at a similar prevalences in the HIV–infected individuals from Italy, Uganda, and South Africa (table 1). In particular, they were detected in 40 (13.2%) of 302 Italian, 15 (10.8%) of 139 Ugandan, and 19 (13.9%) of 137 South African individuals, respectively; the differences were not statistically significant ($P = .71$). The median and the range of reciprocal antibody titers were also similar among the study groups. Zero of 240 serum samples tested from HIV–negative individuals (132 from Italy, 74 from Uganda, and 34 from South Africa, respectively) had detectable anti-Tat IgG.

To identify epitope-specific antibodies and to further characterize the extent of cross-recognition of the BH-10 (B clade) Tat protein, 7 synthetic overlapping peptides spanning the entire Tat sequence (aa 1–86) of the BH-10 clone were used (table 2). The most frequently recognized epitope was located within the 1–20 aa region of Tat (42.5% [17/40], 20.0% [3/15], and 42.1% [8/19] of the anti-Tat–positive patients from Italy, Uganda, and South Africa, respectively). Additional epitopes were identified within residues 36–50 (12.5% [5/40], 13.3% [2/15], and 5.3% [1/19] for Italian, Ugandan, and South African anti-Tat–positive

<table>
<thead>
<tr>
<th>Country, patient group</th>
<th>IgG positive/total (%)</th>
<th>Titer, median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Italy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV+</td>
<td>0/132 (0)</td>
<td></td>
</tr>
<tr>
<td>HIV+</td>
<td>40/302 (13.2)</td>
<td>100 (50–102.400)</td>
</tr>
<tr>
<td><strong>Uganda</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV+</td>
<td>0/74 (0)</td>
<td></td>
</tr>
<tr>
<td>HIV+</td>
<td>15/139 (10.8)</td>
<td>100 (100–800)</td>
</tr>
<tr>
<td><strong>South Africa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV+</td>
<td>0/34 (0)</td>
<td></td>
</tr>
<tr>
<td>HIV+</td>
<td>19/127 (13.9)</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

**NOTE.** The prevalence of anti-Tat antibodies in HIV-positive (+) vs. HIV-negative (−) individuals from each country was statistically significant ($P < .0001$, $P = .002$, and $P = .01$ for Italy, Uganda, and South Africa, respectively) but did not significantly differ among the 3 geographic areas ($\chi^2 = 2.09; P = .35$). See Patients and Methods for experimental details. Pearson’s correlation coefficient ($\rho = 0.69$; $P = .71$) indicated no statistical difference of anti-Tat antibody prevalence among serum samples from HIV-infected individuals from the 3 countries.
Table 2. Epitope mapping of anti–Tat-positive serum samples of human immunodeficiency virus (HIV) type 1–infected individuals from Italy, Uganda, and South Africa, stratified according to peptide recognition.

<table>
<thead>
<tr>
<th>Country (total no. of anti-Tat–positive sera), no. (%) of serum samples</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italy (40)</td>
<td>+</td>
</tr>
<tr>
<td>13 (32.5)</td>
<td>+</td>
</tr>
<tr>
<td>3 (7.5)</td>
<td>+</td>
</tr>
<tr>
<td>1 (2.5)</td>
<td>–</td>
</tr>
<tr>
<td>22 (55.0)a</td>
<td>+</td>
</tr>
<tr>
<td>Uganda (15)</td>
<td>+</td>
</tr>
<tr>
<td>2 (13.3)</td>
<td>+</td>
</tr>
<tr>
<td>1 (6.7)</td>
<td>+</td>
</tr>
<tr>
<td>9 (60.0)a</td>
<td>–</td>
</tr>
<tr>
<td>South Africa (19)</td>
<td>+</td>
</tr>
<tr>
<td>5 (26.3)</td>
<td>+</td>
</tr>
<tr>
<td>2 (10.5)</td>
<td>–</td>
</tr>
<tr>
<td>1 (5.3)</td>
<td>–</td>
</tr>
<tr>
<td>1 (5.3)</td>
<td>+</td>
</tr>
<tr>
<td>9 (47.3)a</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE. Serum samples were tested (1:100 dilution) by ELISA 2 against a panel of partially overlapping synthetic peptides spanning the entire linear Tat sequence originated from the BH-10 clone of the IIIB strain (clade B) of HIV-1 (aa 1–86). See Patients and Methods for experimental details. Shown is the no. (%) of serum samples that recognized ≥1 peptides or none, according to the country of origin. –, Negative; +, positive.

a No peptide recognition.

patients, respectively) and at residues 73–86 (2.5% [1/40], 6.7% [1/15], and 10.5% [2/19] for Italian, Ugandan, and South African anti-Tat–positive patients, respectively). Other epitopes were recognized at a lower frequency by serum samples from South African and Italian patients. Overall, the epitope-specific anti-Tat antibodies were found in 45.0%, 40.0%, and 52.7% of the Italian, Ugandan, and South African patients, respectively, that recognized ≥1 Tat peptides. The remaining 55.0%, 60.0%, and 47.3% of the Italian, Ugandan, and South African serum samples, respectively, did not recognize the Tat peptides. In addition, the epitope-specific antibodies were present at low titers (≤200) in most of the serum samples (data not shown), including those with high titers of antibodies against the native Tat protein. This suggested that most of the anti-Tat reactivity is due to conformational antibodies. In fact, detection of anti-Tat antibodies in the same serum samples, using an oxidized and inactive form of Tat as the antigen, showed much lower titers of specific antibodies than the same samples tested with the native and biologically active Tat protein (data not shown).

To investigate HIV subtype diversity in the 3 study groups, phylogenetic analysis based on the p6/p7-encoding gag sequence was done for samples from 74 individuals (20 from Italy, 27 from Uganda, and 27 from South Africa). All Italian samples clustered within the B clade. HIV subtypes A and D were the most frequently represented in the Ugandan group (subtype D, 12 [44.4%] of 27; subtype A, 10 [37%] of 27), whereas other subtypes (B, F, and G) represented 18.5% of the total local subtypes (5/27). In South Africa, subtype C was the most common (92.6% [25/27]), whereas other subtypes (A and F) were found at very low frequency.

The diversity of the predicted Tat protein sequences from circulating viruses with respect to BH-10 Tat was unevenly distributed, with most of the first exon–encoded sequence being more conserved and similar to the BH-10 sequence than the second exon–encoded portion of the protein. According to Kimura’s 2-parameter model (table 3), similarity with the entire BH-10 Tat ranged from an average of 76.2% for the B clade sequences to 52.3% for the A clade sequences, with an inter-subtype average of 68.2% (table 3, aa 1–86). The other 2 most frequently represented subtypes (C and D) averaged 72.8% and 59.5%, respectively. When exons 1 and 2–encoded portions of Tat (table 3, aa 1–72 and 73–86, respectively) were considered separately, the exon 1–derived sequence from each subtype was more conserved to the corresponding BH-10 sequence than the
exon 2–encoded portion of Tat. In fact, the similarity in the 1–72 region ranged from 84.1% for clade B sequences to 59.2% for clade A sequences, with an intersubtype average of 74.5%. In contrast, the 73–86 aa region showed a very poor conservation with corresponding BH-10 region (from 38.6% of clade C sequences to 19.3% for clade A sequences, with an intersubtype average of 31.2%). Within the exon 1–encoded region, a conserved sequence (aa 1–58) that contained most of the immunogenic epitopes and the functional regions of the protein was identified. In this portion (table 3, aa 1–58), similarity with the corresponding BH-10 region (from 38.6% of clade B to 69.3% of clade A sequences, with an intersubtype average of 79.4%).

The degree of similarity between Tat from circulating viruses and the BH-10 Tat sequence was then investigated for the 2 previously identified major B cell epitope–containing regions (1–20 and 36–50) (figure 1). Figure 1A shows the similarity of the 1–20 region from each sequence with the corresponding BH-10 Tat region. The average similarity ranged from 92.3% of clade B subtypes to 76.6% of clade A subtypes, which confirms the high conservation of this region among all virus clades. In addition, only amino acids at positions 7 and 19 were relatively polymorphic, whereas all the other amino acids were well conserved with both the BH-10 strain and among all the local viruses, with the exception of clade F and G subtypes, for which a relatively greater polymorphism was present in position 12. Although less conserved than the 1–20 region, the 36–50 region from the circulating viruses showed a greater similarity with the BH-10 corresponding region than the entire Tat protein (figure 1B). In fact, average similarity ranged from 85.2% of the B clade strains to 68.9% of the D clade strains. Polymorphic amino-acid positions showed a lower degree of conservation, compared with the 1–20 region. In fact, at least 3 different amino acids (39, 40, and 42) were polymorphic, with residues 39 and 40 being the most variable.

The relationship between the similarity the of Tat sequences from HIV-infected individuals versus BH-10 Tat and the presence or absence of anti-Tat antibodies was then investigated in the samples from 74 patients from whom the Tat sequences were obtained. The average similarity of these Tat sequences with BH-10 Tat did not significantly differ between anti-Tat antibody–positive (20 patients) and –negative (54 patients) individuals. In fact, the average similarity with BH-10 Tat was 70.27% and 67.77% in individuals with or without anti-Tat antibodies, respectively, and the difference between the averages were not statistically significant (P = .52). The relationship between the recognition of the BH-10 Tat by anti-Tat antibodies and the degree of similarity of the predicted Tat sequences from the local virus strains with BH-10 Tat was also assessed in matched samples where the distribution of the optical density values of anti-Tat antibodies was compared with the level of similarity with the BH-10 Tat. As shown in figure 2, there were no significant differences in the median optical density values according to a similarity ≥75% or <75% (0.129 and 0.121 for ≥75% or <75% similarity, respectively; Mann-Whitney U test, P = .13). When anti-Tat antibody production was considered to be a dichotomous variable (i.e., the presence or absence of antibodies), the OR obtained from the unvaried analysis showed that the anti-Tat response tended to be associated with similarity, but also, in this case, the difference observed was not statistically significant (crude OR, 2.22; 95% CI, 0.78–6.30; P = .46). The adjusted ORs obtained from multivariate analysis did not change significantly.

### DISCUSSION

Preventive and the therapeutic phase 1 vaccine trials using the biologically active BH-10 Tat protein are starting in Italy, and phase 2/3 trials are being planned in Italy, Uganda, and South Africa. To investigate the feasibility of vaccine trials with the Tat vaccine, the prevalence of total and epitope-specific anti-Tat antibodies was determined in HIV-1–infected individuals from Italy, Uganda, and South Africa using 2 ELISAs based on

### Table 3. Similarity of aa 1–86, 1–72, 73–86, and 1–58 Tat sequences from Italian, Ugandan, and South African human immunodeficiency virus (HIV) subtypes with the BH-10 Tat sequence.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Country (no. of isolates)</th>
<th>aa 1–86</th>
<th>aa 1–58</th>
<th>aa 1–72</th>
<th>aa 73–86</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Uganda (10), South Africa (1)</td>
<td>52.3</td>
<td>69.3</td>
<td>59.2</td>
<td>19.3</td>
</tr>
<tr>
<td>B</td>
<td>Italy (20), Uganda (2)</td>
<td>76.2</td>
<td>87.2</td>
<td>84.1</td>
<td>38.5</td>
</tr>
<tr>
<td>C</td>
<td>South Africa (25)</td>
<td>72.8</td>
<td>82.2</td>
<td>82.3</td>
<td>38.6</td>
</tr>
<tr>
<td>D</td>
<td>Uganda (12)</td>
<td>59.5</td>
<td>74.2</td>
<td>64.4</td>
<td>21.7</td>
</tr>
<tr>
<td>F</td>
<td>Uganda (2), South Africa (1)</td>
<td>75.3</td>
<td>84.2</td>
<td>78.1</td>
<td>32.0</td>
</tr>
<tr>
<td>G</td>
<td>Uganda (1)</td>
<td>73.1</td>
<td>79.1</td>
<td>79.2</td>
<td>37.4</td>
</tr>
<tr>
<td>Average similarity</td>
<td>68.2</td>
<td>79.4</td>
<td>74.5</td>
<td>31.2</td>
<td></td>
</tr>
</tbody>
</table>

The relationship between the similarity of Tat sequences and the BH-10 Tat sequence was then investigated for the 2 previously identified major B cell epitope–containing regions (1–20 and 36–50) (figure 1). Figure 1A shows the similarity of the 1–20 region from each sequence with the corresponding BH-10 Tat region. The average similarity ranged from 85.2% of the B clade strains to 68.9% of the D clade strains. Polymorphic amino-acid positions showed a lower degree of conservation, compared with the 1–20 region. In fact, at least 3 different amino acids (39, 40, and 42) were polymorphic, with residues 39 and 40 being the most variable.

The relationship between the similarity the of Tat sequences from HIV-infected individuals versus BH-10 Tat and the presence or absence of anti-Tat antibodies was then investigated in the samples from 74 patients from whom the Tat sequences were obtained. The average similarity of these Tat sequences with BH-10 Tat did not significantly differ between anti-Tat antibody–positive (20 patients) and –negative (54 patients) individuals. In fact, the average similarity with BH-10 Tat was 70.27% and 67.77% in individuals with or without anti-Tat antibodies, respectively, and the difference between the averages were not statistically significant (P = .52). The relationship between the recognition of the BH-10 Tat by anti-Tat antibodies and the degree of similarity of the predicted Tat sequences from the local virus strains with BH-10 Tat was also assessed in matched samples where the distribution of the optical density values of anti-Tat antibodies was compared with the level of similarity with the BH-10 Tat. As shown in figure 2, there were no significant differences in the median optical density values according to a similarity ≥75% or <75% (0.129 and 0.121 for ≥75% or <75% similarity, respectively; Mann-Whitney U test, P = .13). When anti-Tat antibody production was considered to be a dichotomous variable (i.e., the presence or absence of antibodies), the OR obtained from the unvaried analysis showed that the anti-Tat response tended to be associated with similarity, but also, in this case, the difference observed was not statistically significant (crude OR, 2.22; 95% CI, 0.78–6.30; P = .46). The adjusted ORs obtained from multivariate analysis did not change significantly.

### DISCUSSION

Preventive and the therapeutic phase 1 vaccine trials using the biologically active BH-10 Tat protein are starting in Italy, and phase 2/3 trials are being planned in Italy, Uganda, and South Africa. To investigate the feasibility of vaccine trials with the Tat vaccine, the prevalence of total and epitope-specific anti-Tat antibodies was determined in HIV-1–infected individuals from Italy, Uganda, and South Africa using 2 ELISAs based on
Figure 1. The similarity of 2 Tat regions from circulating subtypes containing major B cell epitopes with the regions corresponding to strain IIIB of human immunodeficiency virus type 1 (BH-10) is shown. Viruses are grouped by clades. The horizontal stretches of amino-acid residues represent the BH-10 1–20 (A) and 36–50 (B) Tat sequences, respectively. Amino-acid substitutions are indicated below each amino acid of the BH-10 sequence. Amino-acid residues are accompanied by the percentage of their frequency at a specific position in the alignment (shown as a subscript numeral). Dashes indicate amino-acid deletions.

The highly purified, native and monomeric, biologically active Tat protein derived from the BH-10 clone of the IIIB strain of HIV-1, which has the same characteristics of the Tat produced in GMP for phase 1 clinical trials. In addition, the similarity of the predicted Tat protein sequence from the local viruses with the BH-10 Tat sequence was determined, and the relationship between the anti-Tat antibody response and the sequence similarity was analyzed.

The results indicated that serum samples from Italian, Ugandan, and South African patients, who are mainly infected with HIV-1 subtypes A, B, C, and D and, to a lesser extent, F and G, recognize the Tat vaccine at similar levels (i.e., prevalence and titers of anti-Tat antibodies). No anti-Tat antibodies were detected in samples from HIV-negative blood donors or healthy individuals from each country. The relatively small proportion of individuals with anti-Tat antibodies is intriguing. One reason may be that Tat is produced in very small amounts and mainly during the early phases of infection [21, 24]. Furthermore, our recent and as-yet-unpublished data from novel vaccination studies that combined Tat with structural viral genes suggested that the presence of structural viral antigens reduces or abolishes the responses to Tat. The same could occur in the natural infection. In this regard, it is tempting to speculate that the diversion of immune response from Tat is part of the strategies elaborated by HIV-1 to escape effective immune control.

Epitope mapping done using BH-10–derived Tat peptides revealed the presence of epitope-specific antibodies in 45.0%, 40.0%, and 52.7% of the anti-Tat–positive serum samples from Italy, Uganda, and South Africa, respectively. The presence of epitope-specific antibodies in only a fraction of the Tat-positive serum samples, the low reciprocal titers, and the much lower anti-Tat antibody titers consistently detected when using an oxidized Tat protein in ELISA tests indicate that a large portion of the humoral response against Tat is directed against conformational epitopes whose detection requires a native, monomeric Tat protein. In agreement with previous observations [35–38] (V.F., S.B., B.E., unpublished data), 2 immunodominant B cell epitopes were found at residues 1–20 and 36–50. Overall, these data indicate that the laboratory strain–derived BH-10 Tat protein (B clade) is equally recognized by individuals infected with distantly related and presently circulating HIV-1 subtypes.

This notion is reinforced by the results of sequence conser-
Figure 2. The relationship between antibody levels and the degree of similarity of the predicted Tat protein sequence is shown. Box plots show the relationship between antibody level, expressed as optical density (on the Y-axis), and the degree of similarity of the Tat-predicted amino-acid sequence from the corresponding virus subtypes with strain IIIB of human immunodeficiency virus type 1 (BH-10) Tat, calculated by the Kimura test and used as a dichotomous variable (on the X-axis; < or ≥75%).

vation analyses. Comparison of the BH-10 Tat sequence with those obtained from the large variety of HIV-1 subtypes we describe [39–48] demonstrate that the predicted amino-acid sequence of Tat is conserved among circulating viruses that belong to distinct HIV-1 clades and has a relatively high degree of similarity with the BH-10 Tat sequence. Specifically, similarity is high in the first exon–encoded portion of Tat, particularly in the 1–58 region, which contains key functional domains of Tat and most of the B, T helper, and cytotoxic T lymphocyte (CTL) epitopes so far identified [38, 49–51]. In particular, the 2 regions containing the B cell epitopes identified in the present study (1–20 and 36–50 residues) are highly conserved among the different HIV-1 subtypes. Notably, the 1–20 sequence is key for the transactivating function of Tat [22, 52] and contains both a major B cell epitope [38] (V.F., S.B., and B.E., unpublished data) and a CTL epitope [49]. Similarly, residues 36–50 include the core region of the protein, which is also key for Tat functional activities and contains both a B cell epitope [38] and a major CTL epitope that was recently identified in subtype C [51]. Furthermore, previously identified B, CTL, and T helper epitopes are also present in the highly conserved aa 1–58 portion of Tat [35, 38, 49, 50, 51, 53–55]. Conversely, the portion of Tat coded by exon 2, which is dispensable for Tat transactivation, is much less conserved and has a lower degree of similarity with the corresponding BH-10 region.

The degree of similarity of the predicted Tat protein sequence from circulating viruses with the Tat BH-10 sequence does not vary significantly between samples from anti-Tat–positive and –negative individuals when using the titers of Tat-specific antibodies as a continuous variable. When the anti-Tat humoral immune response was dichotomized (i.e., as positive or negative), a tendency toward an association was observed, although it did not reach statistical significance. This suggests that an increased variability might indeed slightly influence the specific immune response. However, the response was not associated with clade distribution. In fact, in the multivariate analysis, the proportion of anti-Tat responders infected with different clades (i.e., clade B vs. non-B) did not change significantly.

It should be noted that the results of immune cross-recognition and sequence similarity have been obtained using a Tat protein derived from one of the first HIV laboratory strains (IIIB strain of HIV-1), which was isolated almost 20 years ago [56]. Because of HIV variability dynamics, it is certainly of relevance that individuals presently infected by different HIV-1 strains can equally recognize the distantly related BH-10–derived Tat protein.

Taken together, these results indicate that the immunogenic and functional domains of Tat are highly conserved among distinct HIV-1 subtypes and have a high degree of similarity with the corresponding sequence of BH-10–derived Tat that is being tested for use as a vaccine. The present data provide further support to the emerging notion that Tat may represent an optimal target for specific immunization against multiple HIV-1 subtypes.

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