

Fine Epitope Specificity of Anti-erythropoietin Antibodies Reveals Molecular Mimicry With HIV-1 p17 Protein: A Pathogenetic Mechanism for HIV-1–Related Anemia

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Background. Circulating autoantibodies to endogenous erythropoietin (anti-Epo) are detected in human immunodeficiency virus type 1 (HIV-1)–infected patients and represent a risk factor for anemia. The aim of this study was to map the B-cell epitopes on the Epo molecule.

Methods. Serum samples from HIV-1–positive patients and healthy individuals were tested against overlapping peptides covering the entire sequence of Epo.

Results. Serum samples from anti-Epo–positive patients exhibited significant binding to Epo epitopes spanning the following sequences: amino acids 1–20 (Ep1), amino acids 54–72 (Ep5), and amino acids 147–166 (Ep12). Structural analysis of erythropoietin revealed that the immunodominant epitopes, Ep1 and Ep12, comprise the interaction interface with Epo receptor (EpoR). Autoantibodies binding to this specific region are anticipated to inhibit the Epo-EpoR interaction, resulting in blunted erythropoiesis; this phenomenon is indicated by the significantly higher Epo levels and lower hemoglobin levels of anti-Ep1–positive patients compared with anti-Ep1–negative individuals. The region corresponding to the Ep1 epitope exhibited a 63% sequence homology with the ³⁴LVCASRELERFAVNPGLLE⁵² fragment of the HIV-1 p17 matrix protein.

Conclusions. These results suggest that the main body of anti-Epo is directed against a functional domain of Epo, and that the presence of anti-Epo can be considered to be a result of a molecular mimicry mechanism, which is caused by the similarity between the Ep1 region and the p17 protein.

Human immunodeficiency virus type 1 (HIV-1)–related anemia has serious implications because it is associated with quality-of-life decrements, accelerating progression of the disease, and increased risk of death [1]. Although the pathophysiology of this dreadful complication is multifactorial and not well understood, it

appears that dysregulation of erythropoietin (Epo) plays a pivotal role, since early studies have shown that HIV-1–related anemia is associated with inadequate production of Epo and a blunted response to its physiologic action [2].

HIV-1 infection is characterized by a plethora of autoimmune phenomena; a high prevalence of circulating autoantibodies against a constellation of antigens has been reported in infected patients [3–5], but their clinical significance is unclear [6]. In a previous retrospective study [7], we demonstrated that circulating autoantibodies to endogenous erythropoietin (anti-Epo) were present in a substantial percentage (23.4%) of patients with HIV-1 infection. This observation prompted a prospective study which revealed that the presence of anti-Epo is an independent risk factor for anemia [8], a finding which suggests that autoimmunity might contribute to the pathogenesis of HIV-1–related anemia.

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Several mechanisms have been proposed to explain autoimmune manifestations of HIV-1 infection: immune activation by HIV-1 per-se [9], dysregulation involving T and B cells or both [10], and increased expression of autoantigens. Molecular mimicry is thought to be one of the major mechanisms that might initiate an autoimmune response, in the case of sequence or structural antigen similarity between the host and infectious agent [11]. Viral peptides can induce autoimmune responses by cross-reaction of specific viral antigens with self-proteins through the stimulation of autoreactive T cells [12].

The aim of the present study was to define the fine specificity of antibodies to Epo in HIV-1-infected patients, using 20-mer overlapping synthetic peptides that spanned the entire sequence of the molecule.

METHODS

Patient Serum Samples

Serum samples ($n = 32$) from HIV-1-positive patients in Greece were obtained. All patients were diagnosed and followed up in the Department of Pathophysiology, Medical School, National and Kapodistrian University of Athens. Sixteen patients were anti-Epo-positive and 16 were anti-Epo-negative at different stages of disease. Most patients were male ($n = 27$), and the median age was 39 years (range, 27–54 years). Sixteen serum samples from age- and sex-matched healthy blood donors were used as negative controls. None of patients and healthy control individuals had been treated with erythropoietin before sampling time. Anemia was defined as a hemoglobin (Hgb) level of <13 g/dL for male patients and <12 g/dL for female patients, respectively [13]. The study was approved by the institutional review board of the Laiko General Hospital in Athens, Greece. All participants gave their informed consent.

Peptide Synthesis

Twelve sequential 20-mer peptides, which overlapped each other by 7 amino acids and covered the entire sequence of the mature Epo protein molecule (EPO Human; UniprotKB/Swiss-Prot), were prepared using automated Fmoc (*N*-[9 fluorenyl] methoxycarbonyl) solid-phase synthesis (Biosynthesis). Each peptide was purified by reversed-phase high-performance liquid chromatography and exhibited a single peak at its predicted molecular weight by mass spectroscopy.

Erythropoietin Measurements

Epo serum levels were measured by radioimmunoblot assay with a commercial kit (^{125}I RIA Kit; EPO-Trac, Stillwater, MN) at the same time that blood samples were collected for anti-Epo.

Detection of Antibodies to Epo

Recombinant human Epo (rHuEpo) purified by analytical gel filtration and characterized by amino acid composition and NH_2 -terminal analysis (Cilag AG) was used as an antigen. Serum

samples from patients with HIV-1 infection that contained anti-Epo, as defined in our previous study [8], were used as positive controls. Serum samples from 40 healthy blood donors were used as reference controls. Anti-Epo was detected using the enzyme-linked immunosorbent assay (ELISA) technique described elsewhere [14]. Briefly, 96-well polystyrene plates (Nunc) were coated with 10 μg of rHuEpo in phosphate-buffered saline (PBS; pH 7.2). Optimum blocking conditions for nonspecific binding were achieved by adding to each well 100 μL of 5% bovine serum albumin (BSA)–Tris–NaCl (pH 7.2) and incubating at 4°C overnight. After washing 3 times with PBS, serum samples were added in duplicate, at a 1:25 dilution, in PBS containing 2% BSA and 0.2% Tween 20. After 1 hour of incubation at 37°C, the plates were washed 5 times with PBS and subsequently incubated with goat anti-human immunoglobulin G (IgG) conjugated with alkaline phosphate, at a 1:2000 dilution, for 1 hour at room temperature. The substrate buffer (*P*-nitrophenyl phosphate disodium; 2 mg/mL; Sigma Chemicals) was added, and after 30 minutes of incubation at 37°C, the final reaction was stopped with a solution of NaOH 10% (2.5 mol/L) and then read at 405 nm using a Dynatech UR 4000 ELISA reader. The cutoff point for positive samples was calculated as the mean optical density for the reference controls plus 3 times the standard deviation. Intra- and inter-assay coefficients of variation were $<8\%$ and $<15\%$, respectively. The specificity of the method has been evaluated with homologous and cross-inhibition assays [14].

Epitope Mapping

Microtiter polystyrene plates (Nunc) were coated with 100 μL of peptide solution (5 $\mu\text{g}/\text{mL}$) with b-carbonate buffer (pH 9.6) and kept at 4°C overnight. After blocking the remaining binding sites with 2% BSA and 0.2% Tween 20 in PBS, the plates were incubated at 37°C for 1 hour. Afterward the plates were washed 3 times with PBS and serum samples were added in duplicate, at a 1:40 dilution, in PBS containing 2% BSA and 0.2% Tween 20 (200 μL per well). Following incubation at room temperature for 2 hours and 3 washes, goat anti-human IgG conjugated with alkaline phosphate was added at a 1:1200 dilution, and the plates were kept for 1 hour at room temperature. Finally, after 5 washes, 100 μL of substrate buffer was added and the reaction was read at 405 nm after 10 minutes. The cutoff point for positive samples was calculated as the mean optical density for the reference controls plus 5 times the standard deviation.

Homologous and Heterologous Inhibition Assays

To evaluate the specificity of the reaction, inhibition ELISA experiments were performed. Each serum sample (1:100 dilution) was preincubated overnight at 4°C with different concentrations (5–30 $\mu\text{g}/\text{mL}$) of the peptides Ep1, Ep5, and Ep12 and a control peptide. A peptide corresponding to amino acids 250–257 of *Leishmania* glycoprotein gp63 (IASRYDQL) was used as control peptide. Subsequently, all serum dilutions were examined

by ELISA for reactivity against the peptides, having been incubated for 2 hours at room temperature. Heterologous inhibition assays were performed following exactly the same steps as those for the homologous inhibition assays, apart from the different dilution used for each serum sample (1:160) and the concentration of the peptides (10–40 µg/mL).

Computer Predictions and Homology Search

The peptide sequences of the immunodominant epitopes were compared against the UniProtKB database (version 2010_10). The similarity search was performed using the National Center for Biotechnology Information BLASTP algorithm (version 2.2.17) and scored with PAM30 matrices. A 63% sequence homology was observed between the Ep1 peptide (amino acids 1–20; APPRLICDSRVLELYLLEAK) and the amino acids 34–52 domain of the HIV p17 matrix protein (LVCSARELERFA-VNPGLE). None of the other peptides showed any homology with human, bacterial, or viral proteins that were unrelated to Epo.

Statistics

Continuous variables were reported as mean (SD) and compared using unpaired 2-tailed Student's *t* tests. Differences between groups were considered to be statistically significant with a *P* value of <.05. The distribution of the normality of the control samples was tested by the D'Agostino *K*² test. The sensitivity and specificity of the ELISAs with the constructed peptides were calculated using the following formulas:

$$\text{Sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false negatives}}$$
$$\text{Specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}}$$

Data were analyzed using the Prism software package (GraphPad Software).

RESULTS

Epitope Mapping

Twelve 20-mer peptides, overlapping by 7 amino acids and covering the entire sequence of Epo as determined by Lai et al [15], were synthesized. The amino acid sequences of the peptides are listed in Figure 1. Epitope mapping was performed against serum samples from 3 different groups: HIV-1–infected patients with anti-EPO (group 1; *n* = 16), HIV-1–infected patients without anti-Epo (group 2; *n* = 16), and age- and sex-matched healthy blood donors (group 3; *n* = 16). Serum samples from anti-Epo–positive patients exhibited significant reactivity with the following Epo epitopes: Ep1, Ep5, and Ep12. The antibody-binding patterns for all tested peptides are shown in Figure 2. The Ep1 (amino acids 1–20) and Ep12 (amino acids 147–166) peptides both exhibited significant reactivity with 13 of the 16 serum samples from group 1, with high sensitivity (81.2%) and

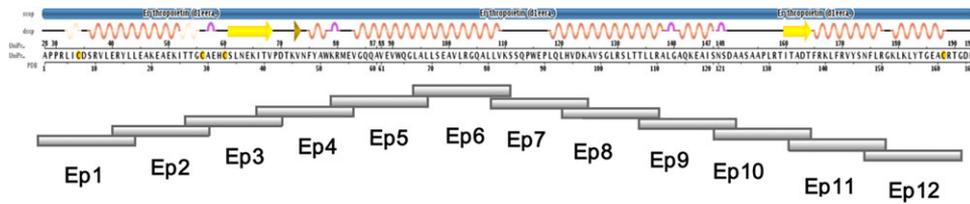
specificity (93.7%). The Ep5 peptide, corresponding to the amino acids 53–72 region of Epo, also reacted significantly with 14 of the 16 serum samples from group 1 (Figure 2), with high sensitivity (87.5%) and specificity (93.7%). Minor epitopes of the molecule were also defined. In this regard, peptide Ep7 (amino acids 53–72) was recognized by 5 of 16 serum samples, whereas all other peptides reacted with very few serum samples (Figure 2). Serum samples from anti-Epo–negative patients had no significant reactivity against any peptide; Ep4 was positive for 2 of the 16 serum samples, and Ep1, Ep3, Ep5, Ep7, Ep9, Ep11, and Ep12 reacted with only 1 of the tested serum samples. Finally, serum samples from healthy individuals gave low absorbance values for all tested peptides (Figure 2). The tendency of some group 2 serum samples to recognize peptides to a greater extent than did controls could be attributed to higher levels of background binding in HIV-1–positive serum samples due to immune activation, B-cell stimulation, and autoantibody production caused by chronic HIV-1 infection [16]. Overall, 3 distinct peptides presented significant binding reactions regarding the absorbance differences of the serum groups. The results from anti-peptide ELISA assays for Ep1, Ep5, and Ep12 are depicted in Figure 2.

Inhibition of the Antibody Binding to the Antigenic Peptides

The binding specificity of antibodies to the major epitope analogues was assessed by inhibition experiments. Several serum samples from anti-Epo–positive patients were preincubated with all 3 synthetic peptides, which led to partial inhibition in a dose-dependent manner (Figure 3). No inhibition was observed when the control peptide was used as an inhibitor. Although the levels of homologous inhibition reached a moderate level (50%–60%), this was significantly higher (~10 times) than the level of inhibition produced by the control peptide. This is common in peptide ELISAs because soluble synthetic peptides are small, flexible molecules that exist in a variety of conformations and cannot compete efficiently with the immobilized peptides that are recognized bivalently (with greater avidity) by antibodies.

Structural Features of Epo Immunodominant Regions

The 3-dimensional structural analysis of the Epo molecule [17] allowed the exact identification of the major B-cell epitopes of Epo. Ep1 and Ep12 are located at the amino (NH₂) and the carboxy (COOH) terminus of the molecule, respectively (Figure 4), and form a consecutive antibody interface stabilized with 1 disulfide bond linking Cys 7 of Ep1 and Cys 161 of Ep12 (Figure 4). An essential precondition for the biological activity of Epo is its binding to the erythropoietin receptor (EpoR), which exists as a preformed dimer (Figure 4) [18]. Epo possess 2 distinct receptor binding sites, 1 of high affinity and the other of low affinity. The high-affinity receptor binding site, located in the carboxy terminus, includes the residues Asn 147, Arg 150, and Gly 151, which belong to Ep12 epitope, whereas the low-affinity receptor binding site includes the residues Val 11,



| Peptide Name | Amino-acid residues | Sequence |
|--------------|---------------------|-----------------------|
| Ep1 | [aa1-20] | APPRLICDSRVLERYLLEAK |
| Ep2 | [aa14-33] | RYLLEAKEAENITTGCAEHC |
| Ep3 | [aa27-46] | TGCAEHC SLNENITVPDTKV |
| Ep4 | [aa40-59] | TVPDTKVN FYAWKRMEVGQQ |
| Ep5 | [aa53-72] | RMEVGQQAVEVWQGLALLSE |
| Ep6 | [aa66-85] | GLALLSEAVLRGQALLVNSS |
| Ep7 | [aa79-98] | ALLVNSSQPWEPLQLHVDKA |
| Ep8 | [aa92-111] | QLHVDKAVSGLRSLTLLRA |
| Ep9 | [aa105-124] | LTLLRALGAQKEAISPDDA |
| Ep10 | [aa118-137] | AISPDDAASAAPLRTITADT |
| Ep11 | [aa131-150] | RTITADTFRKLFVYSNFLR |
| Ep12 | [aa147-166] | NFLRGKCLKLYTGCACTGDR |

Figure 1. Secondary structure of erythropoietin molecule and the amino acid sequences of erythropoietin synthetic peptides.

Arg 14, and Tyr 15, which belong to Ep1 epitope [19]. Ep5 and Ep7 (Figure 4) are composed of amino acid residues that play no direct functional role in the receptor binding [20]. Because Ep1 and Ep12 comprise the interaction interface with EpoR (Figure 5), it is highly likely that binding of anti-Epo to this specific region can block the Epo-EpoR interaction, resulting in blunted erythropoiesis.

Sequence Homologies

Identification of the primary structure of Epo epitopes allowed the use of protein databases to search for sequence similarities with proteins of unrelated origin. The search results revealed that one of the major identified targets belonged to HIV proteins. In this regard, the epitope APPRLICDSRVLERYLLEAK (amino acids 1–20) had a 63% (12 of 19 amino acids) sequence homology to a fragment (³⁴LVCASRELERFAVNPLLE⁵²) of the HIV-1 Gag polyprotein. This amino acid sequence corresponds to a region of p17 matrix protein (MA), which is 1 of the 3 major proteins comprising Gag polyprotein [21].

Study of Ep1 Specificity and Cross-Reactions

The sequence similarity observations prompted us to construct a new peptide derived from HIV-1 Gag, which had the exact sequence of the region (amino acids 34–52) corresponding to matrix protein. A different peptide, with the same amino acid residue content in a scrambled order (VLRE-FARVPENLGESLICAL), was also constructed to be used as a control. Both peptides and Ep1 were tested against selected serum samples (n = 10) from HIV-1-infected patients with and without anti-Epo. All anti-Epo-positive serum samples that reacted with the Ep1 epitope also bound to the peptide derived from HIV-1 Gag protein (Figure 5). In contrast, none of the anti-Epo-negative serum samples recognized Ep1. Among the examined anti-Epo-negative serum samples, 1 of them (S7) exhibited a significant reactivity with HIV-1 Gag, probably as a result of the presence of antibodies against p17 [22]. Finally, none of the tested serum samples exhibited significant reactivity with the scrambled peptide. Because all anti-Epo-positive serum samples that were tested recognized

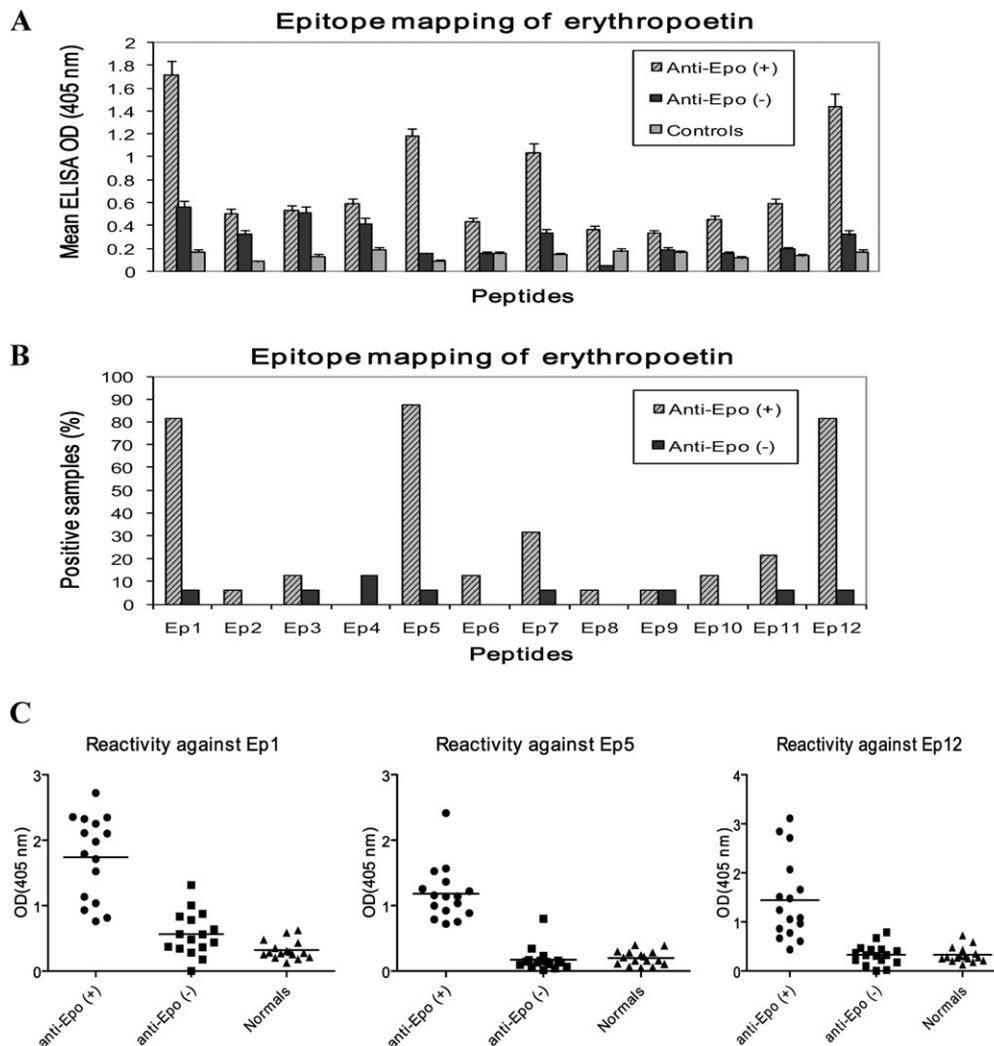


Figure 2. Epitope mapping of erythropoietin (Epo) using 20-mer overlapping synthetic peptides. *A*, Binding of the synthetic peptides to Epo, represented as the mean optical density (OD) at 405 nm, for the 3 groups of patients used in the study; serum samples from human immunodeficiency virus type 1 (HIV-1)-infected, anti-Epo-positive patients reacted significantly with 3 distinct peptides (Ep1, Ep5, and Ep12). *B*, Percentage of HIV-1-seropositive patients ($n = 32$) that reacted significantly with each synthetic peptide. *C*, Binding reaction, expressed in mean OD, of the 3 major Epo epitopes that reacted with serum samples from 16 HIV-1-infected patients with anti-Epo, 16 HIV-1-infected patients without anti-Epo, and 16 healthy blood donors.

both Ep1 and HIV-1 Gag synthetic peptides in the same extent, and to support the existence of molecular mimicry between the 2 peptides, cross-inhibition tests were conducted. In these assays, anti-Ep1-positive serum reactivity with the HIV-1 Gag peptide was inhibited up to 59% and up to 50% when the HIV-1 Gag and Ep1 peptides were used as inhibitors, respectively (Figure 6). The inhibition rates of serum reactivity with the Ep1 peptide in ELISA, when the HIV-1 Gag and Ep1 peptides were used as inhibitors, reached 52% and 62%, respectively (Figure 6). The results of the above experiments indicate a cross-reaction of antibodies directed against both Ep1 and HIV-1 Gag-related peptides.

Relation of Anti-Epo With Anemia and Levels of Erythropoietin

The 2 groups of HIV-1-positive patients were studied with regard to their Epo and Hgb levels. The group of anti-Epo-positive

patients had significantly higher mean (SD) Epo levels (49.33 [26.44] ng/mL vs 24.09 [9.46] ng/mL, respectively; $P < .001$) and lower mean (SD) Hgb levels (11.9 [1.6] g/dL vs 14.1 [1.1] g/dL, respectively; $P < .001$) compared with those of the anti-Epo-negative group of patients (Figure 7). In light of these results, the patients were categorized into 2 different groups using as a criterion the existence of antibodies against the major B-cell epitopes. Because the reactivity levels of anti-Epo-positive serum samples were similar between the Ep1 and Ep12 peptides (Spearman rank correlation coefficient [r_s], 0.93; $P < .001$) (Figure 7), the analyses were performed only for anti-Ep1. On the basis of the Ep1 epitope positivity, 13 patients were found to be anti-Ep1-positive and 19 were found to be anti-Ep1-negative. Patients in the anti-Ep1-positive group had statistically significant higher mean (SD) Epo levels

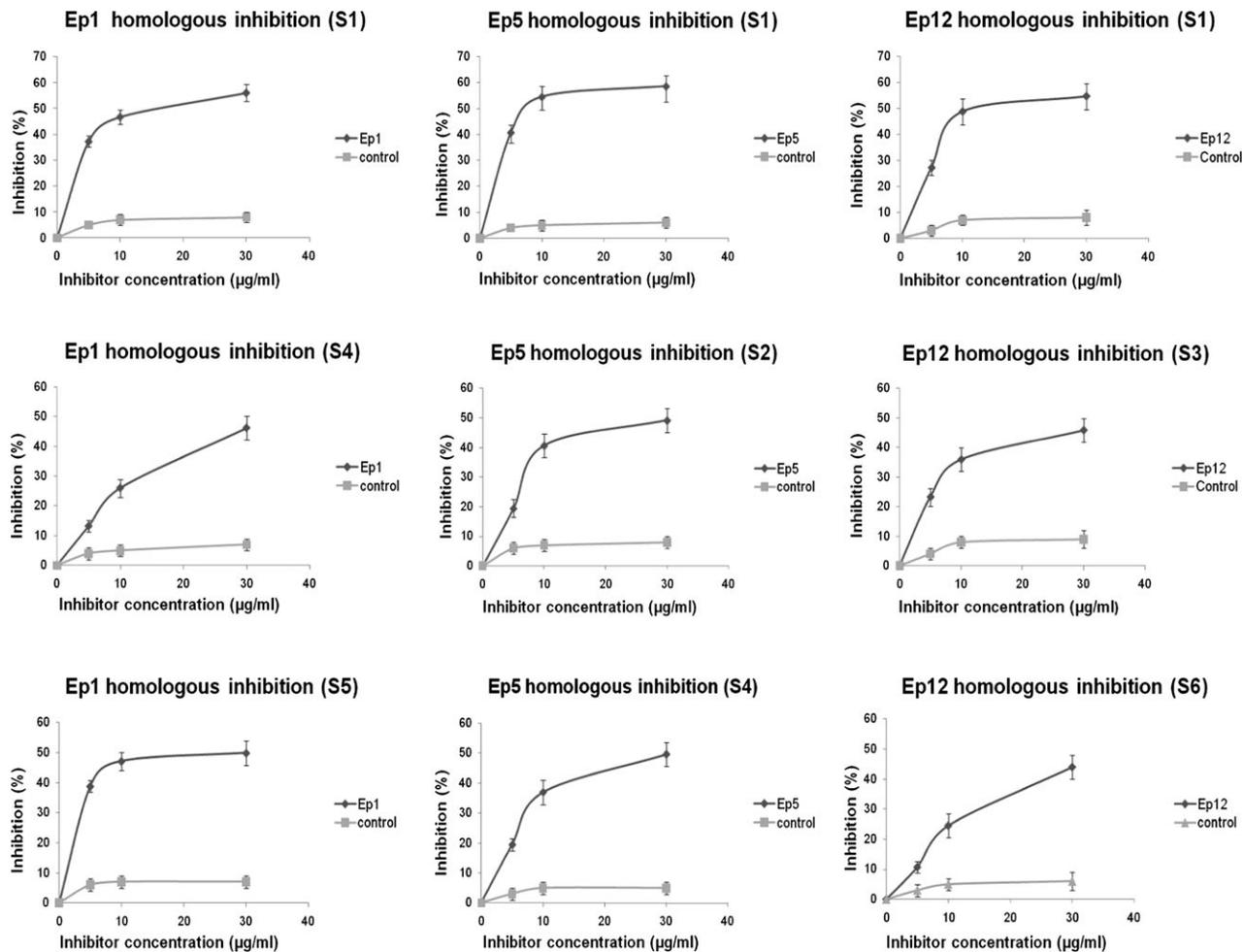


Figure 3. Results from the homologous inhibition tests using serum samples from anti-erythropoietin (anti-Epo)-positive, human immunodeficiency virus type 1 (HIV-1)-infected patients and the Ep1, Ep5, and Ep12 synthetic peptides as inhibitors. In the presence of all 3 peptides, the binding was inhibited for several serum samples (S1 - S6) in contrast to the control peptide.

(53.63 [27.32] ng/mL vs 25.15 [9.49] ng/mL, respectively; $P < .001$) and lower mean (SD) Hgb levels (11.8 [1.7] g/dL vs 14 [1.4] g/dL, respectively; $P < .001$) in comparison with the anti-Ep1-negative group (Figure 7). Furthermore, 9 (69%) of 13 anti-Ep1/13-positive patients were anemic, compared with 4 (21%) of the 19 patients in the other group ($P < .05$).

DISCUSSION

In this study, we have demonstrated that the vast majority of circulating antibodies against Epo in HIV-1-infected patients is directed against the following linear epitopes: amino acids 1–20 (Ep1), amino acids 54–72 (Ep5), and amino acids 147–166 (Ep12). Moreover, epitopes Ep1 and Ep12 belong to the functional domain of Epo, which interacts with its receptor EpoR. The Ep1 epitope showed a sequence homology with the ³⁴LVCASRELERFAVNPGLLE⁵² region of the HIV-1 p17 matrix protein. Further binding and inhibition tests verified the specific binding of Ep1 and HIV p17 Gag analogue. These data suggest

that molecular mimicry is a mechanism that contributes to the pathogenesis of anti-Epo and HIV-1-related anemia.

Anemia is the most common hematologic abnormality among HIV-1-infected patients [23], with significant implications on their quality of life, morbidity, and mortality [1]. Several pathophysiologic mechanisms have been identified as contributors to HIV-1-related anemia [24]. Among those is the blunted response to Epo, in which even very high levels of Epo fail to stimulate erythropoiesis adequately [2]; the pathophysiology of this phenomenon remains unexplained. In our previous study [8], it was demonstrated that the presence of anti-Epo in HIV-1 [24] infected patients is associated with an increased risk of anemia and elevated levels of Epo, but the role of anti-Epo was not clearly elucidated. The present study was conducted to investigate whether anti-Epo antibodies contribute to the pathogenesis of anemia in HIV-1 [24] infected patients or, like other circulating autoantibodies, have no clinical significance [6], but rather is an indicator of the persistent activation of the immune system [25].

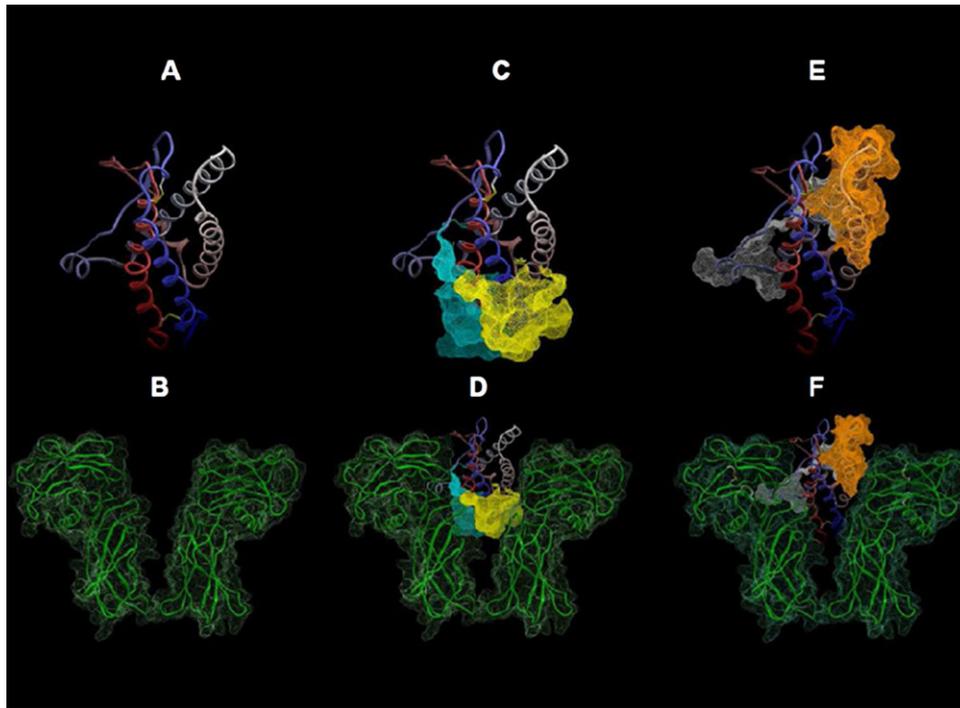


Figure 4. Topology of erythropoietin (Epo), Epo receptor (EpoR), and the Ep1 and Ep12 peptide regions in a 3-dimension configuration. *A*, Overall topology of Epo, a left-handed 4-helix bundle. *B*, Structure of the EpoR dimer. *C*, Surface representation of the 2 major antigenic epitopes of Epo, Ep1 (blue) and Ep12 (yellow). *D*, Surface representation showing the interaction of Ep1 and Ep12 with the EpoR dimer. *E*, Surface representation of Ep5 (orange) and Ep7 (gray). *F*, Surface representation showing that Ep5 and Ep7 are incapable of interacting with EpoR.

A key element in the study of the biological role of auto-antibodies is the definition of their fine specificity through the identification of their B-cell epitopes on the target autoantigen. In the present study, B-cell epitope mapping of Epo was

performed using 20-mer overlapping peptides, and the results disclosed a preferential binding of anti-Epo-positive serum samples on 3 distinct epitopes, covering the sequences of amino acids 1–20 (Ep1), amino acids 54–72 (Ep5), and amino acids 147–166 (Ep12) on the surface of the Epo molecule. The specificity of the antibody binding was confirmed by homologous inhibition experiments.

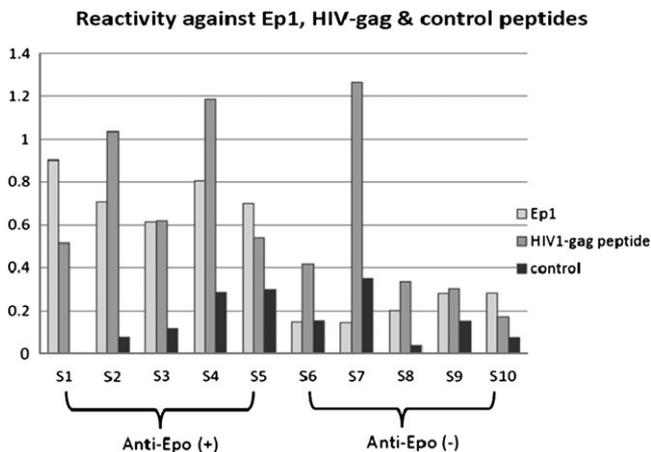


Figure 5. Levels of anti-erythropoietin (anti-Epo) production, represented as mean optical density (OD) at 405 nm, against the Ep1 peptide analogue of Epo, the human immunodeficiency virus type 1 (HIV-1) Gag fragment peptide, and the control peptide. Tested serum samples from HIV-1-infected patients were either positive (S1–S5) or negative (S6–S10) for the presence of anti-Epo.

To gain more insights on the biology of the epitopes, structural features of the Epo immunodominant regions were studied. The 3-dimensional structure analysis revealed that Ep1 and Ep12, the 2 major antigenic epitopes, are located at the amino terminus and carboxy terminus of the molecule, respectively, and come in proximity, covalently associated via a disulfide bond, thus forming a putative consecutive antigenic interface for antibody binding. The Ep1 and Ep12 epitopes are located in the region of Epo that was previously found to interact with EpoR [17], so the binding of anti-Epo to this specific domain of the erythropoietin molecule is anticipated to interrupt the Epo-EpoR interaction, leading eventually to blunted erythropoiesis.

These observations suggest that the mechanism of action of anti-Epo in HIV-1-infected patients is probably different from that described by Casadevall et al [26] in non-HIV-1-infected patients with pure red-cell aplasia during treatment with rHuEpo. In that case, the antibodies had a neutralizing effect on Epo, leading to the destruction of the molecule and undetectable

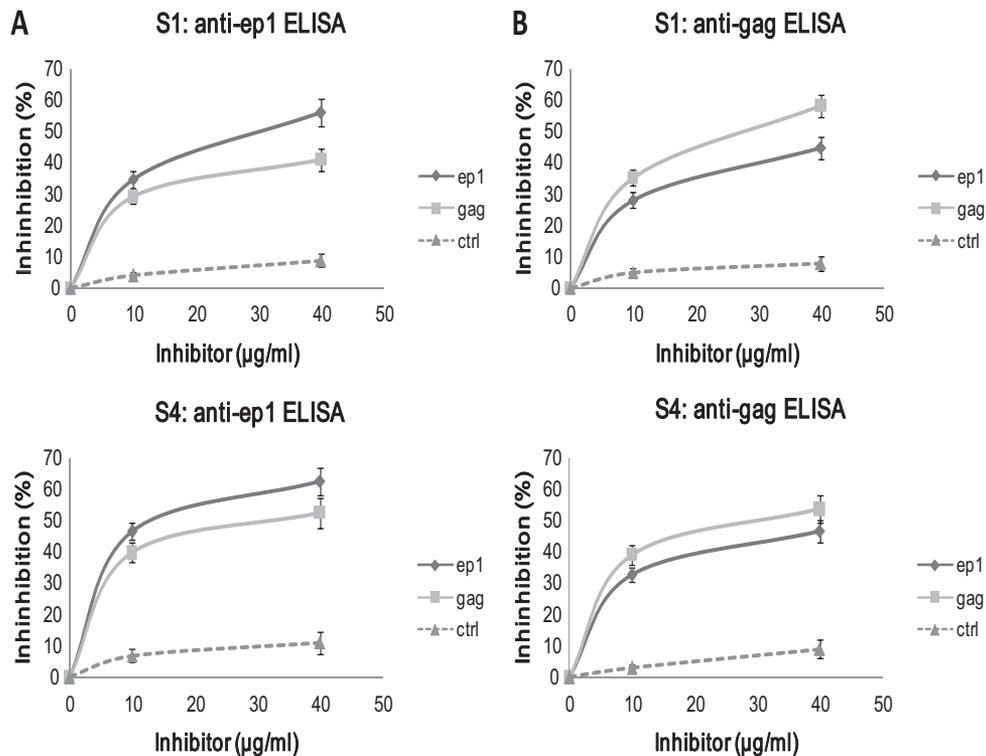


Figure 6. Cross-inhibition of serum reactivity against the antigenic peptides. *A*, Serum anti-Ep1 reactivity inhibition with the Ep1, human immunodeficiency virus type 1 (HIV-1) Gag fragment, and control peptide; *B*, serum anti-HIV-1 Gag reactivity inhibition with the Ep1, HIV-1 Gag fragment, and control peptide.

plasma levels in the majority of studied patients. In our study, similarly to a previous one [8], HIV-1-infected patients who were positive for the presence of anti-Epo exhibited significantly higher serum levels of Epo compared with anti-Epo-negative patients; this finding suggests that blockade of the Epo-EpoR interaction leads to blunted erythropoiesis and reactive production of greater amounts of Epo. An alternative hypothesis could be that the higher Epo levels, observed in anemic patients with circulating anti-Epo, are not a function of the antibodies but a physiological response to anemia. However, this issue has been addressed in our previous study [8], in which we compared anemic patients with anti-Epo antibodies ($n = 19$) with anemic patients without anti-Epo antibodies ($n = 8$). We found that anti-Epo-positive patients had significantly higher mean (SD) serum Epo levels (66.4 [83.5] ng/mL vs 24.3 [6] ng/mL, respectively; $P < .05$), whereas mean (SD) Hgb levels were similar between the 2 groups (10.86 [1.51] vs 11.41 [0.47] g/dL, respectively; $P = .8$). These findings support the hypothesis that the high Epo serum levels detected in anti-Epo-positive, HIV-1-infected, anemic patients are not only a result of the normal Epo response to anemia but also a reaction to the blunted erythropoiesis caused by anti-Epo.

Following the identification of major B-cell epitopes on the Epo molecule, a search for sequence homologies was performed in protein databases. It was found that the Ep1 epitope

presents sequence homology with the fragment 34 LVCASRELERFAVNPGLE 52 of the HIV-1 Gag polyprotein, corresponding to a region of the p17 matrix protein [27]. The Gag polyprotein consists of 3 major folded polypeptides, MA (p17), capsid (p24), and nucleocapsid (p7), and 3 smaller peptides (p1, p2, and p6) [21, 28]. MA forms the N-terminal domain of the Gag polyprotein precursor and takes part in several viral functions, including membrane binding/virus assembly and Gag targeting, Env incorporation into virions, and early postentry events [29].

The observed sequence homology between Ep1 and p17 prompted us to investigate the immunologic response against these regions in serum samples from both anti-Epo-positive and anti-Epo-negative HIV-1-infected patients. Our results showed that anti-Epo-positive serum samples contain antibodies against both peptides. Only 1 anti-Epo-negative serum sample exhibited reactivity against the Gag p17 protein, a finding that might be attributed to antibodies directed against this structural protein of HIV-1 [22]. Further inhibition and cross-inhibition assays using the peptides as inhibitors confirmed the specificity of this cross-reaction. Our data suggest that the HIV-1 matrix protein most likely induces the production of anti-Epo through a molecular mimicry mechanism. This is not surprising because several protein sequences that are shared between human and HIV-1 proteins have been previously found to be cross-recognized by autoantibodies: HLA-DR4, HLA-DR2, variable

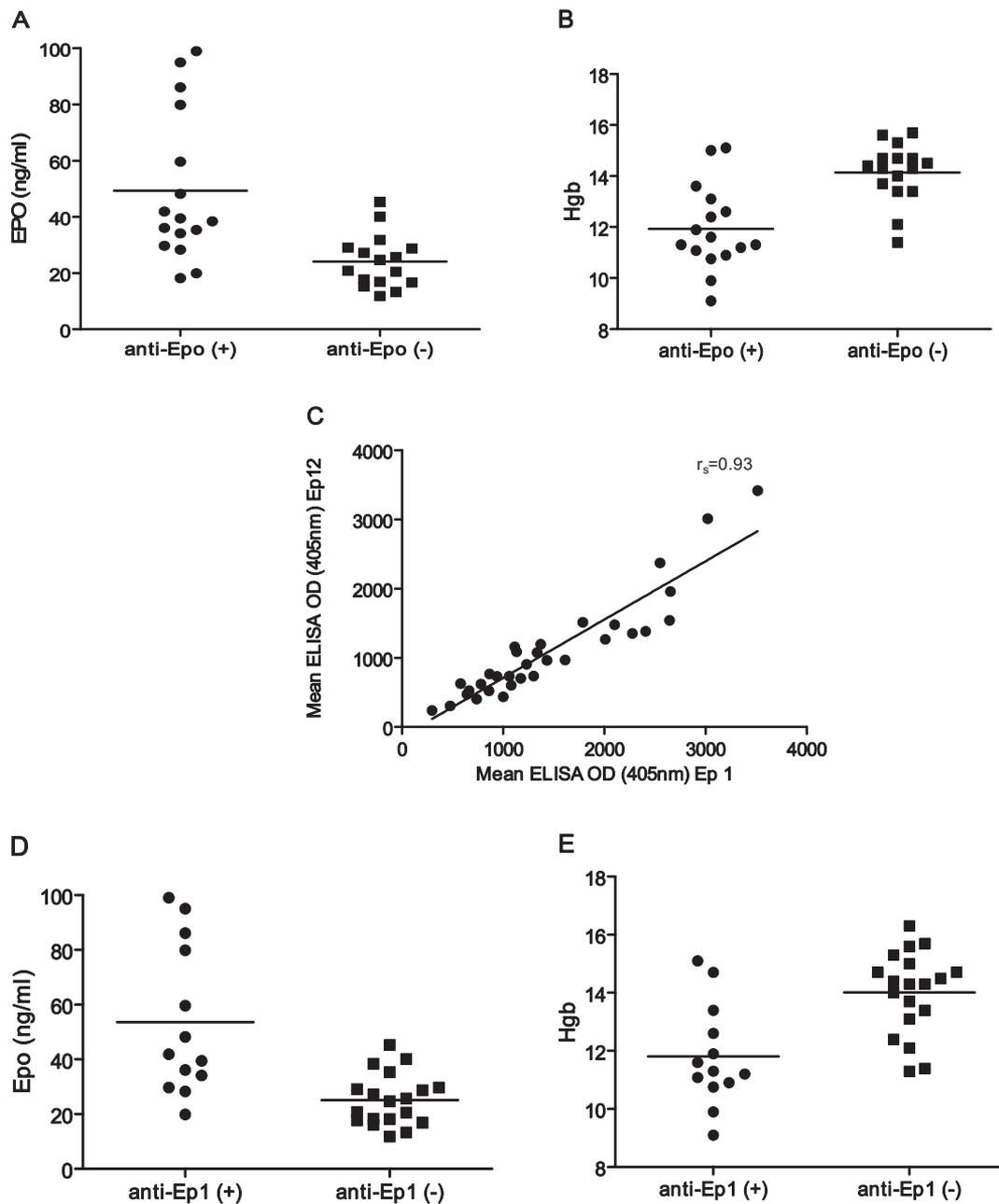


Figure 7. A, Levels of erythropoietin (Epo) in anti-Epo–positive and anti-Epo–negative serum samples. B, Levels of hemoglobin (Hgb) in anti-Epo–positive and anti-Epo–negative serum samples. C, Correlation of anti-Ep1 and anti-Ep12 reactivity levels in anti-Epo–positive serum samples. D, Epo levels in anti-Epo–positive and anti-Epo–negative serum samples. E, Hgb levels in anti-Epo–positive and anti-Epo–negative serum samples.

regions of T-cell receptor, Fas protein, several function domains of IgG and IgA, and anti-GPIIIa all exhibit significant sequence homologies with different HIV-1 proteins [30, 31].

In conclusion, our study demonstrates that anti-Epo antibodies are directed against specific linear B-cell epitopes, located in the NH₂ and COOH termini of the erythropoietin molecule. Those epitopes are located within the binding site of the molecule to its receptor and possess high sensitivity and specificity. One of the Epo epitopes (Ep1) shows molecular homology with

p17 matrix protein; therefore, the presence of anti-Epo in HIV-1–infected patients could be attributed to a molecular mimicry mechanism. Further experiments will aim to shed light on the functional implications of these antibodies.

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