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Simple Enzyme Immunoassay for Titration of Antibodies to the CD4-Binding Site of Human Immunodeficiency Virus Type 1 gp120

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We report the development of an immunoassay for the titration of antibody to the CD4-binding site (CD4BS) of the human immunodeficiency virus type 1 (HIV-1) surface glycoprotein gp120. This assay is a competitive enzyme-linked immunosorbent assay in which serum antibodies compete with labeled F105, a human monoclonal antibody whose corresponding epitope overlaps the conformation-dependent CD4BS, for binding to purified recombinant gp120 coated on a solid phase. Ninety-nine percent (109 of 110) of HIV-1-positive French patients and 91% (51 of 56) of HIV-1-positive African patients had CD4BS antibodies, indicating that the conformational CD4BS epitope is well conserved among different subtypes of HIV-1. Titers of CD4BS antibodies according to clinical status appeared to be not statistically different. A longitudinal study in 21 seroconverters showed that, for the majority of individuals, CD4BS antibodies appeared early and persisted at relatively high titers for several years. None of 21 HIV-2-seropositive patients had CD4BS antibodies in our assay, suggesting that the antibodies produced during HIV-2 infection are not cross-reactive with the CD4BS of HIV-1 gp120.

Individuals infected with the human immunodeficiency virus type 1 (HIV-1) develop neutralizing antibodies which appear to be important components of a protective immune response. Indeed, they were shown to inhibit HIV-1 infection in vitro and to prevent in vivo infection in chimpanzees (9). Although antibodies to all of the major gene products are generated, the surface envelope glycoprotein gp120 is the principal target for antibodies that neutralize virus infectivity (24, 27). Two epitopes located within the gp120 protein are responsible for the induction of the majority of neutralizing antibodies (6, 27). The principal neutralizing determinant is located in the central area of the third hypervariable domain of gp120 (V3) and is part of a disulfide bridged loop. This epitope was shown to be linear and contiguous (13, 26). Because of its variability, antibodies directed against this region usually neutralize only a limited number of HIV-1 strains (13, 20), although a subset of antibodies directed to a conserved hexapeptide in the central position of the V3 loop was shown to neutralize divergent isolates (12). The second class of neutralizing antibodies binds to a discontinuous or conformational epitope of gp120 (18), since the binding of such antibodies is lost when the protein is denatured (10). These conformation-dependent antibodies neutralize a wider range of virus isolates than antibodies directed to linear determinants (27), suggesting the presence of a conserved neutralization epitope(s). Several studies have characterized the conformation-dependent neutralizing epitope as overlapping the CD4-binding region of gp120 (11, 14). An enzyme immunoassay for antibodies blocking gp120-CD4 attachment indicated that conformation-dependent neutralizing antibodies were associated with those blocking antibodies (1). By using monoclonal antibodies (MAbs) to gp120 and mutated gp120s, amino acids important for the binding of these conformation-dependent neutralizing antibodies were identified (16, 28, 29). These amino acids are located within seven discontinuous conserved regions of gp120. Some of them are common to both the CD4-binding site (CD4BS) and the conformation-dependent neutralizing epitope (21).

Analysis of the antibody response to the principal neutralizing determinant is relatively easy by simple immunochemical methods. Immunoassays with synthetic oligopeptides representing V3 sequences have already been developed (30). In contrast, characterization of discontinuous epitope(s) overlapping the CD4BS of gp120 and the study of antibodies that bind to this epitope remain more difficult because of the restricting conditions of conformation in which gp120 must be maintained. Analysis of the antibody response directed to this epitope can be carried out either by testing the ability of antibodies to block CD4 binding to gp120 (1) or by an immunoassay with specific antibody binding to conformational epitopes. In this latter context several human MAbs derived from HIV-1-infected individuals that block gp120-CD4 binding and neutralize virus infection have been identified and constitute valuable tools (11, 16, 29). Among them the human MAb F105 has been broadly characterized (22, 23). F105 is an immunoglobulin G1 antibody that neutralizes a broad spectrum of HIV-1 strains (strains MN, RF, IIIB, and SF2) (22). The F105 epitope has been defined as conformational, including distinct elements of the discontinuous CD4BS. Two important regions for F105 recognition, at positions 256 and 257 and positions 368 to 370, correspond precisely to gp120 amino acids previously identified as important for CD4 binding (21).

Here we report the development of an immunoassay for titration of antibody to the CD4BS (CD4BS antibodies). This assay is a competitive enzyme-linked immunosorbent assay (ELISA) in which serum antibodies compete with labeled F105 for binding to a purified recombinant gp120 produced in Chinese hamster ovary (CHO) cells. The assay was used to study the prevalence of CD4BS antibodies in individuals infected with either HIV-1 or HIV-2. The kinetics of appearance and

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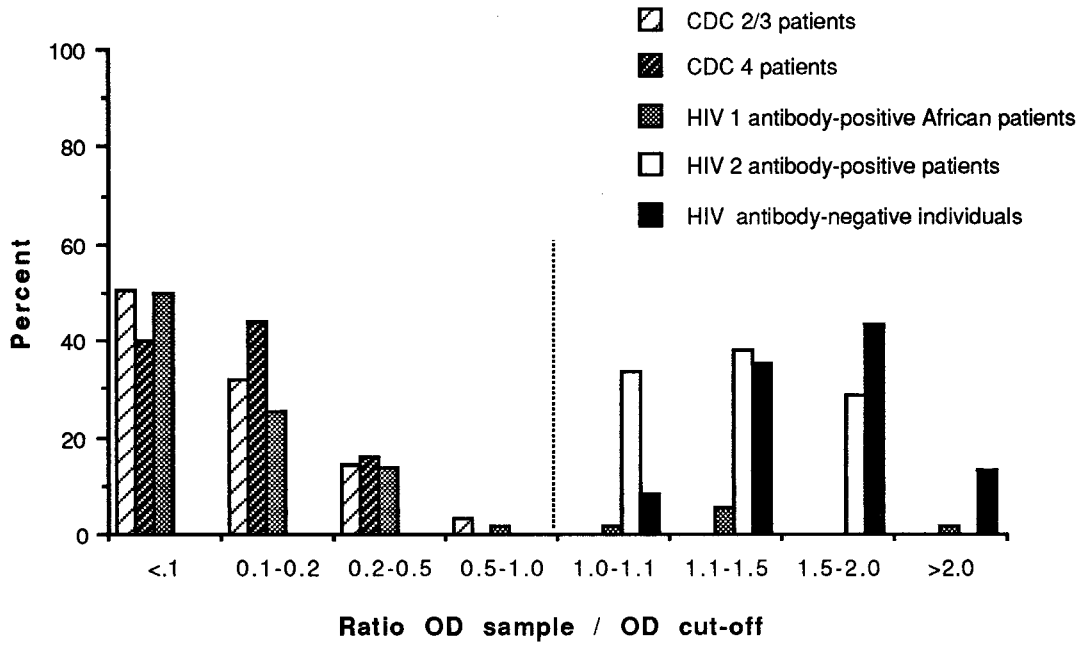

FIG. 1. Distribution of the CD4BS antibody reactivity according to the ratio OD_{sample}/OD_{cutoff} and to the various groups of tested sera. The values on the ordinate correspond to the percentage of samples fitting different categories of reactivities within a population. The dotted line corresponds to the cutoff. It shows the clear cutoff between the reactive sera (to the left of the dotted line) and nonreactive, sera (to the right of the dotted line).

the persistence of these antibodies were further analyzed in 21 seroconverters.

We used a whole purified gp120 derived from an HIV-1 MN-related strain isolated from a Dutch patient and expressed in a recombinant CHO system (gp120 W61D [4]). Biotinylated F105 MAb (Bt-F105) was used as the conjugate. Purified F105 was labeled with biotin-aminocaproate *N*-hydroxysuccinimide ester (Sigma, St. Louis, Mo.). Briefly, 100 μl of F105 at 1 mg/ml was incubated with 300 μg of biotin (2.5 mg/ml diluted in *N*-dimethyl formamide) in 1,800 μl of sodium carbonate buffer (0.2 M; pH 8.8). The ratio of biotin/MAb was approximately 1,000. Labeling was carried out for 30 min and was then stopped with 20 μl of 1 M NH_4Cl (pH 5.6). The solution was then dialyzed overnight in phosphate-buffered saline (PBS; 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.15 M NaCl [pH 7.4]). After dialysis, aliquots were stored at −20°C in 50% glycerol. Preliminary assays were designed to optimize the concentrations of gp120 (0.05 μg per well) and labeled F105 MAb (0.005 μg per well) to be used in the competition assay.

gp120 W61D was adsorbed onto Luxlon plates (CML, Nemours, France) by incubation overnight at 0.5 μg/ml in 100 μl of 50 mM sodium carbonate buffer (pH 9.6). Nonspecific binding sites were saturated for 2 h with 200 μl of a 3% solution of bovine serum albumin (BSA) in PBS at room temperature. The plates were then washed three times with PBS buffer containing 0.5% Tween 20 (PBS-T). Sera were diluted in PBS supplemented with 0.05% Tween 20 and 1% BSA (PBS-T-BSA). Twenty microliters of diluted sera was first added to each well; this was followed by the addition of 100 μl of a Bt-F105 solution diluted in PBS-T-BSA (0.005 μg per well) and incubation for 1 h at room temperature. The plates were washed three times, and subsequent incubation was performed for 30 min at room temperature with 100 μl of a 1:5,000 solution of streptavidin-peroxidase (in PBS-T-BSA). After three washings, a colored reaction was developed with 100 μl of a mixture of H_2O_2 and *o*-phenylenediamine as the substrate. Color development was stopped after 20 min at room temperature with 100 μl of 2 N H_2SO_4. The A_{490} was determined.

The cutoff of the assay was determined by running 184 randomly selected serum samples from HIV-seronegative individuals at a 1:10 dilution. The mean absorbance value (optical density [OD]) and the standard deviation (SD) were 1.564 and 0.333, respectively. Subsequently, 10 of these negative serum samples which gave ODs at about the mean value were pooled, and this pool was then used as a negative control for each run. The cutoff value was calculated for each plate by using the mean OD of four wells corresponding to the negative control, minus 2 SDs (e.g., 0.666). Titration of CD4BS antibodies was done by testing serial dilutions (1:10, 1:50, 1:250, and 1:1,250) of sera from HIV-1 or -2-seropositive patients. The endpoint titer was given by the dilution factor at which the absorbance was equal to the cutoff.

The specificity of binding of Bt-F105 to the CD4BS in our assay was verified by incubating 20 μl of serially diluted recombinant soluble CD4 solution (0.5 to 100 μg/ml, Neosystem, Strasbourg, France) with 100 μl of Bt-F105 by following the same protocol as described above. A typical dose-effect curve was observed when Bt-F105 was incubated with serially diluted recombinant CD4 (rCD4). Approximately 2 μg of rCD4 was able to inhibit 50% Bt-F105 binding.

Different categories of sera were used to study the CD4BS antibodies by our assay. We first analyzed sera from 110 HIV-1-seropositive individuals collected in the infectious diseases unit of the Centre Hospitalier Universitaire Bretonneau. Among them 85 were at CDC stage 2 or 3, and 25 were at CDC stage 4. One hundred nine (99.1%) of them were positive for CD4BS antibodies (Fig. 1). The single CD4BS antibody-negative patient was at CDC stage 2. High titers (>100) of CD4BS antibodies were found at approximately the same percentage in both symptomless patients (92.9%) and patients with AIDS (80.0%) (the difference not statistically significant). Fifty percent of the symptomless patients and 50% of the patients with

AIDS had titers greater than 455 and 356, respectively (difference not statistically significant; Student *t* test). The distribution of CD4BS antibody reactivity, on the basis of the OD_{sample}/OD_{cutoff} ratio, appeared to be slightly different between both groups. Indeed, 50.6% of patients with CDC stage 2 or 3 gave ratios below 0.1 (high reactivity), compared with 40% of patients with CDC stage 4 whereas 31.8% of patients gave ratios with CDC stage 2 of 3 of between 0.1 and 0.2 (moderate reactivity), compared with 44% of patients with CDC stage 4. However, these differences were not statistically significant.

Because distinct subtypes of HIV-1 have been described on the basis of the diversity of the nucleotide sequences (19) and because these subtypes are generally confined to specific geographical regions, we looked for the presence of CD4BS antibodies in sera from 56 HIV-1 infected African patients. Thirty serum samples came from patients living in Burundi (2), and 26 serum samples came from patients living in Côte d'Ivoire (8). On the basis of a recently developed V3 immunoassay for HIV-1 subtyping (unpublished data from our laboratory [F. Barin]), 43 of the serum samples were reactive to subtype C variants, 4 were reactive to subtype D variants, and 9 were reactive to subtype A variants. By contrast, sera from all of our European patients were reactive to subtype B. Fifty-one (91.1%) of the serum samples from African patients were positive for CD4BS antibodies. Although five serum samples were negative, the difference between the prevalences in European or African patients was not statistically significant. Moreover, CD4BS antibody reactivities were quantitatively similar in African patients and European patients (Fig. 1).

Serum samples from 21 HIV-2-seropositive individuals, collected either in France or in Côte d'Ivoire, were also analyzed. Serotyping of HIV-2 infection was done as described previously (3). None of them had CD4BS antibodies in our assay (Fig. 1). However, the distribution of OD_{sample}/OD_{cutoff} ratios were slightly different when comparing HIV-2 antibody-positive sera and HIV antibody-negative sera. Indeed, 71.4% of HIV-2-positive serum samples gave ratios between 1.0 and 1.5, compared with 43.4% of HIV antibody-negative serum samples, whereas 28.5% of HIV-2-positive serum samples gave ratios greater than 1.5, compared with 56.5% of HIV antibody-negative serum samples.

Analysis of 123 sequential serum samples collected from 21 HIV-1 seroconverters allowed us to study the kinetics of CD4BS antibodies. Three different criteria were used to select a patient as a seroconverter. (i) Five hemophiliac patients, whose virological status was regularly followed up, seroconverted between 1982 and 1985. Delays between the last negative HIV serology and the first positive HIV result ranged from 4 to 11 months. For these patients the approximate date of seroconversion was estimated as the mean time between these two dates. (ii) Eight patients presented with a primary HIV-1 syndrome confirmed by a positive p24 antigen assay. Their primary infection was confirmed by analysis of subsequent sequential serum samples showing the appearance of antibodies to HIV-1. (iii) Eight patients presented with a first serum sample showing a typical profile of seroconversion (presence of either antibodies to p24 or antibodies to gp160 or both [25]), and subsequent sera showing an increase in reactivities, confirming the effective seroconversion.

For 12 seroconverters, early sequential serum samples collected shortly after primary infection were available, allowing us to study the delay of appearance of CD4BS antibodies. Among them, six were positive 4 months after seroconversion and four had CD4BS antibodies 12 months after seroconversion. Two individuals, who were negative at 4 months, were subsequently lost to follow-up. For nine seroconverters a long follow-up was available (between 5 and 9 years), allowing us to study the persistence of CD4BS antibodies. All of them developed CD4BS antibodies (Fig. 2), with a maximum titer before the fourth year following seroconversion except in one subject. After reaching this maximum titer, CD4BS antibodies either remained stable or decreased slowly. In only one patient, who responded weakly to CD4BS (maximum titer at year 2 after seroconversion, 66), CD4BS antibodies became undetectable 7 years after seroconversion.

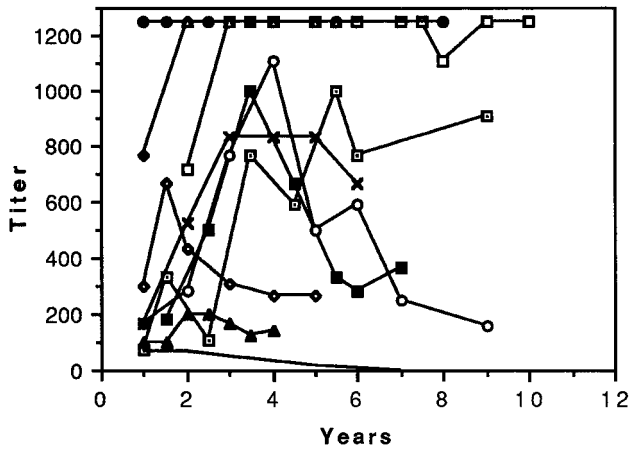

FIG. 2. Persistence of CD4BS antibodies in HIV-1 seroconverters. Each symbol represents a different seroconverter.

By our assay, the presence of CD4BS antibodies was detected in most of the HIV-1-positive individuals tested, in agreement with a previous study which used an assay based on the inhibition of gp120-CD4 attachment (1). These results also agree with data showing that antibodies to the discontinuous CD4BS are prevalent in HIV-1 antibody-positive sera (18). Although CD4BS antibody titers appeared slightly higher in sera from symptomless patients when compared with that in sera from patients at advanced stages of infection, no statistical difference was established between groups of patients at different stages. This lack of correlation between CD4BS antibody titers and disease progression has already been described (1, 5).

The kinetics of CD4BS antibodies were studied in 21 seroconverters: CD4BS antibodies arose early in the majority of seroconverters (no later than 4 months after seroconversion), and relatively high titers persisted for several years in most of them. These longitudinal results are in agreement with the fact that CD4BS antibodies are found in patients at CDC stage 4. This observation suggests that persistent CD4BS antibodies may be unable to stop virus spread or the clinical evolution during natural infection. However, only longitudinal studies will provide precise information on the clinical significance of these antibodies. Our study with sera collected early after infection confirmed previous data by Moore et al. (17), who showed that CD4BS antibodies were among the earliest detectable anti-gp120 antibodies during seroconversion.

CD4BS antibodies were also detected in 91.1% of HIV-1 antibody-positive African individuals. Both prevalence and titers were comparable to those observed for European patients. The four African patients whose sera clearly reacted predominantly to the V3 peptide of the D subtype, described as the most divergent V3 sequence (15), were highly positive for CD4BS antibodies. These data indicate that the conforma-

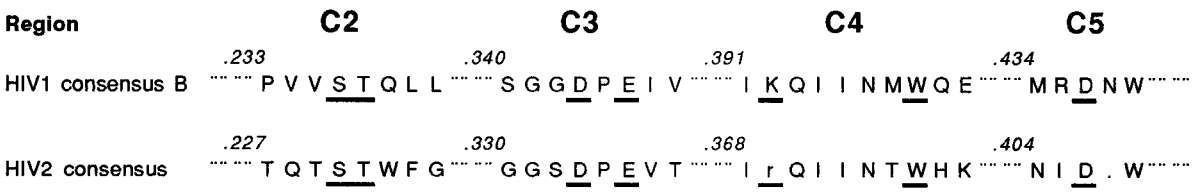

FIG. 3. Alignment of regions involved in CD4 binding according to Thali et al. (29) and Myers et al. (19). Amino acids important for binding of the F105 MAb to HIV-1 gp120 and for gp120-CD4 attachment are underlined. All of them except for the lysine at position 392 are conserved in the HIV-2 surface glycoprotein. The lysine at position 392 is replaced by an arginine in the majority of HIV-2 strains.

tional CD4BS epitope is well conserved among different subtypes of HIV-1 and confirm its importance as an immunogenic target.

In contrast, none of the 21 HIV-2 antibody-positive specimens tested positive for the presence of CD4BS antibodies, suggesting that antibodies generated during HIV-2 infection are not cross-reactive with the CD4BS of HIV-1 gp120. Thali et al. (28) pointed out the amino acids important for the binding of the F105 MAb to the discontinuous epitope overlapping the CD4-binding region of HIV-1 gp120. It is noteworthy that these amino acids are conserved at almost the same positions in the HIV-2 surface envelope glycoprotein (Fig. 3). The fact that antibodies present in HIV-2 antibody-positive sera did not react with the HIV-1 CD4BS in our assay, in spite of the conservation of critical amino acids for CD4 binding between the surface envelopes of both viruses, suggests that these residues should be essential for preserving the conformation of the CD4BS but would not participate at the antibody-binding site. As has been shown previously for picornaviruses, by the "canyon hypothesis," the receptor attachment site might be located in a surface depression and neutralizing immunogenic sites should be mapped on exposed parts (25).

Because of the conformation-dependent nature of the CD4BS, broad studies on the CD4BS antibody response are rare. The first objective of our work has been to develop a simple assay for the titration of antibody to CD4BS. Since previous studies done in our laboratory showed the difficulty in obtaining a sufficiently specific assay for the titration of antibodies blocking HIV-1 envelope-CD4 attachment (unpublished data), we preferred to develop a competitive ELISA using a gp120-coated solid phase and the labeled F105 MAb whose binding epitope has been described to overlap the CD4BS (22, 23, 29). The assay is easy to perform, allows quantitation of the CD4BS antibody response, and permits the analysis of a large number of serum samples. Simple assays able to detect and quantify CD4BS antibodies are necessary both to try to understand the role of these antibodies during natural infection and to study the capacity of prototype vaccines to induce such a potentially important immune response.

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