Variability and Immunogenicity of Human Immunodeficiency Virus Type 1 p24 Gene Quasispecies

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Despite the conserved nature of the human immunodeficiency virus type 1 (HIV-1) gag gene, multiple quasispecies of the p24 gene coexist in HIV-1-infected patients. We cloned and sequenced 31 p24 genes from four HIV-1-infected patients. The intrapatient homology between the p24 genes ranged from 97.1 to 99.1%, whereas the interpatient homology ranged from 91.5 to 93.8%, suggesting a host-specific evolution. Synonymous and nonsynonymous nucleotide changes were evenly distributed in the p24 gene, with 27% and 28%, respectively, located within host human leukocyte antigen class I recognition sites. This would suggest only a minor influence from the host cytotoxic T-cell response on the evolution of the p24 gene. The importance of minor variations within p24 was analyzed by designing DNA-based immunogens from two distinct p24 quasispecies genes simultaneously derived from one patient. In plasmid-immunized H-2b, H-2d, and H-2k haplotype mice, a clear influence from the host major histocompatibility complex was noted on the immune responses, fully consistent with those noted when a recombinant p24 protein is used as the immunogen. The two p24 DNA immunogens did not differ in their immunogenicity, indicating that the limited genetic variability (<1%) had little influence on the immune responses.

The human immunodeficiency virus type 1 (HIV-1) p24 capsid protein is released from the central portion of the Gag polyprotein by two cleavages mediated by the viral protease. The mature form of p24 contains approximately 240 amino acids and constitutes the major subunit of the nucleocapsid. It has become clear that HIV-1 rapidly adapts to a new host by continuously changing the sequence of the viral proteins which are recognized by the host immune system. The cellular immune responses are generally believed to be of critical importance in controlling the HIV infection (4, 8, 10, 11, 20). However, little is known about how the immune system recognizes the virus present in the host.

Most previous studies on HIV-1 immunogens have been performed by using immunogens based on laboratory prototype strains of HIV-1 (6). We know today that the difference between the sequence of a laboratory-based immunogen and that of the virus existing in patients greatly exceeds the variability already present within each patient (3). Consequently, even if an immunogen-specific immune response is elicited within an infected host, there is a high probability that it will not recognize the multiple viral variants or quasispecies present in the host. This might be one of the reasons why all HIV-1 vaccines tested to date have failed to show any clinical benefit (6).

We recently noted that the evolution of the well-conserved p17 gene within an infected host is in part influenced by the contact regions between the virus and the host class I-restricted immune response (3). Recombinant protein immunogens based on two members of the p17 quasispecies from the same patient and with a 92.4% homology were found to have distinct antigenic and immunogenic properties (2). Thus, despite sequence homologies between p17 quasispecies of >90%, these quasispecies have distinct properties. We were now interested to study whether minute sequence variations might influence the immune responses to patient-based genetic immunogens.

MATERIALS AND METHODS

Human serum samples. Plasma samples were selected from four HIV-1-seropositive patients (A, B, C, and D) described in detail previously (3). All patients were monitored at the Division of Infectious Diseases, Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden. All patients were men (age range, 27 to 38 years) who were infected by sexual transmission of HIV-1 subtype B. None had received any antiviral therapy prior to sampling.

HLA class I typing of each patient had been performed previously using sequence-specific primers and PCR (3). The HIV-1 subtype of each patient had earlier been determined by sequencing the variable third domain of gp120. This analysis showed that all studied patients were infected by HIV-1 subtype B (3).

Mice. C57BL/6 (H-2b), BALB/c and B10.D2 (H-2b), B10.M (H-2d), CBA and B10.BR (H-2k), and B10.S (H-2k) mice were purchased from BK Universal, Sollentuna, or Harlan, Oxon, United Kingdom. All mice were used at 4 to 6 weeks of age.

Isolation, amplification, cloning, and sequencing of p24 genes. Virions in plasma were disrupted by treatment with a buffer (pH 7.5) containing 50 mM Tris·HCl, 10 mM EDTA, 50 mM NaCl, 0.5% sodium dodecyl sulfate, and 10 mg of protease K per ml for 60 min. Thereafter, total viral mRNA was isolated by oligo(dT)-coated magnetic beads (Dynabeads; Dynal A.S., Oslo, Norway). Reverse transcription was done at 42°C for 60 min (Moloney murine leukemia virus; Boehringer Mannheim GmbH, Mannheim, Germany) using the downstream primer p24out5: 5'-CTTTGCCACAATTGAAACACTT-3' and the p24out3' primer. Amplification was carried out according to the following protocol: preheating for 4 min at 95°C, followed by 30 cycles at 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Finally, there was an elongation step of 4 min at 72°C. For the second round of PCR, we used the p24start primer containing an EcoRI restriction site: 5'-CGGTTGGAGATCCTGGCTACACCACAGTCGAC-3' and the p24out3' primer. Amplification was carried out according to the following protocol: preheating for 4 min at 95°C, followed by 30 cycles at 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Finally, there was an elongation step of 4 min at 72°C. For the second round of PCR, we used the p24start primer containing an EcoRI restriction site: 5'-CGGTTGGAGATCCTGGCTACACCACAGTCGAC-3' and the p24stop primer containing an XbaI restriction site and a stop codon: 5'-CGGTTGGAGATCCTGGCTACACCACAGTCGAC-3'. The second round of PCR was performed under identical conditions as the first PCR except for a lower annealing temperature (50°C). The PCR fragment corresponding to the expected size of p24 was extracted from the agarose gel (2%) and purified (QIAquick gel extraction kit; Qiagen GmbH, Hilden, Germany).

Amplified p24 genes were cloned in the eucaryotic expression vector pcDNA3.1C/His (Invitrogen, San Diego, Calif.) containing a cytomegalovirus promoter and a ColE1 origin of replication followed by a multiple cloning site.
and an ampicillin resistance gene. The purified gene products and the vector were digested with EcoRI and XbaI (Boehringer Mannheim) for 2 h at 37°C. Ligation of the ampicillin vector with the DNA was performed at a molar proportion of 1:1 using T4 DNA ligase (GBCO Life Technologies, Gaithersburg, Md.) for 30 min at room temperature. For transformation, 2 μl of the ligation mixture was added to 25 μl of DH5α-competent E. coli. Cells were spread on Luria-Bertani agar containing ampicillin (50 μg/ml). Growing clones were selected and checked for the p24-encoding gene by PCR using the p24-start and p24-stop primers. The plasmid DNA of the p24-positive clones was further purified with the QIAprep plasmid kit (Qiagen).

The DNA was thereafter prepared for sequencing according to the dideoxy chain termination method. Sequences were read using the Cy5 AutoRead sequencing kit (Pharmacia Biotech, Uppsala, Sweden) in conjunction with an ALFexpress sequencer (Pharmacia Biotech). Sequencing was performed using the T7 upstream primer (5′-Cy5-TAATACGACTCACTATAGGG-3′) and the Sp6 downstream primer (5′-Cy5-AGCTATGATATAGCAGAATG-3′). Locations of defined human p24 cytotoxic-T-lymphocyte (CTL) epitopes which correspond to the HLA restriction elements of the four patients were derived from the HIV Molecular Immunology Database (http://hiv-web.lanl.gov/immunology/index.html), as previously described (3). Alignment and phylogenetic analysis of the p24 sequence homology were carried out by using the GeneWorks 2.3 (Mountain View, Calif.) software package. Dendrograms were constructed using the unweighted pair group method with arithmetic mean (UPGMA) (GeneWorks 2.3).

DNA-based immunogens and in vitro translation. Protein expression of the p24 protein was analyzed using the T7 coupled ribosomeway system (Promega Corp., Madison, Wis.). In vitro translation of plasmids was performed at 30°C and the translation products were labeled using [35S]Met/selenomethionine (Amersham International plc, Little Chalfont, Buckinghamshire, United Kingdom). [35S]Met-labeled proteins were resolved using sodium dodecyl sulfate–15% polyacrylamide gels. The translation products were visualized by autoradiography on X-ray film (Hyperfilm MP; Amersham) for 18 h.

Recombinant protein and plasmid immunizations. A p24/p17 fusion protein (12) was kindly provided by Darrel L. Peterson, Virginia Commonwealth University, Richmond, Va. Groups of three or four mice were immunized intraperitoneally with 100 μg of p24/p17 protein emulsified in Freund’s complete adjuvant and received boosters of the same dose in incomplete adjuvant 4 weeks later. The mice were bled at 4 and 6 weeks after the first immunization. Titers of antibody to the immunogen were determined using microplates coated with p24/p17 fusion protein at 0.5 μg/ml and assay protocols previously described (22).

Genetic immunizations were performed with groups of four or five mice; mice received inoculations in tibialis anterior muscles 5 days following injections of 50 μl of 10 mM cardiotxin in phosphate-buffered saline (7). Purified plasmids were resuspended in phosphate-buffered saline and were injected at 100 μg of DNA per mouse. The mice were given boosters at weeks 7 and 12.

ELISAs. HIV-1 p24 antibody titers in the serum of the DNA-immunized mice were measured by Abbott HIV-1/HIV-2 3rd Generation Plus enzyme immunoassay (ELISA) (Abbott Diagnostics Division, Chicago, Ill.). Serum samples from each group of mice were pooled and were tested at a final dilution of 1:50. To detect murine antibodies, alkaline phosphatase-labeled goat anti-mouse immunoglobulin G was used (Sigma). The incubation times for the murine and goat antibodies were 45 min. The substrate dinitrophenyldiamine was used to indicate bound substrate, and absorbancies were read at 405 nm. All murine samples received inoculations in tibialis anterior muscles 5 days following injections of 50 μl of 10 mM cardiotoxin in phosphate-buffered saline (7). Purified plasmids were resuspended in phosphate-buffered saline and were injected at 100 μg of DNA per mouse. The mice were given boosters at weeks 7 and 12.

In vitro recall assays. T-cell recall assays were performed as previously described (18, 22). In brief, 3 × 10⁶ spleen cells were suspended in 100 μl of Click’s medium were added to sterile 96-well microtiter plates. The cells were incubated with and without fivefold dilutions of recombinant p24 antigen (human T-cell lymphotropic virus IIIIB [HTLV-IIIIB]) (Intracell Corporation, Cambridge, Mass.) starting at 7.5 μg/ml. After 72 h, 1 μCi of [³H]thyminidine (Amersham) was added to each well and the cells were incubated for another 16 to 20 h. The cells were harvested onto cellulose filters and quenched, and the level of incorporated radiolabeled nucleotide was determined by liquid scintillation in a beta counter.

RESULTS

p24 sequence analysis. Thirty-one clones from four HIV-1 positive patients were sequenced and analyzed, and for 28 clones, full-length p24 sequences could be obtained (Fig. 1). The first 23 and the last 18 nucleotides of each clone corresponded to regions covered by the PCR primers. Thus, the p24 sequence which was used for analysis contained 647 bp coding for 215 amino acids.

Sequence alignment revealed a 97.1 to 99.1% intrapatient sequence homology (Table 1). The interpatient homology ranged from 91.5 to 93.8%. A total of 60 base substitutions were found when the individual p24 sequences were compared with the respective intrapatient consensus sequences. Approximately 0.34% (range, 0.21 to 0.46%) of the total number of analyzed nucleotides showed changes.

Eighteen (30%; range, 23 to 40%) nucleotide mutations were nonsynonymous, whereas 42 (70%; range, 60 to 77%) were synonymous. The frequency of transitions (80%) was higher than that of transversions (20%) and was dominated by the nucleotide change from A to G (50%), followed by C to T (27%) and T to C (17%).

The inter- and intrapatient homologies of the p24 genes were compared to the previously reported p17 gene variabilities of the same patients (Table 1) (3). As expected, the analysis showed that the inter- and intrapatient homology was higher for the p24 gene than for the p17 gene in all patients analyzed. Thus, despite the fact that these are conserved neighboring genes encoding proteins that are not present on the viral surface, they show different degrees of variability.

Analysis of p24 sequence variability in relation to locations of host p24 HLA class I recognition sites. Previously identified CTL epitopes within p24 were matched with the HLA class I restriction element of each patient, as described previously (3). p24 CTL epitopes have been more precisely mapped for the restriction elements HLA-A2, -B7, -B8, -B12, and -B27, which are present among the four patients. An average of 14% (range, 7 to 20%) of the patient-derived p24 sequences corresponded to known host p24 HLA class I recognition sites (Fig. 2). Out of the 18 amino acid changes, 3 (17%) were located inside while 15 (83%) were located outside p24 epitopes recognized by the HLA class I recognition sites of the host. Thus, the distribution of mutations showed no preference for HLA class I recognition sites. One minor observation is that two out of the three mutations detected in the p24 genes of patient C resided within an HLA-A2-restricted recognition site (Fig. 2).

Construction of p24 gene-based plasmid immunogens. Two members of the p24 quasispecies derived from patient A were represented by the clones C and E, which were used for the construction of p24-expressing plasmids (p24DNA-C and p24DNA-E). The clones were selected according to their few but distinct differences in amino acid sequences, residue 47-Pro instead of Ala (p24DNA-C) and residue 79-Glu instead of Gly (p24DNA-E) (Fig. 2). Also, the two clones differed from the HIV-1p17 p24 sequence by two amino acids within an H-2d-restricted T-cell site (21), but all sequences were identical within an H-2d-restricted T-cell site (14) (Fig. 3). The p24-C and p24-E p24 genes were reamplified with primers containing XbaI restriction sites and were inserted into the pcDNA3.1C/His vector for protein expression, as shown in Fig. 2.

The expression of p24 protein was analyzed by in vitro translation using a rabbit reticulocyte lysate assay. Both plasmids express proteins of 24 kDa, confirming the integrity of the cloned genes (Fig. 3). Also shown is the positive 60-kDa control plasmid included in the kit and a plasmid expressing a 70-kDa hepatitis C virus nonstructural 3/4A fusion protein (Fig. 3).

Immune responses to recombinant and genetic p24 immunizations. As a reference for the murine responder hierarchy and the immunogenicity of recombinant p24, groups of H-2d and H-2k mice were immunized with the recombinant p24/p17 fusion protein in adjuvant. The antibody titers were around 1:1,000 in the primary response (after 4 weeks) and from 1:16,667 to 1:129,167 in the secondary response (after 6 weeks) (Fig. 4).

Groups of four or five mice of haplotypes H-2d, H-2k, and H-2s were primed and given boosters with 100 μg of either p24DNA-C or p24DNA-E at weeks 0, 7, and 12. Serum sam-
FIG. 1. Phylogenetic analysis of the 31 full-length p24 sequences derived from four patients infected with HIV-1 subtype B. Dendrograms were generated using the UPGMA algorithm supplied with the GeneWorks 2.3 software package. The two boxed sequences of patient A indicate clones C and E, which were used for the design of DNA immunogens. Also given is the HLA class I type of each patient. Each clone that contains an amino acid change within the HLA restriction elements of the host is underlined, and the respective restriction element is indicated.
TABLE 1. Comparison of the inter- and intrapatient variability of the p17 and p24 genes in four patients infected with HIV-1 of subtype B

| Patient | p17 Nucleotide homology (%) | p24 Nucleotide homology (%) |
|---------|-----------------------------|-----------------------------|
| A       | A 98.2 B 87.9 C 90.2 D 84.9 | A 98.2 B 93.2 C 93.2 D 91.5 |
| B       | A 96.7 B 89.4 C 87.9 D 99.1 | A 99.1 B 93.8 C 92.1 |
| C       | A 93.7 B 84.9 C 98.1 D 93.0 |
| D       | A 97.2 B 97.2 C 97.1 |

ples were collected at 2- to 4-week intervals. Serum samples were pooled, and the presence of anti-p24 antibodies was determined by a commercial EIA. At week 16, all animals were sacrificed, and splenocytes were collected for T-cell proliferation assays. Antibodies to p24 became detectable within 5 to 10 weeks from first immunization (Fig. 4). The H-2b and H-2d haplotypes were the earliest and best responders, while the H-2k haplotype was consistently a slow and low responder. This is fully consistent with the rp24 immunizations (Fig. 4), which confirms the influence from the murine major histocompatibility complex (MHC) genes on the responder status. No difference between the humoral responses to the p24DNA-C and p24DNA-E immunogens was noted, showing that the minor sequence variations between the p24DNA immunogens did not influence the ability to respond to HIV-1 p24. Overall, the responses primed by the recombinant protein immunizations were quantitatively much stronger than those recorded following the DNA-based immunizations (always <1:500).

The p24-specific proliferative responses in the two best antibody responder haplotypes confirmed the immunogenicity of the DNA immunogens on the T-cell level (Fig. 4). p24-specific T-cell proliferation could be recalled at p24 concentrations as low as about 10 to 100 ng/ml regardless of the haplotype and regardless of the immunogen. It should be noted that the primed T cells were recalled by p24 from the IIIB strain, which differs by two amino acids within an H-2b-restricted T-cell site.

FIG. 2. Alignment of all 28 sequenced full-length p24 clones from the four HIV-1-infected patients. The consensus sequence derived from the 28 clones is given at the top of the alignment. Homologies with the consensus are indicated by a dot. Also shown are the locations of known CTL epitopes within p24 and the responsible restriction element. For each patient, the sequence corresponding to an epitope recognized by the host restriction element is boxed. The sequences of clones C and E of patient A, which were used for the design of DNA immunogens, are indicated by arrows.
(Fig. 3) (21), whereas all sequences were identical within an H-2d-restricted T-cell site (14). This sequence difference may explain the slightly less efficient recall of p24DNA-C and p24DNA-C-primed H-2b-restricted T cells than that of the H-2d-restricted T cells (Fig. 4).

DISCUSSION

Due to the high replication rate and low fidelity of the reverse transcriptase, HIV-1 has an extremely high mutation rate, resulting in changes in the antigenicity (9, 16). These factors are likely to be major obstacles in both antiviral therapies and the development of HIV-1 vaccines. By accumulating mutations, HIV-1 may render itself less sensitive to the host humoral and CD4+ and CD8+ cellular immune responses. This has now been evidenced by the failure of all human HIV-1 vaccine trials in humans to prevent or to modulate the infection (6).

Most HIV-1 immunogens so far have been based on viruses, proteins, or genes from laboratory strains of HIV-1. For example, it was recently shown that the sequence differences between the gp120 of the HTLV-IIIB and the SF-2 subtype B strains result in distinct immunogenicities in mice (1). In particular, A.SW mice were antibody responders to gp120 of the SF-2 strain and were nonresponders to gp120 of the IIIB strain (1). This is most likely explained by the fact that these two gp120 proteins have an amino acid sequence homology of only around 83%. We recently found that despite a 92% homology between two members of the HIV-1 p17 quasispecies, the two genes encode proteins which are antigenically and immunogenically distinct in both humans and mice (2). It could therefore be questioned whether it is at all possible to induce immune responses by vaccines based on even slightly varying proteins of HIV-1 that actually recognize a wild-type virus that has adapted to the host immune response. To further address this question we have now performed similar analyses using the highly conserved p24 gene.

Although our analysis is limited by the number of clones sequenced and the incomplete definition of CTL epitopes for all HLA restriction elements, we found that both synonymous and nonsynonymous mutations seem to be evenly distributed throughout the HIV-1 p24 gene. In contrast to the more variable p17 gene (3), no clear clustering of the nonsynonymous mutations was found within host HLA class I-restricted recognition sites within the p24 gene. These observations could, apart from the already-mentioned limitations, also be secondary to the possibility that mutations within p24 may be lethal for the virus or that changes in p24 CTL epitopes occur immediately after infection, thereby adapting the virus to the new host. Several studies have indicated that CTLs to p24 are present within the infected host, which suggests that these types of responses are induced (5, 11, 13). Although both p17- and p24-related CTL epitopes have been proposed to undergo
immune escape, for example through antagonism, no clear correlation has been found between the p24-specific CTL responses and the rate of disease progression (15). CTLs have in some cases even been found to be deleterious for the infected host (13). In contrast, it was recently shown that the p24-specific CD4⁺ proliferative T-cell response correlates with the ability to control HIV-1 viremia (17).

Using two members of the p24 quasispecies from one infected individual, we designed DNA-based genetic immunogens. The translation products from the two plasmids were of

FIG. 4. Immunogenicity of recombinant p24/17 fusion protein in two murine haplotypes (H-2ᵇ and H-2ᵈ). Groups of three or four mice were immunized and given boosters with p24/17 protein as described in Materials and Methods. The mice were bled at weeks 4 and 6, and the sera were tested for specific total immunoglobulin G in serial dilutions by the p24/17 EIA as described in the text. Values are given as the mean endpoint titers (a). Also shown is the immunogenicity of the p24DNA-C and p24DNA-E plasmids in H-2ᵇ, H-2ᵈ, and H-2ᵏ mice as determined by p24-specific antibodies (b) and in vitro recall T-cell proliferation (c). For panel b, the sera from each group of mice were pooled and tested. For panel c, the results were calculated as the mean sample counts per minute minus the mean counts per minute of the medium control. OD, optical density; TdR, thymidine.
identical size and quality, indicating that the two quasispecies may not differ substantially in their biochemical properties. More importantly, when used as genetic immunogens, both quasispecies-based plasmids induced comparable immune responses. The responder hierarchy, with the $H^{-2\beta}$ haplotype as a good responder and the $H^{-2\alpha}$ haplotype as a low responder, was reiterated using both DNA and recombinant protein immunizations, implicating the host MHC as the major determinant in the responder status. This is similar to observations made for other conserved viral proteins, where clear influences from the host MHC on the immune responses can be noted (18, 19). However, in responses to more variable proteins, such as HIV-1 gp120 and p17 or the hepatitis C virus NS4A, a clear influence is also seen from sequence variations between the different viral variants (1, 2, 22). Altogether, the present data suggest that the two quasispecies-based genetic immunogens have comparable immunogenicities, which implies that the minor differences between these two proteins do not have a major impact on the immune response. Collectively, p17 quasispecies-based recombinant proteins with a homology of 92% were antigenically and immunogenically distinct (2), whereas a sequence homology of >99% between p24 immunogens did not seem to cause obvious differences in immunogenicity in the three murine haplotypes tested. We noted that the p24-specific humoral responses were of a low magnitude following DNA-based immunizations as compared to immunizations using recombinant proteins. This is fully consistent with similar studies using HIV-1 or other viral antigens (18). Both humoral and cellular immune responses following genetic immunizations using the hepatitis B virus core and e antigens were of a 100-fold-lower magnitude than the corresponding responses following immunizations with recombinant proteins in adjuvant (18). Thus, the present study further confirms that genetic immunogens are comparatively ineffective in priming these responses.

We have herein not found clear evidence that nonsynonymous mutations cluster within the HLA class I-restricted recognition sites of the HIV-1 p24 protein. However, to further minimize the risk of inducing immune responses which are only vaccine-specific, we have herein described the design and evaluation of two patient-derived p24-based genetic immunogens. The immunological analysis of these genetic immunogens suggests that they have comparable immunogenicities and that the viral variability does not affect the ability to mount an immune response. Similar patient-derived, “personal” vaccines may be the most effective way to ensure the priming of immune responses that actually recognize the virus of the infected host if more variable viral proteins are to be used as immunogens.

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