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THE MOLECULAR EPIDEMIOLOGY OF HIV

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HIV-1 and HIV-2 are members of the lentivirus subfamily. These retroviruses are approximately 50% related at the nucleotide level (1,2). A major difference in their genetic organization is that *vpu*, an accessory gene, is unique to HIV-1 (3), while the *vpx* gene is found only in HIV-2 (4,5). Of the two viruses, HIV-1 is the more widely distributed, accounting for approximately 95% of all HIV infections worldwide. Phylogenetic analyses of sequences from globally circulating strains of HIV reveal a great deal of genetic diversity. Sequence analyses have shown not only that there is genomic heterogeneity between the various strains worldwide, but also that the diversity is unevenly distributed throughout the HIV-1 genome (2). Furthermore, inpatient isolates have less diversity than outpatient isolates, but even within a single individual replicating viruses can differ as much as 10% at the nucleotide level (1,2,6). Given this extensive variability, the development of a classification scheme for all circulating HIV-1 strains became necessary.

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CLASSIFICATION OF HIV STRAINS

Prior to 1992, HIV-1 strains were classified based on their geographic origin, as early phylogenetic analyses indicated that viruses from Europe and North America clustered separately and distinctly from viruses isolated in Africa (7–9). However, as additional sequence data were generated from viruses from around the world, it became obvious that the original classification scheme was insufficient. Further analyses of *env* and *gag* gene sequences indicated the presence of multiple phylogenetic clusters, or clades, that were equidistant from one another (9).

These clades were termed subtypes, which are defined as groups of viruses that closely resemble each other more than they do other subtypes (8,10,11). The viruses originally classified as “European/North American” were re-classified as subtype B, while the African viruses were divided between subtypes A through F, excluding E. Subsequently, subtypes G, H, J, and K were also identified (9). The nine subtypes were organized into one large group of viruses called the group M (major) HIV-1 viruses (9). In 1994, highly divergent HIV-1 viruses that did not cluster with any of the known group M subtypes were identified in Cameroon. These isolates were considered a separate group of viruses, group O (outlier) (12). In 1998, group N (non-M/non-O) viruses were identified in a few individuals from Cameroon (13). Overall, group M viruses account for most HIV infections, while group N and group O viruses are relatively rare. But it must be noted that there is limited detection of group N and O infections because of a lack of specific serological and Western blot profiles available to detect these viruses. This point becomes significant when we consider that because they are not as well detected as group M viruses and may be circulating in larger numbers than we have been able to ascertain, group N and O infections may have a significant impact as antiretroviral regimens become less effective (14).

Group M viruses can be further divided into sub-subtypes (9). Based on full-length sequence data, subtype A has been subdivided into A1, A2 (15), and A3 (16,17), and subtype F into F1 and F2 (18,19). It has also been suggested that subtype K should actually have maintained the name F3; however, for historical reasons, it has been left as a separate subtype (9). Similarly, subtypes B and D should have been reclassified as related sub-subtypes, but have been maintained as separate subtypes (9,20).

Like HIV-1, HIV-2 has been classified into subtypes, designated A–H (21–24). Most HIV-2 infections are from subtypes A and B. At present, the other subtypes are considered rare (24). Each of the HIV-2 subtypes is proposed to represent individual cross-species transmission events (24). In a 2004 paper characterizing HIV-2 subtype H, Damond et al. raised the issue that the HIV-2 nomenclature patterns are inconsistent with those developed for HIV-1 and proposed that the current subtypes of HIV-2 be reclassified as groups (24). Furthermore, the authors suggested that since HIV-2 “groups” C–H had been defined based on single isolates, these groups should be referred to as “putative” (24).

Distinctions Between HIV-1 Groups, Subtypes, and Sub-subtypes

HIV-1 groups, subtypes, and sub-subtypes can be distinguished not only on the basis of phylogenetic analyses, but also by using pairwise genetic distance analyses (2,9–11). The genetic distances between

the groups are relatively large, with groups M and O differing by 53% in the envelope gene (2). Within each of the subtypes and sub-subtypes identified, a range of genetic diversity has also been noted (15,25–28). Subtypes are genetically equidistant from one another with the intersubtype nucleotide distances ranging from 15% to 22% in the *gag* gene and from 20% to 30% in the *env* gene (7,9,15,29). Intrasubtype distances range from 3% to 10% in the *gag* gene and 5% to 12% in the *env* gene, while inter-sub-subtype diversity ranges from 7% to 12% in the *gag* gene and 11% to 16% in the *env* gene (9,11,15,29).

HIV Recombinants

With the increasing number of full-length HIV sequences available for analysis, it has become obvious that, in addition to groups, subtypes, and sub-subtypes, a number of intersubtype recombinants also exist (9). These recombinants are either defined as circulating recombinant forms (CRFs) or unique recombinant forms (URFs). CRFs are defined as recombinants that share an identical mosaic structure, indicating that they are descendants of the same recombinant events (9,20). By the end of 2005, 19 CRFs had been formally recognized, with the most recent additions CRF18_cpx and CRF19_cpx from Cuba (30,31). In addition, a large number of URFs, identified in single individuals or a restricted cluster of individuals, also have been characterized. There have also been reports of intergroup recombinants, composed of a mosaic of group M and group O viruses (32–34). Thus far, although dual infection with HIV-1 and HIV-2 has been shown in a number of studies, there has been no in vivo evidence of an HIV-1/HIV-2 recombinant (2,35).

Origin of HIV Viruses

The prevailing hypothesis is that the presence of groups M, N, and O of HIV-1 is the result of three independent introductions of simian immunodeficiency viruses from *Pan troglodytes troglodytes* chimpanzees (SIVcpz) into the human population (2,36–40). Based on inferences from phylogenetic tree analyses, it is believed that group M viruses originated from SIVcpz in west-central Africa (41,42). The earliest recorded isolate of HIV-1 came from a plasma sample from 1959, suggesting that the pandemic likely started in the twentieth century (2,43,44). Through various phylogenetic analyses and evolutionary modeling, it was estimated that the transfer of the group M virus into the human population occurred sometime during the early part of the twentieth century (45–47). Once group M viruses entered the human population, they diversified rapidly. Analyses of subtype C sequences, within the group M radiation, have indicated that these viruses arose in the mid- to late 1960s (48). Similar analyses have also been done for group O sequences and it was estimated that they likely entered the human population around 1920 (49).

Phylogenetic analyses of several group N isolates revealed that they contain a recombinant mosaic structure, where the *gag*, *pol*, 5'-*vif*, and *nef* genes cluster closely with group M and the 3'-*vif*, *vpr*, *tat*, *vpu*, and *env* cluster with Cameroonian SIVcpz and the SIVcpzUS strain of unknown origin (40). It is unclear if the recombination event between the group M and SIVcpz strains occurred before or following zoonotic transmission to humans. However, considering that the group N viruses exhibit less

diversity than group M and O viruses, it might be argued that the zoonotic transmission of these viruses occurred more recently relative to the transfer of the group M viruses. That would suggest that the recombination would have occurred prior to the cross-species transmission event. Yet, without direct evidence it cannot be ruled out that the group N viruses represent a recombination event that occurred in a human that was dually infected with an ancestor of HIV-1 group M and a SIVcpz virus (40). If the in-human recombination scenario was the source of the group N viruses, then the date of origin is estimated as pre-1930 (46).

HIV-2 is closely related to the SIVs that have been isolated from *Cercocebus atys* sooty mangabeys (SIVsm) from West Africa as well as from several macaques (three *Macaca* species). As HIV-2 is primarily found in West Africa, it has been hypothesized that the cross-species transmission event to humans occurred in that region (37,50). As with HIV-1, it has been estimated that the zoonotic transfer of HIV-2 occurred in the first half of the twentieth century (51).

MECHANISMS OF HIV VARIABILITY

Two major viral mechanisms contribute to the diversity of HIV-1: the error-prone nature of the viral reverse transcriptase and viral recombination. By its nature, reverse transcriptase allows the introduction of mutations into the HIV-1 genome. Combined with the absence of exonuclease proofreading activity and the high replication rate of the virus, it is estimated that there is anywhere from one to ten errors per genome per replication cycle (52,53). It is possible that each new provirus that is generated represents a new mutant strain, distinct at a minimum of one base site (43). Retroviral genomes also undergo insertions, deletions, and frameshifts, and are prone to high rates of G-to-A transitions, or hypermutations (53). In vitro studies have demonstrated that the HIV-1 reverse transcriptase has a higher misincorporation rate than other retroviral reverse transcriptases, being 10-fold and 18-fold more error prone than avian myeloblastosis and murine leukemia viruses, respectively (54,55). Yet the in vivo mutation rates are much lower than those suggested by the in vitro studies (56).

Recombination has been estimated to occur at a rate of 2.8 crossovers per replication cycle (53,57) and is a fundamental property of viruses because of their diploid RNA genome. For recombination between two subtypes to occur, a cell must be dually infected with two distinct viruses. Then, the resulting progeny virions will contain RNA genomes from each of the viruses. During the next round of reverse transcription, as the reverse transcriptase switches between strands, the resulting cDNA will contain sequence from the two different viruses. Although we have ample evidence indicating that intersubtype recombination occurs, we still do not have significant data on the rates and limitations (58–60). Intrasubtype recombination also occurs and has been demonstrated. A 2005 study examining in vitro recombination showed that the recombination rate between two subtype C viruses resembles that between two subtype B viruses. The rate of recombination between subtype B and subtype C viruses is much lower, however, than the rates seen in intrasubtype recombination (58). These differences were attributed to a three-nucleotide difference in the dimerization initiation signal region between subtype B and C viruses (58).

As previously mentioned, for recombination to occur, a person must be dually infected with two variants of the virus. Dual infections can occur either simultaneously as coinfections or sequentially as superinfections of a second strain following an infection with a primary strain (59,60). In most cross-sectional studies in which cases of dual infections have been detected, it is difficult to determine whether the infections occurred simultaneously. Diagnoses of superinfection are hard to make as well. In prospective studies in which samples are available over time, however, superinfection can be identified. A few reports have documented cases of superinfection both with the same subtypes and with different subtypes (59,61–67). Superinfection has even been seen with viruses from two different groups, where a person previously infected with an HIV-1 group O virus then became superinfected with a group M CRF02_AG virus (68). Two papers even documented cases of people who were infected with three different HIV-1 strains (69,70); in these cases, however, the investigators were unable to distinguish between coinfection and superinfection.

To date, while in vivo recombination following superinfection with different subtypes has been postulated, no one has been able to show convincingly recombination between a non-recombinant first and superinfecting second strain within a single individual (71). In a 2004 report, Fang et al. documented the generation of an intersubtype recombinant that took place in a patient originally infected with non-recombinant subtype A who was then suspected to have been superinfected with a subtype C strain (71). While the authors were able to show that the resulting recombinant did contain subtype A sequence from the original infecting strain, they did not show the superinfecting subtype C virus prior to recombination (71).

Finally, selection pressures from the infected host, the environment, or the introduction of therapeutics may also contribute to overall viral diversity (53,72). The mechanisms that generate this diversity result in variants that can evade the host immune system, are resistant to drug therapy, may have an altered cell tropism, or may exhibit a variety of other phenomena that can contribute to disease (2).

GEOGRAPHIC DISTRIBUTION OF HIV-1 GROUP M SUBTYPES

Phylogenetic classification of HIV strains has assisted in tracking the diversity of the globally circulating strains (Figure 4-1). It has been shown that the HIV-1 subtypes exhibit a heterogeneous distribution. Overall, subtypes C and A account for most of the current HIV-1 infections, followed by subtype B and the intersubtype recombinants CRF01_AE and CRF02_AG. While subtype B viruses are primarily found in Europe, the Americas, and Australia, subtype C dominates in sub-Saharan Africa and India (7,8,43,72). It has also been documented that the subtype C viruses are spreading exponentially in Brazil and are slowly outcompeting the predominant subtype B viruses in that country (73). Subtype D viruses are predominantly found in Central and East Africa, with a few cases appearing in southern and West Africa (7,74–77). Although a pure subtype E virus has yet to be found, it is part of the CRF01_AE recombinant form. CRF01_AE has been identified in Thailand, the Philippines, China, and Central Africa. Subtype F has been found in Central Africa, South America, and Eastern Europe. Subtype G has

Figure 4-1. Geographic Distribution of HIV-1 Group M Viruses



been reported in West and East Africa as well as Central Europe. Subtype H has only been found in Central Africa. Subtype J was identified in Central America and subtype K was found in the Democratic Republic of Congo and Cameroon. The recombinant virus CRF02_AG is the most prevalent virus in West Africa (8,72,78–82).

Although certain subtypes appear to be restricted geographically, other subtypes are spreading rapidly and co-circulating as the world becomes more of a global community. As immigration and travel increase, there has been a shift in infection patterns; it has been estimated that anywhere from 25% to more than 40% of the new infections in Europe are non-B variants of African and Asian origin (74,83,84). The United States also has an increasing number of non-B infections. For example, in a 2003 report of a well-studied military cohort, 6% of all new infections were because of non-B subtypes (85).

It is difficult to interpret the variations seen in the distribution patterns of the various subtypes. As over two decades have elapsed since the beginning of the HIV epidemic, the global patterns of spread are a result of a long period of evolution, which is nearly impossible to reconstruct. The geographic distribution of the different subtypes has been determined by a number of factors. Primarily, it is believed that founder viruses were introduced into given populations and rapidly diversified. It is interesting to note that, while the rate of HIV spread is not uniform across the globe, there appears to be an inverse correlation between rate of disease spread and the variety of subtypes in a population (7). More specifically, in areas where the rate of spread has been relatively slow and stable, such as Central Africa, there are a number of circulating subtypes in the population, as opposed to southern Africa, which has

witnessed an explosive epidemic, but has mainly one predominating subtype. It has been speculated that the large variety of subtypes in Central Africa has been due to the relatively low spread of HIV-1 in the population. Furthermore, it is believed that a number of subtypes can continue to coexist, possibly because of the low crossover between networks of risk groups (7).

IMPLICATIONS OF GENETIC DIVERSITY

The genetic diversity found in HIV is much greater than has been found in any other virus infecting the human population. Whereas with certain viruses a single amino acid change can result in a global pandemic, there can be as much as 10% genetic diversity in the HIV-1 viruses within a single individual (10,86). This diversity has been shown to have an impact on viral phenotype at the level of transmission patterns, pathogenicity, and immunology. In addition, this diversity plays a role in responses to treatment and vaccines.

Studies focusing on the differences between HIV-1 and HIV-2 have shown the clearest evidence of the impact genetic diversity has on the biological phenotype of the virus. While HIV-2 is transmitted through the same modes as HIV-1, the HIV-2 transmission rates are significantly lower, with the most common modes being perinatal and heterosexual (1,87–89). HIV-2 has also been shown to be less pathogenic than HIV-1, with a progression to AIDS significantly longer than that with HIV-1 infection (90,91). In addition, viral load levels are lower with HIV-2 than with HIV-1 (92,93), which likely contributes to the lower pathogenicity of HIV-2. A 2006 in vitro study comparing the viral kinetics of HIV-1 with those of HIV-2 also indicated that, unlike HIV-1, which continuously produces virions at a steady state, HIV-2 had an initial burst of replication and then retired to a latent state with an absence of virion production (94). Interestingly, HIV-2 isolates have also been shown to be more promiscuous in their use of coreceptors; however, the significance of this ability to use additional coreceptors is unclear (95).

Just as with the studies comparing HIV-1 and HIV-2, various reports have shown differences between HIV-1 group M subtypes with regard to important biological characteristics. Evidence has suggested a relationship between subtype and modes of transmission. Studies in South Africa (96), Finland (97), Thailand (98,99), and Australia (100) found that most subtype B strains were associated with homosexual transmission while non-B strains were associated with heterosexual transmission. Similarly, in a study of U.S. military personnel, Brodine et al. found that people infected with non-subtype B HIV were more likely to report heterosexual contact than those with subtype B infection (85). Finally, a study of injection drug users in Thailand found a significantly higher transmission probability associated with subtype E than with subtype B (101), suggesting the certain viruses may be more efficient at certain transmission modes.

It had previously been suggested that certain subtypes were more effectively transmitted through mucosal routes than others and that perhaps certain properties of non-B subtypes facilitate transmission of HIV through the heterosexual route (102), perhaps explaining the exponential spread of disease in parts of Asia and Africa. Furthermore, it has been proposed that since HIV-1 subtype B strains had primarily been transmitted and passaged parenterally and through rectal intercourse, it might have undergone a period of “counterselection,” where it lost the genetic sequence material that would have

enhanced its capacity to be transmitted through a heterosexual route while other non-B subtypes did not experience this negative selection (102,103). It must be noted, however, that these original findings have subsequently been challenged (104–107), underscoring the difficulty of elucidating the role of subtypes in transmission.

Infection with certain subtypes has also been associated with increased risk of vertical transmission. A study conducted on mother-child pairs in Tanzania revealed that mothers infected with HIV-1 subtype A, subtype C, and intersubtype recombinants were more likely to transmit virus to their infants than mothers infected with subtype D (108). A previous study by the same group found that among perinatally transmitted C/D recombinant viruses, the V3 regions (*env*) were always from subtype C and never from subtype D, suggesting that viruses containing subtype D-V3 may have reduced fitness compared to viruses with subtype C-V3 (109). Conversely, a study conducted in Kenya indicated that women infected with subtype D were more likely to transmit than women who had any other sequence combinations in the *env* and *gag* (110).

Various studies have demonstrated differences among the HIV-1 subtypes with regard to disease progression. Kanki et al. found that women infected with a non-A subtype were eight times more likely to develop AIDS than those infected with subtype A (111). Similarly, Kaleebu et al. reported that subjects with subtype A had a slower progression to disease than those with subtype D (26,112). One study conducted in Brazil even found a difference in disease progression within a subtype (113,114). Specifically, individuals infected with serotype B-Br (Brazilian B) progressed to AIDS more slowly than individuals with non-Brazilian serotype B infections (113). Conversely, a cross-sectional study in London that compared HIV-1-infected African immigrants and non-African Londoners found no difference in progression by subtype (115).

Clinical and immunological differences also have been found between subtypes. In Kenya, where subtypes A, C, and D were all co-circulating within the same population, Neilson et al. found that higher plasma RNA levels and lower CD4+ counts were significantly associated with subtype C infection (116). In a prospective study conducted at a methadone treatment clinic in Thailand, people infected with CRF01_AE were found to have higher viral loads in early infection than those infected with subtype B (117). This difference decreased over time, however, to the point that the viral loads were similar at 12, 18, and 24 months post-seroconversion (117). Similarly, a study in our laboratory indicated that women infected with CRF02_AG had a significantly higher viral load during the early stage of infection than women not infected with CRF02_AG (118). Kaleebu et al. found that subjects infected with subtype D had a lower average CD4+ T cell count over the period of follow-up than those infected with subtype A (112). Conversely, a study in Thailand found no major differences in the degree of immunosuppression or the rates of opportunistic infections between people infected with subtype B' (Thai B) or CRF01_AE (119). Finally, infection with multiple subtypes has also been associated with higher viral load and lower CD4+ T cells counts (120).

A few reports suggest that the different subtypes may vary with regard to chemokine coreceptor usage and tissue tropism. It had been well documented that coreceptor use evolves during subtype B

infection from use of CCR5 in the earlier stages of infection to use of CXCR4 during late stages of disease. While in vitro studies have shown that subtype A and CRF01_AE viruses have a similar evolution in coreceptor usage as subtype B viruses, subtype C and D viruses do not (121–124).

Subtype variation has also been associated with different levels of interaction with HIV-2. For instance, Sarr et al. showed that the in vivo interaction between HIV-1 and HIV-2 is influenced by HIV-1 subtype (125). They found that the prevalence of A3 viruses was significantly higher in dually infected individuals than in women who were singly infected with HIV-1 (125). Some cross-sectional studies have failed to demonstrate biological or clinical differences in genetically diverse viral strains. Alaeus et al. found no difference in the rate of CD4+ decline, clinical progression, or plasma HIV-1 RNA levels between individuals infected with subtypes A, B, C, or D (126). Laurent et al. found no difference in survival, clinical disease progression, or CD4+ decline between those infected with CRF02_AG and those infected with other viral strains (127). In addition, in a 2002 study comparing differences between subtypes A and D in mother-to-child transmission, subtype did not appear to influence infant survival (128).

Several in vitro studies have shown that subtypes differ on genetic components that might affect transcriptional efficiency. Subtype C viruses were shown to contain an extra NF- κ B site in the long terminal repeat (LTR) region. The presence of the additional binding site is believed to render subtype C more responsive to p65/RelA than subtype B, which has two NF- κ B binding sites (129). Subsequently, HIV-1 subtype C isolates were shown to have an elevated responsiveness to TNF- α , which correlated with increased NF- κ B copy number (130). In a related study, subtype E LTRs were shown to have only one functional NF- κ B site (131). Based on these in vitro results, it was suggested that the presence of an additional NF- κ B site might confer an adaptive advantage on subtype C viruses, particularly in regions in which incidence of sexually transmitted infections that stimulate the production of pro-inflammatory cytokines are high (130).

Viral fitness is the relative replicative adaptation of a virus species to its environment, especially in the presence of a competitor (132,133). It depends not only upon the ability of the virus to replicate, but also on its longevity, its potential for transmission, and its ability to cause disease (134). It is not clear whether viruses of different subtypes and sub-subtypes have differing levels of replicative capacity. Several studies have conducted ex vivo assays to determine the relative fitness of two viruses. For example, in dual competition analyses, it was shown that subtype B viruses were capable of outcompeting subtype C viruses in infecting HIV-uninfected cells (135). A similar analysis, comparing CRF02_AG isolates to other subtype A isolates, indicated that CRF02_AG appears to be more fit in an ex vivo setting (136). Similarly, competition assays comparing HIV-2 to HIV-1 group M and group O viruses showed that the group M viruses were most fit, with HIV-2 isolates 100-fold less fit than all the group M viruses and group O viruses 100-fold less fit than HIV-2 (137). Although it is important to take caution in interpreting in vitro data from individual isolates, it is interesting to note that the order in replicative and transmission fitness of the isolates in the Arien et al. study closely mimic the distribution of subtypes in the global epidemic (137).

Finally, another important reason to characterize and understand genetic diversity is that knowledge of the predominant HIV-1 subtypes, sub-subtypes, and CRFs in a given population may be important in

designing effective HIV vaccines (138). Although the importance of matching a vaccine candidate to regional circulating strains is yet unclear, incorporation of local strains might maximize the efficacy of a potential vaccine candidate (138).

As mentioned earlier, several studies could not detect differences between subtypes with regard to various disease parameters. The reasons for these discrepancies are still under investigation. It is worth noting that a number of the studies that did not find associations between subtype and correlates of disease and pathogenesis were compromised by a cross-sectional design. Overall, given the complexity of HIV genetic diversity, it is possible that a single characteristic such as subtype will not entirely account for the differences in transmission and pathogenesis. Rather, it might be one of many virologic, immunologic, and host factors that contribute to a particular phenotype or outcome.

Viral Diversity and Antiretrovirals

There is increasing evidence in the literature that subtype diversity might influence susceptibility and resistance to antiretroviral therapy (ART). Although the *pol* gene is the most conserved region of HIV-1, with a variation of approximately 10% (139,140), it possesses sufficient variability to allow for phylogenetic classification of all subtypes and CRFs (141). The *pol* gene encodes three enzymes: protease; reverse transcriptase; and integrase. Antiretrovirals (ARVs) that target reverse transcriptase and protease belong to three classes—nucleoside analogue reverse transcriptase inhibitors (NRTIs); non-NRTIs (NNRTIs); and protease inhibitors (PIs).

Significance of Diversity for Therapy

Widespread use of highly active antiretroviral therapy (HAART) has greatly reduced morbidity and mortality among HIV-infected people in industrialized nations (142). ART is increasingly playing a major role in controlling the pandemic as many developing countries have recently launched treatment programs (143–145), allowing more people access to drugs. A major obstacle to the efficacy of such large-scale treatment programs is the inevitable development of drug-resistance mutations (DRMs). These mutations consist of two varieties: a primary resistance mutation that reduces the susceptibility of the virus to drugs by itself, while a secondary mutation requires the presence of another mutation or mutations to exert its resistant phenotype (146). The development of drug resistance limits treatment options, facilitates viral rebound, and ultimately leads to immunologic decline and the development of opportunistic infections. In addition to the selective pressure from therapy, drug resistance may also be acquired through the transmission of drug-resistant HIV strains. This phenomenon, known as primary drug resistance, has a prevalence of 6% in Switzerland (147), 11% in the United Kingdom (148), 18% in Australia (149), and 6% to 21% in the United States (56).

Genetic variation within the reverse transcriptase and protease may greatly influence viral replication and fitness as well as susceptibility to therapy and the development of drug resistance (146). The evolutionary dynamics within individuals involves the interplay between advantageous natural selective forces and deleterious mutations, resulting in the formation of a heterogeneous ensemble of geneti-

cally distinct, yet related variants called quasispecies. Strains with higher replicative advantage in the face of these factors will outcompete the other quasispecies to become the dominant virus in the individual. ART modulates this balance by suppressing the replication of the majority of the quasispecies, including, in most cases, the dominant strain, while sparing some variants that possess mutations that enable them to replicate in the face of drug pressure. These initially “less fit” drug-resistant variants then become the predominant viral strains within the individual’s quasispecies population. It is important to note, however, that although many drug-resistant viruses are less fit than wild-type strains, the effect of resistance on fitness depends on the drug used. For example, some NNRTI mutations, such as K103N and Y181C, do not reduce viral fitness significantly, and the variants containing these mutations rapidly become the dominant quasispecies in treated patients (150,151).

Although the relationship between genetic diversity and clinical outcome is complex, various studies have demonstrated that the clinical response of non-subtype B viruses to the available ARVs is similar to that seen in subtype B infection (152–154). Various studies have described genotypic and phenotypic resistance in non-subtype B infections (144,152–158). Most of the studies described the lack of major drug-resistance-conferring mutations, but a high prevalence of secondary mutations; one 2005 study reported considerable overlap between subtype B resistance mutations and mutations associated with at least one non-B subtype (144). The non-subtype B sequences included subtypes A, C, D, F, and G as well as CRF01_AE and CRF02_AG. Each of the 55 known subtype B drug-resistance mutations occurred in at least one non-B isolate, and 44 (80%) of these mutations were significantly associated with ART in at least one non-B subtype. Conversely, 61 of 67 mutations associated with ART in at least one non-B subtype were also associated with ART in subtype B isolates.

Studies examining the relationship between HIV subtype and response to ART have been limited by small samples sizes, leading to general comparisons of subtype B to other subtypes grouped together. It is conceivable that the widespread use of ARVs will exert extra selective pressure on viral reverse transcriptase and protease and that this pressure will result in the development of different mutations by subtype. It is also possible that preexisting polymorphisms may determine the precise mutation pathways used by the viruses to achieve drug resistance; that is, some codons are closer to a resistance phenotype than others even if the amino acid involved is identical.

Impact of Evolutionary Forces on Drug Resistance

Development of drug resistance depends on the extent of viral replication during therapy, the ease of acquiring a particular mutation or set of mutations, recombination, the effect of mutations on drug susceptibility, the presence of viral reservoirs, and viral fitness (159,160). It is unclear whether resistant viruses are pathogenic or would get transmitted at different rates than those with reduced replicative capacity. Following therapy, mutations that confer drug resistance lead to an initial decrease in fitness (124,161). With continued drug pressure, however, secondary or compensatory mutations that partially restore the activity of viral enzymes may result in a rebound in viral fitness.

Drug-resistant viruses are able to persist in CD4+ T lymphocytes, follicular dendritic cells, macrophages, and various other cells and tissues long after initiation of therapy (162). These reservoirs serve as potential sources of viral particles for the circulating viral pool. In addition, even when viruses are at undetectable levels, low-level replication and viral evolution are likely still occurring. Studies have shown that the replication of zidovudine-resistant HIV-1 increases multiplicatively during therapy, and it has been suggested that an intentional increase in mutation rate leading to lethal mutagenesis of the HIV-1 genome may be another viable approach to ART (161).

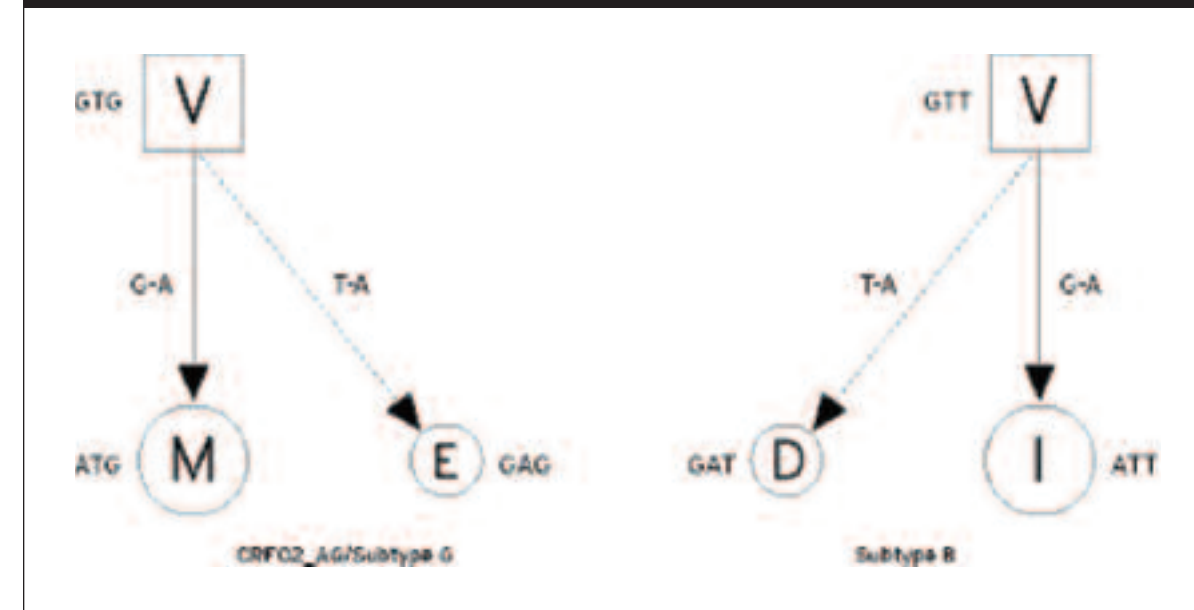
Subtype-Specific Polymorphisms and the Pathways to Resistance

Subtype-specific polymorphisms, defined as specific mutations occurring in the majority of sequences from a particular subtype, may play functional roles in determining the mutation pathway to drug resistance. The degeneracy of the genetic code may mean that different mutational pathways are possible even when the codon encodes the same amino acid. This relationship between codons that specify the same amino acid but achieve a different pattern of amino acid substitution by a single nucleotide change has been termed quasi-synonymy (163). Quasi-synonymous mutations within the virus may dictate different mutational pathways, which may have bearing on the development of drug resistance and viral escape from the immune response (139,144,163). Figure 4-2 shows an example of subtype-specific codon differences and predictions of how they may influence mutational routes to the development of drug resistance (144). The amino acid valine at position 179 in the reverse transcriptase is encoded by GTT in subtype B and by GTG in CRF02_AG and subtype G. Although these valine codons are synonymous, a single nucleotide change at the first position to adenine results in isoleucine (ATA) and methionine (ATG), respectively. Similarly, a single nucleotide change to adenine at the second codon position results in formation of glutamate for CRF02_AG and subtype G, while subtype B viruses encode aspartate at the same position. Thus, subtype B viruses are more likely to have V179I mutations while CRF02_AG viruses are more predisposed to forming V179M mutations in response to NNRTIs (164).

The Immune Response and Pol Diversity

Recent studies have demonstrated some interplay between the selective pressures exerted by ART on reverse transcriptase and protease and the emergence of viral escape mutants secondary to CD8+ T lymphocyte (CTL)-mediated immune pressure (165–167). Karlsson et al. demonstrated that among individuals who had developed PI mutations, the wild-type epitope was strongly recognized and bound by CTLs, while the mutant epitope (V82A mutation) was poorly recognized by the wild-type-specific CTL (165). V82A is a common PI mutation (146), suggesting that it may act both as a CTL and PI escape mutant. Within reverse transcriptase, the V179I mutation confers intermediate levels of nevirapine resistance (146) and has been reported to reduce HLA-B35 recognition and binding affinity (168). In addition, Mason et al. demonstrated that common drug resistance mutations sustained or even enhanced the antigenicity and immunogenicity of common HIV-1 *pol* CTL epitopes presented by com-

Figure 4-2. Prediction of Subtype-Specific Drug Resistance Mutations at HIV-1 Reverse Transcriptase Position 179 from Codon Bias and Nucleotide Substitution Matrix Data



Resistance mutations may be influenced by quasi-synonymy and genetic cost of mutations. Examples of predicted mutations at position V179 in RT are shown. The wild type amino acids are enclosed within quadrilaterals, while mutant amino acids are enclosed with ovals. The predicted relative abundance of mutants is proportional to the size of the oval. The preferred codon bias is indicated adjacent to each amino acid. Codon usage was determined from consensus sequences from our samples and subtype B consensus. The thickness of the arrows indicates the ease of substitution. G-A substitutions are more likely to occur than T-A substitutions. CRF02_AG and subtype G viruses are predicted to preferentially develop V179M and V179E mutations in RT while subtype B viruses will develop V179I and V179D mutations under drug pressure.

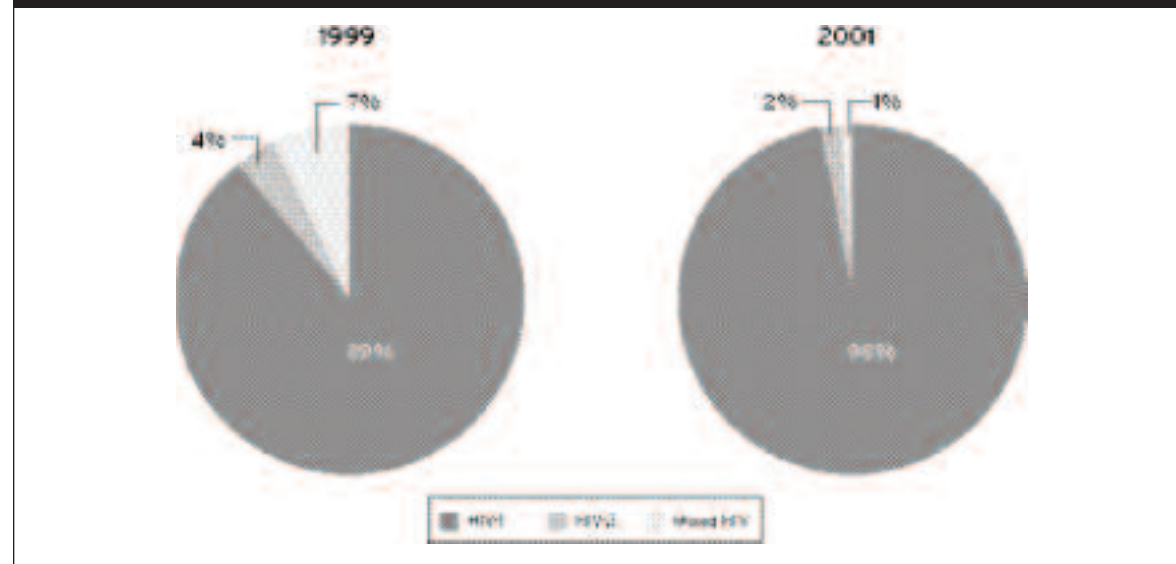
mon HLA molecules (165). These findings suggest that certain viral signatures may be under both drug- and immune-driven selective pressures that could be exploited to combat the HIV-1.

Emerging data support the hypothesis that some subtype-specific polymorphisms in various HIV-1 genes, including *pol*, may be immune escape variants that have persisted in the populations where these epitopes and subtypes predominate (164,169). HIV-1 subtypes are predominant in different regions of the world (74), and many epitopes have ethno-geographic bias (170). It is therefore also possible that differences in HLA frequencies between populations will contribute to this phenomenon.

Subtypes and Vaccines

Whether a candidate vaccine against HIV-1 should be based on the dominant subtypes within a given geographic region has been the subject of much speculation and debate. To address the issue appropriately, one must consider the relationship between the HIV-1 genetic subtypes and immune responses. Since effective neutralizing antibodies appear to function across genetic subtypes (171–173), it has been argued that a vaccine targeting the humoral immune response by focusing on genetic subtypes seems illogical. A vaccine that targets more conserved regions of the genome and that is cross-protective might be more efficacious. As Moore et al. (10) point out, given that subtype designations were not made based on antigenic or immunogenic properties of the virus and do not correspond to neutralization serotypes,

Figure 4-3. Proportions of HIV-1 and HIV-2 Infection among Pregnant Women During the 1999 and 2001 National Sentinel Surveys in Nigeria



it might not be as relevant to generate a subtype-specific vaccine, but rather one that is broadly cross-reactive (71).

Many researchers are still interested in generating subtype-appropriate vaccines for given regions. The belief is that a subtype-specific vaccine candidate might increase the number of potentially cross-reactive epitopes by augmenting the level of similarity between the vaccine and the endemic virus. For those designing subtype-specific vaccines, there are two major approaches: the isolate-based approach and the consensus or ancestral-sequence-based approach (86). The first approach involves selection of an isolate from the geographic region to which the vaccine is directed, while the second approach requires the construction of either a consensus or ancestral sequence using all available sequence data and an evolutionary model (86). When considering the possibility of using an isolate-based vaccine, researchers must decide not only which subtype to use, but also which geographic region from which an isolate should be drawn. Given the great diversity within subtypes and the fact the geographic restrictions of viruses are slowly disappearing, this is a difficult choice. The use of a polyvalent vaccine containing isolates as well as group M consensus sequence is a promising approach (86). A number of multiclade vaccines are now under evaluation.

MOLECULAR EPIDEMIOLOGY OF HIV IN NIGERIA

Both HIV-1 and HIV-2 circulate in Nigeria (174–178). Serologic data from HIV screening centers and published results in the early to mid-1980s showed slightly higher rates of HIV-2 infection than HIV-1 infection (174,175,179–182). The HIV-1 infection rate steadily increased during the late 1980s, however, accounting

for approximately 60% of infections during the late 1980s and early 1990s; since then, HIV-1 has accounted for more than 95% of total HIV infections and almost 99% of all AIDS cases (Figure 4-3). While the pattern of the two types of HIV infection in the country can be partly explained by the higher infectivity and transmission efficiency of HIV-1 (183,184), widespread use of rapid testing procedures without follow-up confirmatory Western blotting and inadequately trained personnel for HIV testing may also account for the reportedly low rate of infection of HIV-2 in the country (Kanki P, *personal communication*).

The initial indication that HIV strains circulating in Nigeria may differ from the HIV-1 subtype B viruses that circulate in Western Europe and North America came from the work of Olaleye et al., which showed differential amplification rates of fragments of the HIV genome using primers designed from the HIV-1 subtype B genome (184). Initial sequence analysis of the *env* gene of the isolate designated HIV-1 IbNg (for Ibadan, Nigeria) showed it to be a variant of HIV-1 subtype A (185). Later work indicated that the IbNg isolate was, in fact, a recombinant of HIV-1 subtypes A and G. In addition, another HIV-1 strain from an infected person in Jos, while subtype G based on sequencing of the *env* gene (186), was also found to be a recombinant of subtypes A and G following full-length sequencing. Collectively, these subtype A and G recombinants are classified as CRF02_AG.

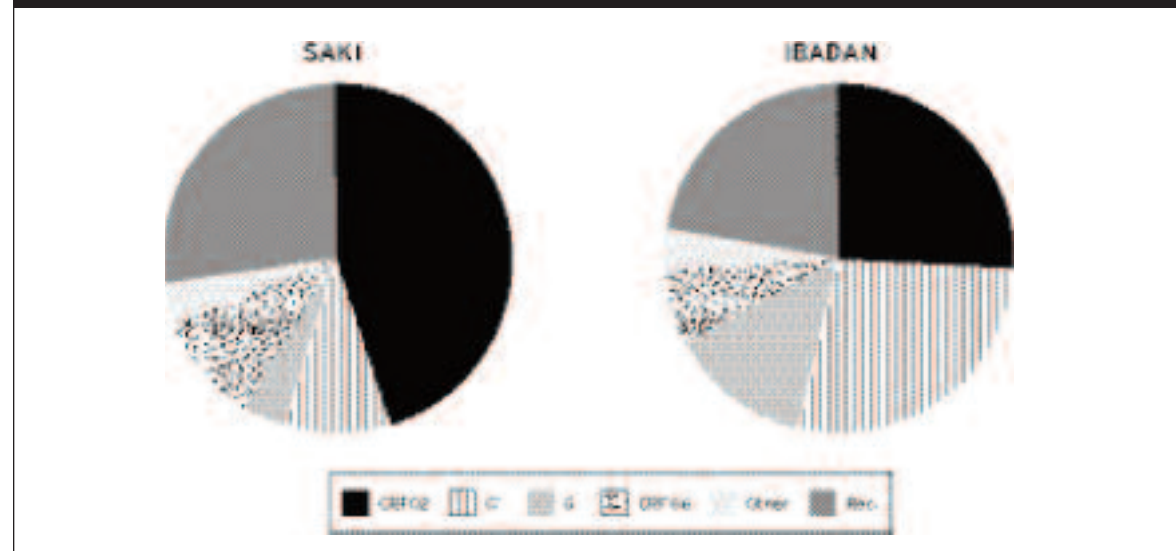
The identification of an HIV-1 subtype A variant from the southwest region and a subtype G virus from the north-central region led to the initial speculation of regional differences in the distribution of HIV-1 subtypes in the country (184,185). Subsequently studies found HIV-1 subtype G in most parts of northern Nigeria, but CRF02_AG and subtype A were found in the middle southern parts (82). However, these studies were seriously limited by their small sample size, as well as the relatively low specificity of subtyping due to use of HMA or peptide assays for genotyping.

In recent years, more efforts have been committed to identifying the various HIV-1 subtypes circulating throughout the country. A 1998 study found serologic evidence of HIV-1 group O in the southeastern and northeastern parts of Nigeria (187). Also, using a peptide-based subtyping assay, Odaibo et al. reported circulation of HIV-1 subtypes A, B, and C, and group O in different parts of the country (188,189). Genotypic analyses of samples previously subtyped using the peptide assay have confirmed the circulation of subtypes A, B, and C in Nigeria (190).

Unlike most previous HIV-1 subtyping studies, which were based on samples collected from hospital patients, genotyping of HIV-1 isolates from a community-based HIV project in Oyo State established the HIV subtypes among asymptomatic and symptomatic people at the community level (191). Sequence analyses based on consensus sequences from *env* and *gag* gene fragments of approximately 100 HIV-1 isolates collected from 2001 to 2003 showed the presence of several HIV-1 variants, including multiple CRFs. Approximately 35% of the isolates were CRF02_AG, and another 35% were subtype G. Interestingly, a number of the subtype G samples clustered as a distinct group, previously described as G' (80,82,191,192). Further analysis of several of these samples using full-length sequence data revealed that some people were in fact infected with full-length G' viruses (193).

Other CRFs and subtypes were identified for the first time in Nigeria, including CRF06_cpx, CRF01_AE, CRF11_cpx, and A3. The results of the study also indicated some differences in the distribu-

Figure 4-4. Proportions of Various HIV-1 Subtypes and Circulating Recombinant Forms in Two Communities in Oyo State, Nigeria (2001–2003)



Abbreviations: CRF: circulating recombinant form; Rec.: recombinant

tion of the two major subtypes, G and CRF02_AG, between the two communities. Detection of subtype G in Ibadan was twice that in Saki, a city about 200 kilometers away (Figure 4-4). In similar studies, multiple HIV-1 subtypes and CRFs were found in other parts of the country (194,195).

Although the biological consequences of HIV diversity are not well understood, the implication for diagnosis, ART, and vaccine development are enormous. It is known that the antigens of most of the HIV testing kits are primarily targeted to detect HIV-1 subtype B, the major circulating strain in North America and Western Europe. These test kits may not accurately detect infections due to some non-B subtypes of the virus (196). The problem may even be greater with primer mismatch in the molecular detection or amplification of HIV nucleic acid fragments or genes (184). It is now well established that recombinant forms of HIV are common in Nigeria (191), which may influence the prevalence of drug resistance as data from a study in Ibadan and Saki, in Oyo State, show that the complex CRF06_cpx harbors more DRMs than other subtypes found in antiretroviral-naïve individuals (164).

Drug Resistance Mutations in Nigeria

The same study of antiretroviral-therapy-naïve HIV-1-infected subjects in Ibadan and Saki found a significant degree of primary and secondary DRMs in the reverse transcriptase and protease, as well as polymorphisms at positions of previously characterized DRMs (164). For instance, 6 of 35 (15%) reverse transcriptase sequences harbored primary NRTI/NNRTI mutations, including M41L, V118I, Y188H, P236L, and Y318F. Notably, three of these individuals were infected with viruses that clustered with CRF06_cpx in the reverse transcriptase. In addition, three of the four CRF06_cpx reverse transcriptase samples harbored primary DRMs, compared with 11% of the other variants combined ($p = 0.011$). This

level of primary drug resistance is high for a drug-naïve cohort, but it is noteworthy that half the sequences with drug-naïve DRMs are CRF06_cpx, which constitutes only about 10% of viruses in Nigeria (164,191). These findings suggest the possibility that CRF06_cpx sequences harbor natural polymorphisms that are drug resistant rather than acquired after therapy initiation. It is possible, however, that individuals with these DRMs were infected with drug-resistant viruses, possibly from individuals not adherent to therapy, or on suboptimal ARV regimens.

No primary DRMs were observed in protease. Secondary DRMs, however, were observed in all protease sequences. Three secondary PI resistance mutations—L101V, M36I, and L63P—were detected. The K20I and M36I secondary PI mutations were found in all subjects, regardless of subtype, while all the subtype G viruses harbored the V82I polymorphism in the protease. Similar patterns have been found in the other parts of the country (197,198). This subtype-specific polymorphism at a DRM site raises concerns about second-line and salvage-therapy regimens, which typically contain PIs.

CONCLUSION

The global HIV epidemic exhibits a great deal of genomic heterogeneity. A number of groups, subtypes, sub-subtypes, and recombinants have been identified and characterized. Interestingly, the distribution of the various strains is also heterogeneous, with the greatest diversity represented in sub-Saharan Africa. The worldwide spread of the various subtypes can primarily be attributed to the increased travel and movement of populations. Various studies indicate that the subtypes differ in biological properties, but many of the findings are still controversial and require further research. It is conceivable that future studies that make more accurate distinctions between subtypes and sub-subtypes may better clarify findings regarding the association between viral genotype and biological phenotype. This diversity has some obvious implications for ART as well as vaccine development. The further characterization of the predominant HIV-1 subtypes, sub-subtypes, and CRFs in a given population will enhance our understanding of viral diversity critical to the informed design of interventions, therapies, and vaccines. Although the importance of matching a vaccine candidate to regional circulating strains is yet unclear, incorporation of local strains might maximize the efficacy of a potential vaccine candidate.

Analyses of the sequence diversity in Nigeria have revealed that the number of variants circulating in the country is increasing. Furthermore, it has become apparent that a growing number of infections are due to recombinant forms of the virus, and one of the recombinants, CRF06_cpx, is associated with primary drug resistance mutations. These factors should be taken into consideration as treatment regimens are designed for a given population. Continued monitoring will enhance our understanding of the scope of diversity in Nigeria as well as the role that diversity plays in the ongoing epidemic.

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