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Genetic Variation of HIV Type 1: Relevance of Interclade Variation to Vaccine Development

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ABSTRACT

Accumulating data in human immunodeficiency virus (HIV)-infected individuals support the hypothesis that in primary human immunodeficiency virus type 1 (HIV-1) isolates of different clades and phenotype (syncytium inducing [SI] and nonsyncytium inducing [NSI]) common antigenic structures must exist that can stimulate the immune response to produce a broad spectrum (cross-clade and cross SI and NSI) neutralization response. Certain vaccination regimens in chimpanzees and human volunteers with clade B SI type HIV-1 derived candidate vaccines induce neutralizing antibodies against intraclade B SI type primary HIV-1 isolates, but not against intraclade B NSI type of viruses. To be effective against the full antigenic spectrum of primary HIV-1 isolates (cross-clade—SI and NSI) candidate vaccines should contain immunogens of primary isolates representative of the whole antigenic spectrum of HIV-1. There is an urgent need to identify these immunogens and to improve their immunogenicity. As long as we have not yet characterized these cross-HIV-1 spectrum conserved immunogens, candidate vaccines against the more prevalent clades C, A, and E should be developed for evaluation in developing countries. In support of the follow-up and evaluation of the hopefully increasing number of phase 1, 2, and 3 HIV-1 vaccine trials in humans, it is considered a high priority to develop a high throughput neutralization assay, to further expand the use of a limited number of key primary HIV-1 isolates as a surrogate for neutralization of the entire HIV-1 antigenic spectrum (cross-clade—SI and NSI), to develop high throughput subtyping as well as a rapid system to monitor the immunogenic relatedness of different HIV-1 clades.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV) exhibits an extremely high genetic variation with rapid turnover of virions.[1] On average, 50% of the virions are replaced every 60 hours. HIV-1 generates on average, one error per 10^4 nucleotides, which is the size of its genome. This means that each provirus is a new mutant strain. Consequently, an individual is infected with a swarm of closely related but nonidentical viruses. The blood and lymphoid tissue of human adults in late-stage disease contain between 10^9 and 10^{10} virions. With 3×10^7 HIV-infected patients worldwide, there may be as many as 3×10^{17} HIV genetically unique strains in circulation.[2] This reservoir of genetic HIV variants will increase.

So far, two major viral types have been characterized in humans: human immunodeficiency virus type 1 (HIV-1), the predominant HIV type throughout the world and HIV type 2 (HIV-2), less widespread and still primarily found in West Africa. HIV-1 strains have been classified into a number of subtypes, alternatively termed clades or genotypes, which have been designated by letters A through J, and that constitute the major group or group M of HIV-1.

In addition, some divergent strains or outliers, have been categorized as group O.[3] HIV-2 has also been classified into at least six subtypes (A, B, C, D, E, F).[4,5] However, despite these enormous genetic differences, some of their biological and/or phenotypic characteristics are much more conserved. For example, in addition to the use of the CD4 molecule to enter host cells, nonsyncytium-inducing (NSI) isolates tend to use the C-C chemokine receptor CCR5 exclusively for viral entry (HIV-

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1 R5 type of viruses). Viruses capable of using CXCR4 (HIV-1 X4 type) or both second receptors (HIV-1 X4R5) are characterized as syncytium inducing (SI). This relation has been observed across all HIV-1 genetic subtypes in group M as well as in group O.[6] This relation has also been observed for HIV-2 type of viruses.[7] This suggests that some epitopes and/or conformational structures involved in CD4 and coreceptor binding must be conserved throughout the different clades of HIV-1 and even among the different types HIV-1 and HIV-2.

Although the correlates of protective immunity are unknown, it is still a valid hypothesis that an effective HIV vaccine will need to induce neutralizing antibodies and $CD8^+$ cytotoxic T-lymphocyte (CTL) activity, to prevent transmission and/or to control progression to disease.

This hypothesis is based on the observation that most viral vaccines that have been successful induce protection that limits virus replication, prevent disease, and facilitate clearance of the infection. Few, if any of the successful vaccines are able to completely prevent virus infection (sterilizing immunity).[8] The efficacy of the vaccine is the result of stimulating the humoral as well as the cellular immune response, both of which are needed to eliminate free and cell-associated virus. The creation of a mixture of immunogens able to induce a broad cross-clade potent neutralizing antibody as well as CTL response, which can induce protection against all different HIV-1 strains is the ultimate goal. Studies of the precise nature of the epitopes involved in the induction of such humoral and cellular immune responses, should be made the highest priority. In this article we discuss the relevance of interclade variation to HIV-1 vaccine development as well as suggest future research to overcome the genetic variability barrier in HIV-1 vaccine design and evaluation.

GENETIC VARIABILITY AND HIV-1 SUBTYPE DISTRIBUTION

Excellent review articles have been published in which the phylogenetic relation among HIV-1 strains, the global distribution of HIV-1 subtypes, methods of detecting genetic variation and classification as well as the mechanisms that generate the diversity, have been extensively discussed.[2,3]

Most HIV-1 subtypes have been detected in or associated with Africa. The greatest genetic variation has been detected in Central Africa. An accurate estimate of the subtype prevalence rates is not available yet, due to lack of representative sampling, as well as the labor-intensive and time-consuming work necessary to identify subtypes. One study describes the geographic distribution of HIV-1 subtypes based on genetic analysis of 669 HIV-1 strains originating from 39 countries. Approximately 50% of all strains in this study belong to clade B. Clades A (16%), C (9%), D (10%), and E (10%) represent 45% of the global total. Clades F, G, and H account for only 5% of the global total.[2,9] Subtype B is most frequently observed in the Americas, Europe, and some Asiatic countries. In another study, the distribution of HIV-1 subtypes in 21 different African countries, based on a total of 1161 HIV-1 strains, revealed that subtype A is by far the most commonly recorded strain (44%) in Africa. Subtype A was most prevalent in West and Central Africa, subtypes A and D in East Africa and subtype C in Southeast Africa.[3]

Overall, the estimated prevalence of HIV-1 subtypes worldwide is for subtype C: 30%; A: 23%; B: 16%; E: 13%; D: 11%; others: 7% (Osmanov S, UNAIDS, 1997, personal communication). For the period 1980 through 2000, the estimated global incidence will be highest for clades E and C, followed by clades A, D, and B.[2] Since 1995, HIV-1 dual-infection and intersubtype recombinant in one individual by two different HIV-1 subtypes have been described.[10,11] Retrospectively, already in 1995, 10% of the reported sequences in the HIV sequence database, showed evidence for intersubtype recombinant or mosaic genomes.[11]

Intersubtype recombination is an important additional source of HIV-1 genetic variation globally. Analysis of full genome sequences and subsequent phylogenetic analysis revealed that A, B, C, D, F, and H can be considered as true subtypes, but subtypes E and G consisted so far of recombinant viruses A/E, G/A and A/G.[12,13] The A/E subtype is common in Thailand as well as in the Central African Republic. Intersubtype recombinants A/C, A/D, A/D/I/?, A/C, G/H, A/G/I/?, A/G/H, B/F have also been described.[2,14,15] In close collaboration with Salminen and McCutchan, we have recently generated two full-length genomes identified as subtype F strains, and two F/D recombinants. Full-length genomic sequence allowed us also to identify one homogenous subtype H strain and four apparently multiple recombinants. These observations suggest that in the near future, the emergence of new intersubtype recombinant HIV-1 variants is to be expected.

IMPACT OF HIV-1 GENETIC VARIATION ON FUTURE VACCINATION DESIGN STRATEGIES

Design of vaccines based on representative prevalent HIV-1 strains circulating in a given geographical area

Since 1988, 27 candidate vaccine formulations have been evaluated in more than 2000 subjects in phase 1 and a few in phase 2 clinical trials. Majority of the candidate HIV vaccines were envelope gene-encoded products such as glycoprotein 160 and 120, derived from subtype B strains such as HIV-1 IIIB, HIV-1 MN, and HIV-1 SF2.[16,17] Since, as was shown in the previous paragraph, less than 10% of the 30 million HIV-infected individuals are infected with subtype B, there is an urgent need to design candidate vaccines that contain immunogens derived from representative strains of more prevalent subtypes such as C, A, and A/E. These will be the vaccines of choice for in developing countries. It is encouraging to note that at least a clade A/E gp120/MF59 vaccine is being developed and will be tested in Thailand.[17,18] A number of clade A/E vaccine candidates derived from primary HIV-1 clade A/E viruses (viruses not grown in T cell line adapted cells), including oligomeric gp140 and gp160 constructs, pseudovirions, virus-like particles, and DNA are imminent.[19]

Design of HIV-1 vaccines based on cross-clade conserved epitopes able to induce potent cross-clade neutralization responses

Evidence for conserved neutralization epitopes across clades in HIV-1 infected individuals:

The "neutralization type" does not correlate with the genetic subtype. Several studies comparing the capacity of panels of polyclonal sera from individuals infected with viruses representing genetic subtypes A to I in group M and representatives of group O (neutralization checkerboards) to neutralize primary HIV-1 isolates representing clades A–I have been performed.[20–23]

Mathematical analysis of interclade and intraclade neutralization data reveals neutralization clusters, which do not correlate with the known genetic subtypes.[21,23] One exception was the study of Mascola[24] in which polyclonal sera from patients infected with clades B and E do preferentially neutralize viruses of the homologous clade. However, some of the sera do show low level cross-neutralization with viruses of heterologous clades.

Some primary group M HIV-1 isolates, regardless of their genetic subtype are highly sensitive to neutralization by some polyclonal sera of HIV infected individuals, while others are less sensitive and some are completely resistant. Similarly, some polyclonal sera are potent while others are either weak or have no neutralizing capacity. Generalist sera are capable of neutralizing a large variety of genetically diverse primary isolates of different clades. Specific neutralizing sera are only able to neutralize a restricted range of isolates or a particular strain.[22]

Limited number of primary key isolates as a surrogate for neutralization of the entire spectrum of primary HIV-1 isolates. We have identified three primary key isolates, HIV-1 MN, HIV-1 VI525, and HIV-1 CA9, further designated as MN (subtype *gag* B/*env* B; X4 coreceptor type), VI525 (*gag* H/*env* G and *gag* A; X4R5), and CA9 (*gag*/*env* group O: R5).[22] Recently, six biological clones of VI525 have been generated. Five out of six were subtype A and one subtype G in *env*. One *env* A and one *env* G biological clone were phylogenetically classified as subtype A and H, using sequence information from the p7 region (230bp fragment) of the *gag* gene. Consequently, VI525 is at least a mixture of two different intersubtype subsequently designated as *gag* H/*env* G and *gag* A/*env* A (Beirnaert E, Institute of Tropical Medicine, Antwerp, Belgium, personal communication). If sera can neutralize these key isolates, then they will also be active against 16 primary HIV-1 isolates representing subtypes A–H and three group O primary isolates.[22] This was initially observed with 14 polyclonal sera of HIV infected individuals and confirmed by examining a total of 67 sera (66 HIV-1, 1 HIV-1 + 2, 51 men, 14 women, 2 of unknown gender). Sera that neutralized all three key isolates in fresh phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) were then shown to be able to neutralize 14 (12 group M, subtypes A-H (*env*); 2 group O) other primary isolates. The potency of a serum is defined as the logarithm of the geometric mean of the neutralizing antibody titers. Fifty percent inhibitory doses were defined as the highest serum dilution that produced 50% reductions in antigen production.

Seven patients (6 female, 1 male) with broad cross-neutralizing capacity were identified. The neutralizing activity was immunoglobulin G (IgG) mediated, because it could be absorbed to and eluted from a protein G column. Pre-incubating sera with recombinant gp160 (MN) reduced the neutralizing capacity, indicating that the broad cross-neutralizing antibodies are at least partially directed against gp160.[25]

These results confirm our initial observations, that potent sera that neutralize the three key isolates (MN, VI525, CA9) also neutralize at least 14 other primary HIV-1 isolates representing group M (A–H) and group O. Sera lacking a neutralizing effect on the three key isolates failed to neutralize the 14 other primary HIV-1 isolates. These findings suggest that epitopes present in Env of the key isolates may be of major importance in interacting specifically with the cross-clade neutralizing antibodies. Such key isolates could be used to screen sera derived from immunized human volunteers as well as animals for cross-clade neutralizing activity of primary HIV-1 isolates in group M and group O.

The observation that some potent polyclonal sera of HIV-1 infected individuals neutralize primary HIV-1 isolates representing clades (A–H) and representatives of group O suggests that in the envelope of primary HIV-1 isolates some conserved neutralization epitopes must be present that are able to interact with certain neutralizing antibody populations, present in polyclonal sera of HIV-infected individuals.

Potent cross-clade neutralizing sera were observed in HIV-1 individuals, infected with different HIV-1 subtypes. That means that in HIV-1 infected individuals the lymphocyte B-cell repertoires exist, which are able to induce cross-clade anti-HIV-1 neutralizing antibodies. This favors the hypothesis that in primary HIV-1 isolates of different clades common antigens or immunogens must exist that can induce cross-clade anti-HIV-1 neutralizing antibodies. However, in terms of vaccine design, one should keep in mind that an antigenic epitope, i.e., one that binds neutralizing antibody, is not necessarily immunogenic in the same context.

Use of human monoclonal antibodies to reveal conserved cross-clade neutralization epitopes. Accumulating data obtained with human monoclonal antibodies (HuMabs) further strengthen the hypothesis for the existence of conserved epitopes involved in the cross-clade neutralization process.

A panel of 13 Mabs tested with cells infected with viruses from clades A–E permitted the identification of shared epitopes, as well as clade-restricted and clade-specific epitopes and revealed that some regions on the viral envelope (e.g., gp41) that may not be exposed on the surface of intact virions, are well exposed on the surface of infected cells.[26]

Three HuMabs, IgG1b12, 2G12, and 2F5, derived from persons infected with clade B isolates potently neutralized from four to six from a total of nine subtype B primary HIV-1 isolates. Their neutralizing potency was sufficiently high to suggest that passive immune therapy with a combination of these three HuMabs, could be a valid approach.[27]

Other reports showed that each of these three HuMabs could also neutralize selected primary HIV-1 isolates from clades A to E as well as HIV-1 group O isolates, giving further support for conservation of neutralizing epitopes.[28,29] What is the nature of these conserved neutralization epitopes? Epitopes present in the envelope of primary HIV-1 isolates recognized by these three HuMabs are: CD4 binding region, a conformational

epitope dependent on C2, C3, V4 and C4 domains, and an epitope ELDKWA in gp41.[30]

The HuMab IgG1b12 is directed toward the CD4-binding domain in gp120 oligomers. Current evidence indicates that the CD4-binding domain is most likely a group of closely related discontinuous determinants.[29] A combination of IgG1b12 and the anti-gp41 Mab 2F5 in *in vitro* neutralization assays has demonstrated a synergistic neutralization of primary HIV-1 isolates, stressing the importance of both the $CD4^-$ as well as the ELDKWA epitope in gp41 (Kessler JA II and Conley AJ, unpublished data, 1996). The presence of the ELDKWA epitope in gp41 in primary HIV-1 isolates correlates with the neutralization of these isolates by HuMabs 2F5. This 2F5 neutralizes a broad range of viruses.[31,32]

Among the 16 primary HIV-1 isolates used in the neutralization checkerboard, we have documented the ELDKWA sequence only in HIV-1 MN (key isolate) and HIV-1 MAL. At the same position ALDKWA was observed in key isolate HIV-1 VI525 and ELDEWA in key isolate HIV-1 CA9.[33]

Recently, Buratti et al.[34] reported a conserved-reactive IEEE motif in the 735–752 region of gp41 as well as in the 104 to 107 region of the HIV-1 p17 matrix protein. This motif is of particular interest, because it was found to be present in isolates representing different genetic clades, as well as to be the target of broadly neutralizing antibodies and cytotoxic T lymphocyte response against HIV-1.[34–38]

Furthermore, other studies have reported the ability of an Mab (Mab 1575) to neutralize HIV-1 strain SF33, which has a IEEG motif in the 735–752 region. We have examined the presence of these motifs in the *env* and *gag* regions of 17 primary HIV-1 isolates representing clades A–H in group M and three HIV-1 group O isolates, which were used in our previously described neutralization checkerboard experiments.[33]

We observed that the IEEE and IEEG motifs were common to isolates of different genetic subtype as previously reported[35,36,38] and these motifs were common to isolates in the different clusters. It was striking to observe that the IEEE quadruplet was present in 67% (4/6) of the generalist and only in 30% (3/10) of the specialist isolates. The fact that these motifs have been shown to be capable of eliciting high titers of neutralizing antibodies against different strains[35,36,38] suggest that they may be part of the epitopes playing a role in the broad cross-neutralization observed in our study.[33]

Of the same 17 primary HIV-1 isolates used in the above-mentioned checkerboard neutralization experiments, their SI capacity in MT2 cells and coreceptor usage (CCR5 and CXCR4) on GHOST cells (Hos cells with CD4, CCR5, or CXCR4 and green fluorescein protein [GFP] as the indicator gene) was determined. The SI properties correlated well with coreceptor usage. NSI isolates were exclusively R5, while SI primary isolates were either X4 or X4R5. Generalist potent sera neutralized HIV-1 isolates irrespective of their coreceptor usage. Potent specialist sera of individuals infected with an X4 or X4R5 type of primary HIV-1 isolate neutralized specialist X4 and X4R5 type better than R5 type of viruses, and vice versa.[39]

These observations further confirm that the grouping of isolates into the respective neutralization clusters by spectral map analysis does not just occur by chance, but that they are ruled by certain biological properties common to the viruses belonging to the clusters defined by spectral map analysis.[22] Meanwhile, biological clones of these 17 primary HIV-1 isolates have been isolated and are being used to generate a neutralization checkerboard to correlate neutralization clusters with second receptor use. The availability of cloned virus should allow us to better correlate gp160 structure (primary, secondary, tertiary) with sensitivity to neutralization by polyclonal and monoclonal antibodies, as well as with their coreceptor binding.

By competitive binding assays with neutralizing HuMabs involving binding of IgG1b12 and 2G12 to gp120 it was shown that gp120 interacts directly with CCR5. The binding site on gp120 for CCR5 has been narrowed down to a fragment retaining the CD4-binding site, overlapping epitopes, and epitopes in the V3 loop.[40,41] Consequently, one can hypothesize that the capacity of HuMabs and polyclonal sera to neutralize primary SI and NSI viruses, may be partially driven by neutralizing antibody-mediated blockade of the epitopes in *env* involved in CD4 and second-receptor usage.

At least, work with HuMabs and T cell line adapted (TCLA) viruses has shown that antibodies that inhibit the gp120-CD4 binding as well as antibodies that inhibit post-gp120-CD4 binding (interaction with second receptors and fusion) are playing a role in *in vitro* neutralization.[30,42] However, *in vitro* neutralization data with the same polyclonal sera and/or HuMabs may differ significantly between primary and TCLA strains. Primary isolates are considerably less sensitive than TCLA strains to neutralize by soluble CD4 and by most potent cross-clade neutralizing Mabs. One of the reasons is the difference in the degree of association between the surface glycoprotein gp120 (SG) with the transmembrane protein gp41 (TM). The TMs of TCLA viruses shed their SG more rapidly than those of primary isolates (PI). Therefore, incubation at a constant temperature of identical amounts of TCLA and PI virions will result, after a certain time, in less number of SG associated at the TM proteins in TCLA as compared with PI. Assuming the neutralizing antibody has the same affinity for the SG, a larger quantity of NA will be needed to neutralize the same number of PI compared with TCLA virions. Consequently, TCLA virions are more sensitive to neutralization. Also other factors such as the affinity of the neutralizing agent to native *env* oligomers, the minimum number of SG-TM molecules required for binding and fusion, the affinity of SG-TM molecules for cell receptors, as well as the receptor density at the surface of the host cell, may all contribute to differences in the capacity of TCLA and PI viruses to become neutralized by either polyclonal or Mabs.[43–46] Although many factors can rule the neutralization process, it does not exclude that one fundamental neutralization mechanism exists in which some of the factors are contributing more than the others. Spectral map analysis of the checkerboard neutralization data obtained with 16 primary HIV-1 isolates and polyclonal sera of individuals infected with subtypes (A–H) and group O, showed that the *in vitro* neutralization using a PBMC-PHA stimulated cell-based neutralization assay, was ruled by four structural factors that contributed 37%, 23%, 14%, and 9% of the variance of the neutralization spectra, respectively.[22] Whatever these factors may be, they are at least limited in number.

Why is the prevalence of polyclonal sera as well as HuMabs derived from HIV infected individuals with a potent cross-clade neutralizing capacity low? Initially, we reported that 4 of 14 (28%) polyclonal sera of individuals infected with

group M (A–H) (n = 11) or group O (n = 3) neutralized 16 of 16 (100%) primary HIV-1 isolates tested, representing group M (A–H) (n = 13) and group O (n = 3).[22] In a follow-up study in our laboratory, only 12% of a total of 67 polyclonal sera of HIV-infected individuals tested showed evidence of being able to potently neutralize 14 primary HIV-1 isolates of clades A–H in group M and 3 primary HIV-1 isolates of group O.[25] Studies in the United States revealed that only 10% (2/21) of polyclonal sera of individuals infected with clade A, B, C, D, and E were able to neutralize 21 primary HIV-1 isolates, representing clades A–E.[47] None of the 24 sera representing individuals infected with clade A, B, C, F, and I were able to neutralize 14 primary isolates representing subtype A, B, C, F, and I.[21] From these studies the overall estimated prevalence of polyclonal sera showing cross-clade neutralizing capacity is 10% (13/126).

It is probable that the epitopes inducing cross-clade neutralization are conserved but might be of low immunogenicity in the course of HIV-1 infection. This was supported by studies using an IgG1b12, specifically selected, peptide phage display, and polyclonal sera from HIV-infected individuals, which showed that antibodies capable of competing with the Mab IgG1b12 peptide ligand binding are not present in most infected humans.[29]

Thus it is possible that potent neutralizing capacity of polyclonal sera of HIV-infected individuals are related to the synergistic action of different antibody populations directed toward different conserved epitopes with low immunogenicity.

To generate a sufficiently high level of neutralizing antibodies in order to be measurable, the weak conserved epitopes have to be repeatedly exposed to the immune system. It can be that these epitopes are shielded by the major gp120 variable loops or by N-linked sugars, and that they only become exposed after binding of the virus to the CD4 receptor, which induces a change in the conformation of Env.[48,49]

Acceptance of this idea, leads to the suggestion that within an individual host a gradual increase in the capacity of antibodies to cross-neutralize primary HIV-1 isolates is a function of time after moment of infection. This concept was supported by the observation that neutralizing activity against primary HIV-1 isolates representing clades A, D, and E, both by frequency and titer, increased over time in long-term nonprogressors, but decreased in the group of fast progressors.[50] A gradual increase in the breadth of intraclade cross-neutralizing capacity against primary HIV-1 isolates in function of time after moment of infection has been observed in 18 HIV-1 infected subjects, in whom autologous and heterologous neutralizing antibody responses were monitored 1 to 8 months up to more than 2 years after seroconversion.[51] We observed the same phenomenon in a chimpanzee naturally infected with an HIV-1-like SIVcpz-ant. Serum taken 41 months after the start of the study neutralized all nine primary SIVcpz-ant isolates taken with a 4-month interval after start of the study. However, serum collected at the start of the study did not neutralize the time paired primary SIVcpz-ant isolate, neither the SIVcpz-ant isolates taken later. On average, neutralization escape mutants emerged after 15 months and mounted a neutralization response approximately 8 months later.[52] In terms of vaccine design it would be of great help to identify the conserved weak immunogenic determinants in epitopes that can induce neutralization response against a broad spectrum (inter- and intraclade, SI and NSI) of primary HIV-1 isolates and to improve the immunogenicity.

Are neutralizing antibodies against interclade and intraclade primary HIV-1 isolates inducible by HIV-1 candidate vaccines?: In chimpanzees intranasal immunization with life recombinant adenoviruses containing a gp160 insert derived from a clade B HIV-1 MN strain, followed by one or more boosts of Chinese hamster ovary (CHO) cell-derived clade B HIV-SF2 gp120, delivered intramuscularly with MF59 adjuvant, induced neutralizing antibodies to the homologous HIV-1 MN and HIV-1 SF2 primary isolates and to two (HIV-1 SF33, HIV-1 BZ167) of three heterologous clade B primary isolates. The presence of neutralizing antibodies correlated with protection against intravenous high-dose challenge with a PBMC grown HIV-1 SF2. All of the clade B viruses used in these chimpanzee experiments use CXCR4 as a coreceptor (X4 type) and two of the heterologous strains (SF33, 92HT593) also use CCR5 and thus are dual tropic or X4R5 type of viruses. One of the X4R5 type viruses (92HT593) was not neutralized. Given that the V3 loops of the four neutralized viruses were quite divergent from those of the immunizing strains, it is unlikely that the majority of the induced neutralizing activity was directed mainly at the V3 loop part of the envelope. These results suggest that the clade B X4 type of HIV-1 immunogen induced neutralizing antibodies to conserved epitopes shared by several, but not all, primary clade B X4 type of viruses.[53]

This study with chimpanzees and similar studies with a different prime-boost regimen with humans are the first to suggest that clade B X4 type HIV-1 vaccines can induce neutralizing antibodies against a limited range of HIV-1 clade B primary isolates of the X4 or X4R5 type.[54]

Sera from human volunteers vaccinated with ALVAC gp160MN and/or gp120 SF2, both vaccine components being derived from laboratory-adapted (SI) clade B strains, were able to neutralize four of eight clade B SI primary isolates, but none of five clade B NSI primary isolates. So far, these data answered our question partially, in short, HIV-1 candidate vaccines can induce in humans and chimpanzees neutralizing antibodies against intraclade, but not yet against interclade primary HIV-1 isolates, belonging to the same phenotype (SI) as the virus from which the vaccine was derived. In addition, these data also suggest that for the induction of neutralizing antibodies to a broad array of HIV-1 primary isolates, immunogens should be used that share some conserved immunologic and phenotypic characteristics with the viruses from which the vaccine constructs were derived.[55]

For future design of vaccines these data suggest that in order to induce neutralizing antibodies to the entire spectrum (intersubtype and intrasubtype as well as X4, X4R5, and R5 type of viruses), immunogens of primary isolates representative for the whole spectrum, should be incorporated into the vaccine.

Zolla-Pazner and colleagues[55] suggested a model that represents the antigenic spectrum of HIV as a line, with the SI viruses X4 and/or X4R5 type at the extreme left and the NSI (R5 type) at the extreme right-hand side.

Will the limited number of primary key isolates we have identified represent the HIV-1 antigenic immunogen spectrum? Will HIV-1MN (clade B, SI, X4 type) be at the left side, VI525 (*gag* H/*env* G and *gag* A/*env* A, X4R5, SI type) at the middle

and HIV-1 CA9 (group O; NSI, R5 type) at right-hand side of the line, representing the antigenic spectrum of HIV?

Will immunogens derived from the first key isolate HIV-1MN, SI, X4 type induce neutralizing antibodies, which will be able to neutralize a limited number of antigenically related SI, X4 type of viruses and will not neutralize R5 type of viruses? The data of S Zolla-Pazner[55] seem to support this hypothesis. It has still to be determined if sera of chimpanzees or human volunteers vaccinated with HIV-1MN-derived immunogens will neutralize X4 type of viruses of clades different from clade B. The same question can be posed for the second key isolate HIV-1 VI525. Will animals vaccinated with immunogens derived from the second key isolate HIV-1 VI525 induce antibodies that can neutralize SI, X4R5 type of viruses that are antigenically sufficiently close to HIV-1 VI525?

The same question can also be posed for the third key isolate HIV-1 CA9 (group O, NSI, R5 type). Will animals vaccinated with immunogens derived from the third key isolate induce antibodies that will neutralize the homologous as well as heterologous NSI R5 type of primary isolates sufficiently antigenically related to the HIV-1 CA9? Will the sera induced by HIV-1 CA9 not neutralize SI or X4 type of virus? If for the sake of simplicity we assume that the immunogen spectrum of HIV-1 only consists of these three prototypes of immunogens, HIV-1 MN, HIV-1 VI525, and HIV-1 CA9, would a vaccine containing these three immunogens induce neutralizing antibodies against the entire HIV-1 spectrum? Arguments in favor for the inclusion of at least an HIV CA9 group O derived immunogen into the future vaccine candidate, comes from our previous studies in which we have shown that most HIV-1 group M sera (9/16) neutralized group O viruses, whereas fewer group O sera (3/13) only weakly neutralized primary HIV-1 group M viruses (clades A-H).[56] This alludes perhaps to the existence of a too large antigenic and/or immunogenic difference between primary HIV-1 strains of group M and group O. We have demonstrated that cross-neutralization between HIV-1 and SIVcpz isolates by their sera is more extensive compared with the infrequent and low-titered cross-neutralization observed between HIV-1 and HIV-2. This pattern of neutralization seems to correlate with the phylogenetic classification that distinguishes these viruses.[57] These observations suggest that a candidate vaccine that should induce protection against a broad spectrum (cross-clade HIV-1, HIV-2, SI and NSI) HIV infection, should at least contain HIV-1 cross-clade group M (SI and NSI), HIV-1 cross-group O (SI and NSI), and HIV-2 cross-clade (SI and NSI) derived immunogens.

To verify if the three key isolates we have identified can induce a broad spectrum neutralization response, inactivated key isolates or immunogens derived from them, will be prepared and inoculated into rabbits. The inactivation protocol used was previously described.[58] Individual sera, as well as combinations of the immune sera will be tested on their capacity to neutralize the full spectrum of primary HIV-1 isolates (cross-clade, X4, R5, X4R5 type). As a start, rabbits were inoculated with the inactivated HIV-1 VI525 key isolate with and without Ribi adjuvant. Immune serum taken at 9 weeks postinoculation have so far remained negative for antibodies that can neutralize the HIV-1 VI525 primary isolate, using a PHA stimulated human PBMC-based neutralization assay. Further immunization experiments are still ongoing and immune sera will be retested with more sensitive neutralization assays (Beirnaert E, Institute of Tropical Medicine, personal communication, 1998). It is eminent that for a vaccine to be effective against the full spectrum of HIV-1 isolates (X4, R5, X4R5, cross-clade) it should contain immunogens representative for each of the prototype immunogens as well as immunogens conserved through the full spectrum (cross-clade, X4, R5, X4R5).

SUGGESTIONS TO FACILITATE FUTURE RESEARCH TO OVERCOME THE GENETIC VARIABILITY BARRIER IN HIV VACCINE DESIGN AND EVALUATION

High throughput neutralization assay

Protection against infection in vaccinated chimpanzees correlated with the presence of neutralizing antibodies against homologous cell-line adapted viruses and against homologous and heterologous primary isolates grown in PBMCs and tested in a variety of neutralization assays. The neutralization antibody titer associated with protection was dependent on the virus strain and neutralization assay conditions used.[53] Consequently, it is not simple to assign an absolute value for titers of neutralizing antibodies that correlate with protection. Another disadvantage is that most of the neutralization assays used so far are labor-intensive and time-consuming. Reduction in p24 Ag levels was measured most often at 6 to 7 days postinfection.[53] Because an effective candidate vaccine will have to induce a strong neutralization response to a broad spectrum of HIV-1 strains and hopefully very soon many more phase 2 and phase 3 trials with candidate vaccines will start, a well-standardized high throughput neutralization assay is urgently needed.

An approach to better standardize the host cell used in the neutralization assay is the use of engineered cells that are transfected with the necessary genes to express the cell-receptors required for TCLA and primary HIV-1 entry and a reporter gene to monitor quickly the active growth of HIV-1 in his host cell. Recently, human osteo sarcoma (HOS) cells expressing CD4, and the coreceptor CCR1, CCR2, CCR3, CCR5, CXCR4, Bob or Bonzo and a green fluorescence protein (GFP) as the indicator gene have been used in neutralization assays with both TCLA and primary HIV-1 strains. Reduction in virus growth due to the presence of neutralizing antibodies was measured by monitoring a reduction in the number of green fluorescencing cells.[59] The GHOST cell assay takes 2–4 days compared with the blast assay, which takes 6–7 days.

For measuring the neutralization of TCLA cytopathic HIV-1 strains a high throughput fully automated screening assay can be designed that operates according to the principles used for monitoring anti-HIV-1 viral drug screening. This system measures with a colorimetric assay, quantitatively the reduction in the number of viable MTT treated MT4 cells.[60]

HVST-cells, which are PBMCs immortalized by infection with herpes virus saimiri, offer the possibility for a more standardized continuous supply of host cells very close to the PBMCs of human donors used in neutralization assays.[61] HVST-cells retain the phenotypic cellsurface markers and cytokine profile of activated mature T lymphocytes. They were permissive to the replication of both HIV-1/2 TCLA viruses and primary HIV-1 isolates. Infection in these cells was persistently productive for up to 5 months. The downregulation of

surface CD4 was delayed and virus yields exceeded those obtained in T cell lines. The HVST-cells need further testing as potential host cells in high throughput screening of antibodies with a neutralizing capacity against a broad spectrum of primary HIV-1 isolates.

CEMx174 LTR SEAP cells transfected with a *tat* responsive promoter from HIV-1 NL4-3 linked to a gene coding for alkaline phosphatase. Concentration of alkaline phosphatase secreted from the cell over 48–72 hr after infection correlated with the dose of virus. As such the time required to quantify neutralization is reduced by about 50%. The enzyme is released into the supernatant and monitored by chemoluminescence. This potentially allows full automation and high throughput spin-off. This system worked well with the HIV NL4-3 laboratory-adapted virus and one primary HIV-1 isolate. Can neutralization of a wide range of primary HIV-1 isolates (X4, R5, X4R5 type, cross-clade) be monitored in sera of vaccinated humans and/or animals with this system?[62]

Based on the earliest intracellular synthesis of nascent HIV-1 long terminal repeat (LTR) fragments, a heminested polymerase chain reaction (HNPCR) amplification of the 5'LTR sequences (LTR-HNPCR) was developed. Replication-competent HIV could be detected in PBMCs, 16–18 hr postinfection. *In vitro* neutralization of five primary HIV-1 isolates with six Mabs and an enriched IgG from an HIV-infected individual, was documented. If this system will work for the full spectrum of HIV-1 strains (X4, R5, X4R5 type, cross-clade) it offers potential utility in evaluating immune response to candidate vaccines.[63]

Because binding and fusion are essential steps for HIV-1 to enter in its host or target cells, development of a test to monitor rapidly the inhibition of binding of the full spectrum of primary HIV-1 isolates on PBMCs, could be a valuable alternative. Preliminary data have shown that IgG enriched fractions of potent polyclonal sera of HIV-positive individuals were able to inhibit binding of intact primary HIV-1 isolates on PHA stimulated PBMCs of human donors. Sera of HIV-infected individuals without neutralizing activity and control sera failed to inhibit the binding of the primary HIV-1 isolates (Beirnaert E, Institute of Tropical Medicine, Antwerp, Belgium, 1998, personal communication).

This type of binding assay allows the neutralizing effect of antibody populations present in sera as well as HuMab to be monitored to intact virions of primary HIV-1 isolates. The binding of intact virions to PBMCs was monitored by p24 antigen detection after lysis of the virion as previously described.[64]

Neutralization of a limited number of primary HIV-1 key isolates as a surrogate for neutralization of the entire spectrum of primary HIV-1 isolates

Our observation that sera of HIV-positive individuals that neutralize the three key isolates (HIV-1MN, X4, SI, clade B; HIV-1 VI525, X4R5, SI, *gag* H/*env* G and *gag* A/*env* A; HIV-1 CA9 R5, NSI, group O) are also capable in neutralizing 14 primary isolates representing clade A–H and three group O strains, should be extended to a larger number of primary isolates representing the known clades. If our initial observation is further confirmed for a wider spectrum of HIV-1 primary isolates, these three key isolates in combination with a high throughput sensitive neutralization assay would allow to screen a large number of sera from vaccinated animals and human volunteers, as well as HuMabs, for their capacity to neutralize cross-clade primary HIV-1 isolates.

Subtype identification

True subtype prevalence rates, especially in developing countries, are lacking and need further investigation.

The V3-loop peptide enzyme-linked immunosorbent assay (PEIA) potentially is a promising test for quickly monitoring the prevalence of subtypes in a particular geographic area because it can be done through serology, a technique currently used in developing countries with the highest variety of HIV-1 subtypes. Subtyping using PEIA in countries such as Thailand where only two subtypes of HIV-1 circulate has been shown to be very effective.[65,66] However, this technique has not been very successful in areas where several antigenically related subtypes of HIV-1 cocirculate, due to a high level of cross-reactivity.[67,68]

Our results indicate that V3-loop PEIA methodologies used in different laboratories correlate poorly with genetic subtyping, and their accuracy in predicting HIV-1 subtypes, varies considerably in sera of Belgian individuals infected with different HIV-1 subtypes (A, B, C, D, F, G, and H). The poor correlation between serotyping and genetic subtyping was partly due to the simultaneous occurrence of subtype-specific octameric sequences at the tip of the V3-loop of viruses belonging to different genetic subtypes. We propose to improve PEIA subtyping by extended collaborative studies in which larger numbers of samples from diverse geographic regions are genetically characterized by phylogenetic analysis of nucleic acid sequences, and used as gold standard in PEIA subtyping. New peptides representative for different genetic subtypes will be synthesized and tested. Statistical methods such as polygram analysis,[52,69] will be used to select amino acid tetramers in the immunodominant regions of Env that are specific for a particular genetic subtype, and can be helpful in the design of new peptides. Peptides will be synthesized containing these amino acid quadruplets. Subsequently, PEIA reactivity patterns of a single serum against multiple peptides will be analyzed by spectral map analysis.[52,69] The latter will allow grouping sera from patients infected with a similar HIV-1 subtype, based on the homology of reactivity patterns obtained in PEIA using different peptides. A similar factor analysis for V3 serotyping has also been described by Karamov et al.[70]

Full-length genomic sequence with breakpoint analysis needs to be expanded in order to document the true nature of intersubtype recombinant HIV-1 strains.[71]

Rapid monitoring of the immunogenic relatedness of different HIV-1 genetic subtypes

HIV-1 virions will facilitate the design of a vaccine against HIV-1, if the hypothesis holds that conserved antigenic epitopes turn out to be also good immunogens, which can induce a potent broad spectrum neutralization response.

We have studied the antigenic conservation of epitopes of HIV-1 isolates of different clades A, B, D, F, G, and H with a panel of 17 human anti-HIV-1 gp120 and gp41 HuMab. The HuMabs were derived from clade B-infected individuals and were directed against V2, V3, CD4 bd, C5, and gp41. The HuMabs were coated onto microtiter wells followed by addi-

tion of virus. Bound virus was detected after lysis by testing for p24 antigen.[72] This work should be extended including HuMabs derived from nonclade B-infected subjects, such as HuMab 1324 E. This Mab, made from the cells of a clade E-infected individual, and selected with a peptide of the clade E consensus V3 loop, differs from 20 human anti-V3 Mabs selected from the cells of clade B-infected patients, in that it recognizes an epitope not previously described, which exists primarily in the V3 loops of clades A, C, E, F, and G (but not B). Moreover, Mab 1324 E distinguishes the V3 loops of clades B and F in a manner that anti-V3 Mabs from clade B-infected individuals fail to do. This is but one example of what will surely be a multitude of new, shared antigenic determinants recognized by the human immune system stimulated with non-B clades of HIV. There is the need to further develop Mabs from non-B clade infected patients and study the complete antigenic topography of different HIV-1 clades.[73]

A broad immunochemical cross-reactivity was also noted when HuMabs were tested for their capacity to bind V3 peptides derived from clade A, B, and C viruses. The HuMabs recognize both linear and conformationally dependent epitopes of the V3-loop.

These findings suggest that cross-clade immunity to HIV-1 may be inducible by HIV-1 vaccines.[74]

Making epitopes involved in inducing neutralizing response more accessible to the immune system

Glycosylation of Env, can have a profound impact on the three-dimensional structure of Env as well as on the presentation of epitopes involved in neutralizing antibody binding.[75]

Carbohydrate molecules on the surface glycoprotein of HIV-1 can mask certain epitopes involved in binding to neutralizing antibodies.[76]

Removal of certain glycosylation sites can have a profound effect on the capacity of the envelope to induce a neutralization response as was recently demonstrated in the SIV-macaque model 820.[77] It opens the possibility by removal of the appropriate glycosylation sites, to increase the accessibility of certain conserved epitopes to the immune system, resulting in a much stronger neutralization and/or CTL response, compared with an individual naturally infected with the same HIV-1 strain as used in the vaccine.

Recent studies have shown that the variable loops V1, V2, an V3 can be removed from the external subunit of the HIV-1 envelope, gp120, without too much disruption of the basic core composed of the conserved C1–C5 parts of the molecule. Deletion of V1/V2 and V3 could not destroy the binding of the gp120 molecule to the CD4 receptor.[48]

If such a gp120 molecule, depleted of its variable sites V1/V2 and V3 could be incorporated as an immunogen in a candidate vaccine, it could perhaps induce a strong neutralizing antibody response against a more conserved structure, which is perhaps the same across the entire HIV-1 antigenic and biophenotypic spectrum.

CONCLUSIVE REMARKS (see TABLE 1)

Accumulating data in HIV-infected individuals so far support the hypothesis that in primary HIV-1 isolates of different clades and/or phenotype (SI and NSI) common antigenic structures must exist that can program the B lymphocytes to produce broad spectrum (cross-clade and cross-SI and cross-NSI) neutralizing antibodies. However, only 10% of polyclonal sera of HIV-infected individuals show broad spectrum (cross-clade, SI and NSI) neutralizing activity.

We hypothesise that the epitopes inducing cross-spectrum (= cross-clade + SI and NSI) are conserved but might be of low immunogenicity in the course of HIV-1 infection. For future vaccine design it would be of help to identify these conserved weak immunogenic epitopes and to enhance their immunogenicity by removing certain glycosylation sites as variable loops in Env, in order to make these epitopes more accessible to the immune system (see Table 1).

Studies with vaccinated chimpanzees and studies with a different prime-boost vaccination regimen in humans have shown that clade B, X4-type HIV-1 derived vaccines can induce antibodies that neutralize homologous and some heterologous clade B, X4, and X4R5 type of primary HIV-1 viruses, but not clade B, R5 type of viruses.

It has not yet been proven if these sera of vaccinated chimpanzees and human volunteers can neutralize X4 and X4R5 type of primary HIV-1 viruses of clades different from B.

It is our understanding that for a vaccine to be effective against the full spectrum of HIV-1 isolates (X4, R5, X4R5, cross-clade) it should contain immunogens representing each prototype as well as immunogens conserved throughout the full spectrum (cross-clade, X4, R5, X4R5). There is an urgent need

TABLE 1. POTENTIAL APPROACHES FOR FUTURE HIV-1 VACCINE DESIGN AND SUPPORTIVE RESEARCH TO OVERCOME THE GENETIC AND PHENOTYPIC DIVERSITY BARRIER

Several potential approaches for candidate vaccines are listed below:

- Clade C, A, E specific vaccines in addition to clade B vaccines.
- Immunogens derived from primary HIV-1 isolates.
- Selective deglycosylation of gp120 to generate a conformationally more relevant structure with increased accessibility of conserved cross-clade antigenic sites, which normally are not or difficult to be exposed to the immune system, and which induce a broad spectrum (cross-clade, SI and NSI type) neutralization response.
- Are primary key isolates (HIV-1 MN clade B, SI, X4; HIV-1 VI525 *gag* H/*env* G and *gag* A/*env* A, SI, X4R5; HIV-1 CA9, group O, NSI, R5 type) or components derived from them, able to induce a broad spectrum (cross-clade, X4, X4R5, R5) neutralization antibody response?

In support of the hopefully increasing HIV vaccine phase 1, 2, and 3 trial the following items should be further developed urgently:

- High throughput neutralization assay.
- Neutralization of three primary HIV-1 key isolates as a surrogate for neutralization of the entire spectrum (cross-clade X4, R5, X4R5) of primary HIV-1 isolates.
- High throughput subtyping.
- Rapid monitoring of the immunogenic relatedness of different HIV-1 clades.

to identify the prototype immunogens as well as the conserved immunogens and to improve their immunogenicity drastically (see Table 1). Will the limited number of primary HIV-1 isolates so far identified with promising capability to predict the broad spectrum neutralization nature of polyclonal sera and/or HuMabs, also be good immunogens? Will they represent the limited number of prototype immunogens required to induce a potent broad spectrum neutralization response? We don't know yet, but we are in the process of testing it in the rabbit model (E. Beirnaert, Institute of Tropical Medicine, Antwerp, Belgium, personal communication, 1998).

As long as we have not identified the broad-spectrum inducing epitopes to induce protection, it remains a valuable alternative to design subtype specific candidate vaccines, using HIV-1 strains and/or derived components (see Table 1) that are prevalent in a particular geographical region.

In support of the follow-up and evaluation of the hopefully increasing number of phase 1, 2, and 3 HIV-1 vaccine trials, it is necessary to develop high throughput neutralization assays, to further expand the use of a limited number of key primary HIV-1 isolates as a surrogate for neutralization of the entire HIV-1 antigenic spectrum (cross-clade, X4, R5, X4R5), to develop high throughput subtyping and rapid monitoring of the immunogenic relatedness of different HIV-1 clades.

It is unfortunate that since 1988, only two phase 2 HIV-1 vaccine trials in humans have been documented, and as yet no phase 3 trial. The failure to perform the latter is unethical, because safe HIV-1 candidate vaccines are already available. Only phase 3 trials will allow us to optimize the vaccine as well as to define the correlates of protection.

We must not forget that at least 16,000 new HIV infections occur each day. The history of Sabin's attenuated live vaccine has shown that although at the beginning of the vaccination campaign some prisoners developed polio after vaccination with the first noncloned attenuated polio vaccine batches, less than 6 years later more than 100 million people worldwide had been vaccinated. Sabin's vaccine was officially approved in the United States in 1960, only 6 years after he received the official authorization to test the vaccine in human volunteers!

It is an example that shows that not only science but also politics and decision making are crucial for the development of a vaccine. Genetic and phenotypic diversity of HIV is perhaps less a barrier than politics in the design of a suitable HIV vaccine.

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