

CHAPTER 1

Introduction

The discovery of Human Immunodeficiency Virus

On July 3, 1981, the *New York Times* published an article detailing the diagnosis of a rare form of cancer called Kaposi's Sarcoma among 41 homosexual men primarily in New York City and the San Francisco Bay Area over a period of 30 months (1). Until that time, the nationwide incidence of this disease was one case in every 1.5 million people as reported by the Centers for Disease Control (CDC). Two years later, with 1,350 reported cases, viral DNA from a human T-lymphotropic retrovirus was isolated from the T cells of several patients exhibiting symptoms common to what is now called Acquired Immune Deficiency Syndrome (AIDS) (2). The enveloped virus was named Human Immunodeficiency Virus (HIV), and its discovery earned Drs. Françoise Barré-Sinoussi and Luc Montagnier the Nobel Prize in Physiology or Medicine in 2008. It was classified as belonging to genus *Lentivirus* in the *Retroviridae* family, which encode positive sense single-stranded RNA genomes. The lentiviral classification denotes the protracted period of latency which, in the case of HIV, can exceed seven years before the emergence of opportunistic infections that are collectively referred to as AIDS (3).

Today, HIV is a global pandemic. It is estimated that HIV/AIDS has claimed the lives of 25 million people with an estimated 33 million people living with the disease in 2007 (4). Although the development of life-extending highly active antiretroviral therapy (HAART) in 1996 is credited for the precipitous drop in AIDS-related morbidity and mortality in developed countries such as the United States (5), the costs associated with HAART and the lack of robust health care infrastructures has hindered their widespread use in most developing countries. For example, it was reported that by the end of 2007, the number of people receiving HAART in developing countries had finally reached the

target goal of 3 million – roughly 10% of those in need – two years after the 2005 deadline that WHO/UNAIDS had set in the “3 by 5” initiative proposed in 2003 (4). The combination of economic, logistical, and political barriers to treatment is particularly alarming given that nearly 95% of all HIV-infected people live in developing regions of the world such as sub-Saharan Africa and Asia (6). Education and sexually transmitted disease control programs have proven to be effective strategies for reducing the spread of HIV (7), but the need to develop an inexpensive vaccine capable of preventing or controlling an infection remains one of the most important challenges in combating the pandemic.

The modular nature of antibodies renders them amenable to the construction of antibody-like molecules with architectures that cannot be produced by the natural human immune system. Among others, these architectures include single-chain constructs encoding the variable heavy (V_H) and variable light (V_L) chains joined by short polypeptide linkers (scFv) (8), bispecific antibodies in which the two combining sites bear different specificities (9), and tetravalent bispecific molecules (10). In some cases, these formats have been shown to provide certain advantages over standard antibodies such as increased tissue penetration and more rapid pharmacokinetics (11, 12), multispecificity, enhanced ability to recruit effector cells (13), and increased avidity (10).

The work described here was conducted over a five-year period in the laboratory of Pamela J. Björkman in collaboration with the laboratory of David Baltimore. The primary objective of this work was to utilize existing alternative antibodies architectures and design new ones to uncover principles underlying the mechanisms of HIV neutralization. For example, experiments were conducted to investigate whether cross-

linking epitopes is possible and/or important during neutralization and if other attributes of an antibody, such as size and flexibility, might influence neutralization potency. Attention was also given to the role of antibody-dependent cellular cytotoxicity (ADCC), an issue that has gained greater attention in recent years. Hopefully, the results described here will help guide future work towards the development of new therapeutics and/or vaccines, either in the form of a classical vaccine or an alternative approach such as gene therapy.

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Origin and phylogeny of HIV

When compared to non-human primates (NHPs), the consensus sequence of HIV type 1 (HIV-1) appears to be most closely related to a strain of Simian Immunodeficiency Virus (SIV) that is endemic to *Pan troglodytes troglodytes* (*Ptt*), a sub-species of chimpanzee (14). In the application of various methods of molecular clock analyses that utilize known mutation rates of HIV-1 to the comparison of sequences of RNA extracted from preserved human tissue samples dating between 1959 and 1960, current best estimates for the transfer of HIV-1 into the human population have converged on an initial date of approximately 1910 (15). The simplest and most widely accepted explanation for the crossover from *Ptt* to *Homo sapiens* is the “Hunter Theory”, wherein a period of rapid population growth in sub-Saharan Africa in the early 20th century led to increased contact with and consumption of NHPs (16). The origin of the less virulent and

relatively rare HIV type 2 (HIV-2) is thought to share a similar history with respect to entry into the human population (17). More recently, the evaluation of blood samples collected in 2005 from hunters of NHPs in West Africa have identified new human T-lymphotropic retroviruses similar to HIV-1, suggesting that novel cross-species infection events are ongoing (18). Consequently, measures to reduce the hunting and/or consumption of NHPs may be critical to preventing the crossover of novel retroviruses into the human population.

Because HIV-1 currently accounts for nearly all HIV infections, the term HIV will be used to refer specifically to HIV-1 from this point forward. Among the three recognized phylogenetic branches of the virus, the main branch, group M, accounts for more than 99% of all infections worldwide (19). Distinct lineages within this branch are further subdivided into clades A through K on the basis of sequence diversity (20). Although diversity is found throughout the HIV genome, it is concentrated in the envelope (*Env*) gene, which encodes the gp160 precursor that is subsequently cleaved into gp120 and gp41, the two surface glycoproteins responsible for binding to target cells and catalyzing fusion.

The maximum level of sequence diversity observed between clades is approximately 35% when the HIV branch M-infected population is regarded as a whole, but sequence diversity may reach between 10% and 20% within one chronically infected individual (21, 22). This factor no doubt bears heavily on the ongoing failure to develop a prophylactic or therapeutic vaccine. Further diversity arises from epidemic circulating recombinant forms (CRFs) in which multiple clades are represented within a single isolate (23), presumably arising from recombination events following super-infections of

individuals who are already infected (24).

The global distribution of HIV clades is another factor that may prove critical to the design of a successful vaccine or therapy. Notwithstanding the impact that intercontinental travel has on the spread of HIV clades beyond their geographic origins, specific clades currently tend to be concentrated in well-defined regions. The most common clade today is C, which is primarily found in South Africa, China, and India; clade B is common to the Americas, Europe, Australia, and China; clade E, which is now formally classified a CRF of A and E, is found primarily in Southeast Asia; clades A and D are mostly found in East Africa, and nearly all clades circulate in West and Central Africa including the clades of the other two branches (24-26).

Though classification of HIV isolates by clade is based on general sequence diversity without specific attention to differences in epitopes thought to confer susceptibility to neutralization, at least one study suggests that the cross-reactivity of a particular antibody can be highly clade-specific. Using a small panel of monoclonal anti-HIV IgG subclass 1 antibodies, whose variable regions were derived from the B cells of clade B infected donors, researchers were able to show a statistically relevant clustering between branch M clades A through F on the basis of neutralization (19).

Natural history of HIV infection

In order to infect cells, HIV requires the presence of two receptors on the membranes of target cells: CD4, to which gp120 binds with low nanomolar affinity (27), and one of perhaps as many as a dozen G-protein-coupled receptors (GPCRs) (28, 29). Though CCR5, a principal HIV binding target, is expressed on multiple cell types

including T cells and the epithelial cells of the intestinal tract (30), early methods of characterizing HIV isolates based on the ability to infect different cell lines led researchers to divide them into two groups: R5 virus isolates, which preferentially bind CCR5 and infect activated CD4⁺ memory T cells and macrophages (31, 32), and X4 isolates, which preferentially bind CXCR4 and infect naïve CD4⁺ T cells (32, 33). In addition to CD4 and the dendritic cell (DC) C-type lectin receptors DC-SIGN and DC-SIGNR, these two co-receptors define the tropism of nearly all strains of HIV. Consequently, the primary cellular targets include dendritic cells, macrophages, and naïve, effector, and memory CD4⁺ T cells (28). The microglial cells of the central nervous system expressing the CCR3 GPCR in addition to CCR5 constitute another target as their infection is thought to be an important step leading to the development of AIDS-related dementia by inducing the release of neurotoxins (34, 35).

When initially infected, the majority of people manifest flu-like symptoms and a high viral titer followed by activation of a cytotoxic T lymphocyte (CTL) response and, later, seroconversion (28). Following the early period of acute viremia, the concentration of virus drops to a lower so-called “set point,” a leading prognostic indicator referring to the relatively stable viral titer observed after the early stage of acute viremia, and may remain relatively unchanged for many years without HAART (28). However, the peripheral CD4⁺ T cell count will continue to decay throughout the period of clinical latency and, after dropping below approximately 500 cells/ μ L (28), viral concentration increases as AIDS-defining opportunistic infections emerge, ultimately resulting in death.

With the development of biological models for studying infection such as an *in vivo* model that pairs an HIV/SIV chimera (SHIV) challenge with rhesus macaques,

recent data are converging on the order and nature of early events that occur during mucosal transmission across the vaginal epithelium, the most common mode of HIV infection (reviewed in (36)). Following initial exposure, HIV is believed to penetrate several microns into the stratified squamous epithelium, probably by simple diffusion (37). In this tissue the virus encounters Langerhan cells, a type of DC that migrates within the epithelium and, because of the heavy glycosylation found on gp120, the virus binds to its receptors, DC-SIGN and DC-SIGNR (38-40). Following uptake of the virus, DCs have been observed *in vitro* to form filopodia in response to upregulation of Rho GTPase Cdc42, which then serve to efficiently transfer virus to CD4⁺ T cells via an “infectious synapse” and initiate infection (41). Current evidence suggests that the preceding events probably occur within the first few hours of exposure (36).

After the first few days of infection, the rhesus macaque model revealed that a massive depletion of CD4⁺ memory T cells surrounding the gut-associated lymphoid tissue (GALT) occurs concurrently with a rapid progression to peak viremia (42). The loss of CD4⁺ T cells surrounding the GALT appears to persist in humans even after recovery of the circulating CD4⁺ T cell population observed with HAART (36).

With the development of HAART, it has become possible to derive quantitative information about various steps in the natural history of infection. For example, upon administration of a protease inhibitor called Ritonavir (43) and a high-resolution time course of monitoring virus concentrations and CD4⁺ T cell concentrations among HIV-infected patients, researchers were able to calculate that the average lifetime of a virus particle is ~7 hours, the average life-span of a productively-infected CD4⁺ T cell is ~2 days, and up to ~10 billion new virus particles can be produced per day (44). With such

rapid turnover, the low fidelity of reverse transcriptase (45), and the prevalence of recombination (46), it is not surprising that resistance to individual inhibitors can sometimes be measured in days (47).

Interestingly, despite the sequence variation found in chronically infected individuals, the initial infection can almost always be traced back to a single founder virus whose sequence remains relatively unchanged throughout the initial infection period (48), and for unknown reasons, the co-receptor specificity of these founder viruses is usually for CCR5 rather than CXCR4. Selective pressure from the subsequent cytotoxic T lymphocyte (CTL) response then appears to induce the emergence of divergent virus populations (48).

Unfortunately, the development of potent autologously neutralizing antibodies (NAbs) remains conspicuously absent from the humoral immune responses of most individuals (49, 50). These observations and NHP studies in which depletion of CTLs (*i.e.*, CD8⁺ T cells) led to uncontrolled SIV replication and accelerated death suggest that the early but temporary containment of viral replication that precedes the latency period is largely a function of the CTL response (49, 51, 52). The critical role that the CTL response plays in suppressing viral titer has fueled interest in the development of a CTL-based vaccine that targets conserved MHC class I epitopes but suffers from the fact that it does not address the putative requirement to neutralize cell-free virus particles, which is likely to prove critical to blocking the initial infection. Vaccine candidates have been tested in NHP challenge models (52-56), and the data support the contention that a pre-primed CTL response leads to better viral control (53), but a promising human CTL-based vaccine candidate has yet to emerge.

In contrast to the inherent limitation of a CTL vaccine, passively administered broadly cross-reactive monoclonal NAbS against HIV have been shown to enable NHPs to resist viral challenge as well as suppress viral rebound in humans following interruption of HAART (57-59). These results suggest that a properly designed immunogen capable of eliciting a potent NAb response or efforts to engineer more potent NAbS and antibody-like molecules could prevent an infection and possibly help control an established infection.

Structure of the HIV envelope spike and its mechanism of fusion

The *Env* gene encodes the glycoprotein gp160, which is cleaved into gp120 and gp41 by the cellular protease furin after passing through the Golgi complex (60). A high-resolution X-ray crystal structure of the entire trimeric gp120/gp41 assembly or the heterodimer has yet to be solved. However, sedimentation equilibrium analysis has shown that the mature fusion-competent viral spike present on the virion surface is a 442 kDa assembly of three copies of gp120 non-covalently associated with three copies of gp41 (61), and recent tomographic reconstructions of cryo-preserved HIV and SIV particles derived by electron microscopy have confirmed the three-fold symmetry of the spike (62-64). In addition, high resolution X-ray crystal structures of individual gp120 monomers in different conformational states and a portion of the six-helical bundle of gp41 representing its fusogenic conformation combined with extensive biochemical studies have contributed to the current model for fusion (65, 66).

Two of the high-resolution X-ray crystal structures of monomeric gp120 that are currently available provide substantial clarification to the question of why the human

immune system generally fails to develop potent NAbs against gp120 protein. The first crystal structure, solved in 1998 to 2.5 Å, is of a ternary complex of the hydrophobic core of gp120 derived from strain HxBc2 (also known as IIIB), a soluble form of CD4 composed of the first two domains (sCD4), and a Fab fragment of the modestly effective NAb 17b (67) (Fig. 1). In this ternary complex, nearly all glycosylation has been removed by mutation from gp120, which also carries single GlyAlaGly substitutions for 67 residues of the V1/V2 loops and 37 residues of the V3 loop, and is truncated at the N and C termini. The ability of this altered form of gp120 to still bind a panel of NAbs was confirmed (68).

The authors' analysis of the gp120 interface involved in binding CD4 revealed that the particularly recessed nature of the binding pocket may contribute to its ability to evade the humoral immune response as proposed in the "canyon hypothesis": a conserved binding site is hidden in a pocket that is sterically inaccessible to an antibody combining site while amino acid residues at the surface of the pocket are allowed to mutate with relative freedom (69). It was also observed that many of the contacts with CD4 are mediated by main chain instead of side chain atoms, leaving these residues relatively free to mutate under the selective pressure of the adaptive immune response.

Additional analysis of the interface with Nab 17b, which is known to overlap with the binding site for the chemokine coreceptor, revealed a "bridging sheet" composed of four anti-parallel β -strands, a result that was predicted from the conformational change associated with binding CD4 (27). This prediction appeared to have been validated when the structure of an uncomplexed, nearly fully glycosylated gp120 core from SIV was solved to 4 Å resolution (70) (Fig. 1). When comparing both gp120 crystal structures, it

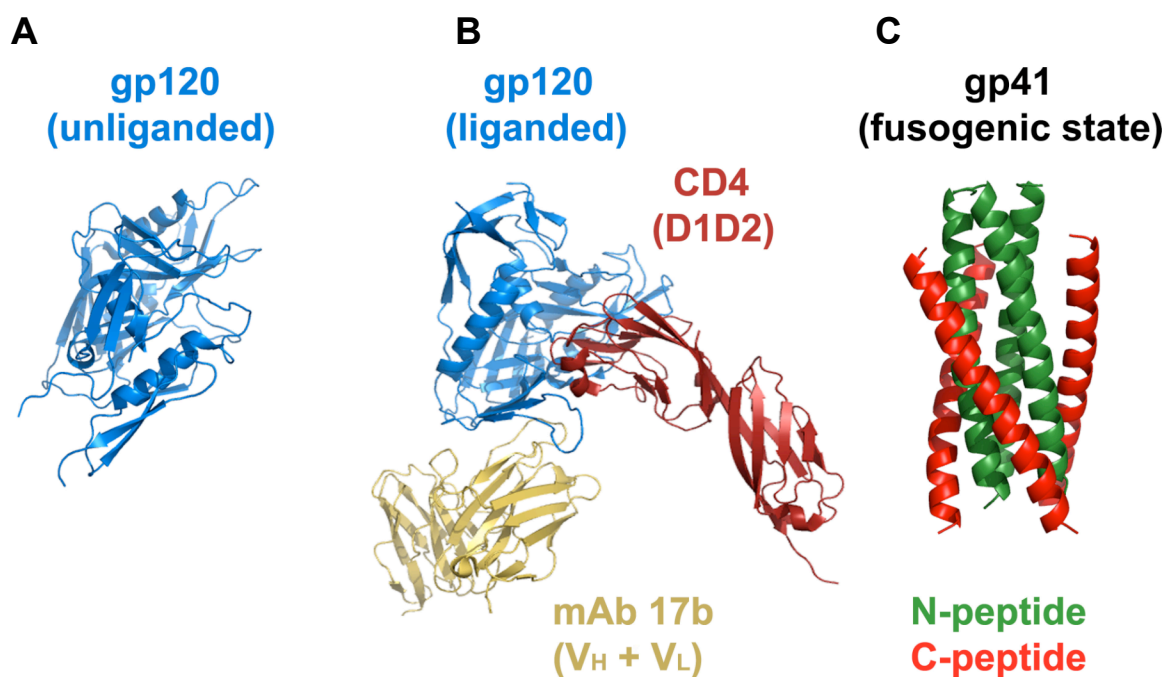


Figure 1. X-ray crystal structures of envelope spike proteins. (A) X-ray crystal structure of unliganded monomeric gp120 (60). (B) X-ray crystal structure of liganded monomeric gp120 bound to CD4 (domains 1 and 2), and mAb 17b (showing the V_H and V_L domains), which overlaps the co-receptor binding site (57). (C) X-ray crystal structure of N-peptides and C-peptides derived from gp41 in the six-helical bundle fusogenic state (71).

can be seen that in the unliganded structure the two β -strands belonging to the V1-V2 region are separated from the other two β -strands of the bridging sheet, β 20 and β 21, by nearly 20 Å, with most of the gap occupied by the amphipathic α 1 helix. Packed against the β 20/ β 21 portion of the bridging sheet is the now malformed CD4 binding loop. Consequently, the CD4 and co-receptor binding interfaces are nearly unrecognizable in this unliganded conformation. These observations are consistent with biochemical data supporting the notion that, concomitant with large enthalpic and entropic shifts derived from isothermal calorimetry measurements (27), gp120 undergoes an extensive conformational rearrangement in response to binding CD4, permitting assembly of the co-receptor binding site (27, 72-76).

Sequence analysis has shown that the gp120 subunit may be separated into five constant regions (C), which form the protein's core and participate in ligand-binding interactions, and five variable regions (V) responsible for shielding the core from immune detection, although studies have shown that V3 is a critical structural component of the chemokine coreceptor binding interface (77). Both gp120 and gp41 have conserved N-linked glycosylation sites; however, gp120 displays an especially high number of glycans, with carbohydrate contributing nearly half of its molecular weight – a feature that is believed to serve as a mechanism to reduce its antigenicity by shielding conserved protein epitopes and epitopes that are subject to selective pressure (78).

The mechanism of fusion is a multi-step process lasting between 15 and 30 minutes (79) for which many reagents exist that specifically target each of the various steps (Fig. 2). In the first step, gp120 engages the CD4 receptor, inducing a conformational change to expose the co-receptor binding site (27), a process that can be

blocked by reagents that target the CD4 binding site including small molecules, antibodies, and soluble forms of CD4 (80, 81). Following CD4 engagement, gp120 binds the co-receptor to form a stable attachment to the target cell, a step that can be blocked by small antibody fragments that can diffuse into the limited volume that separates the virus from the target cell (74).

After attachment, a fusion peptide at the N-terminus of gp41 is inserted into the target cell membrane to form what is called the pre-hairpin intermediate state (82), which can be targeted by peptides derived from the C-terminal heptad repeat region (HR2) of gp41 and by antibodies reactive to the N-terminal heptad repeat region (HR1) or the membrane proximal external region (MPER) (83-86). At some point during this process, the ability of gp120 to “shed” from the envelope spike is required for fusion to proceed (87). However, the precise point at which shedding takes place and whether this might present a rate-limiting step in the mechanism is not yet clear.

In the final step of the fusion mechanism, the three monomers of the C-terminal HR2 individually bind in a parallel orientation to the three grooves formed by the trimeric N-terminal HR1, forming a six-helical bundle or “hairpin” composed of both the N- and C-terminal portions of gp41 (65). This fusogenic conformation is comparable to observations made in structural studies of the envelope spikes of influenza type A and Moloney murine leukemia virus (88). It has further been demonstrated that peptides derived from the HR2 region exhibit a limited ability to partition into lipid environments (89, 90). Consequently, whether formation of the six-helical bundle creates the driving force for membrane fusion or simply represents the final conformation of the envelope spike following lipid mixing induced by these hydrophobic sequences is still debated (91, 92).

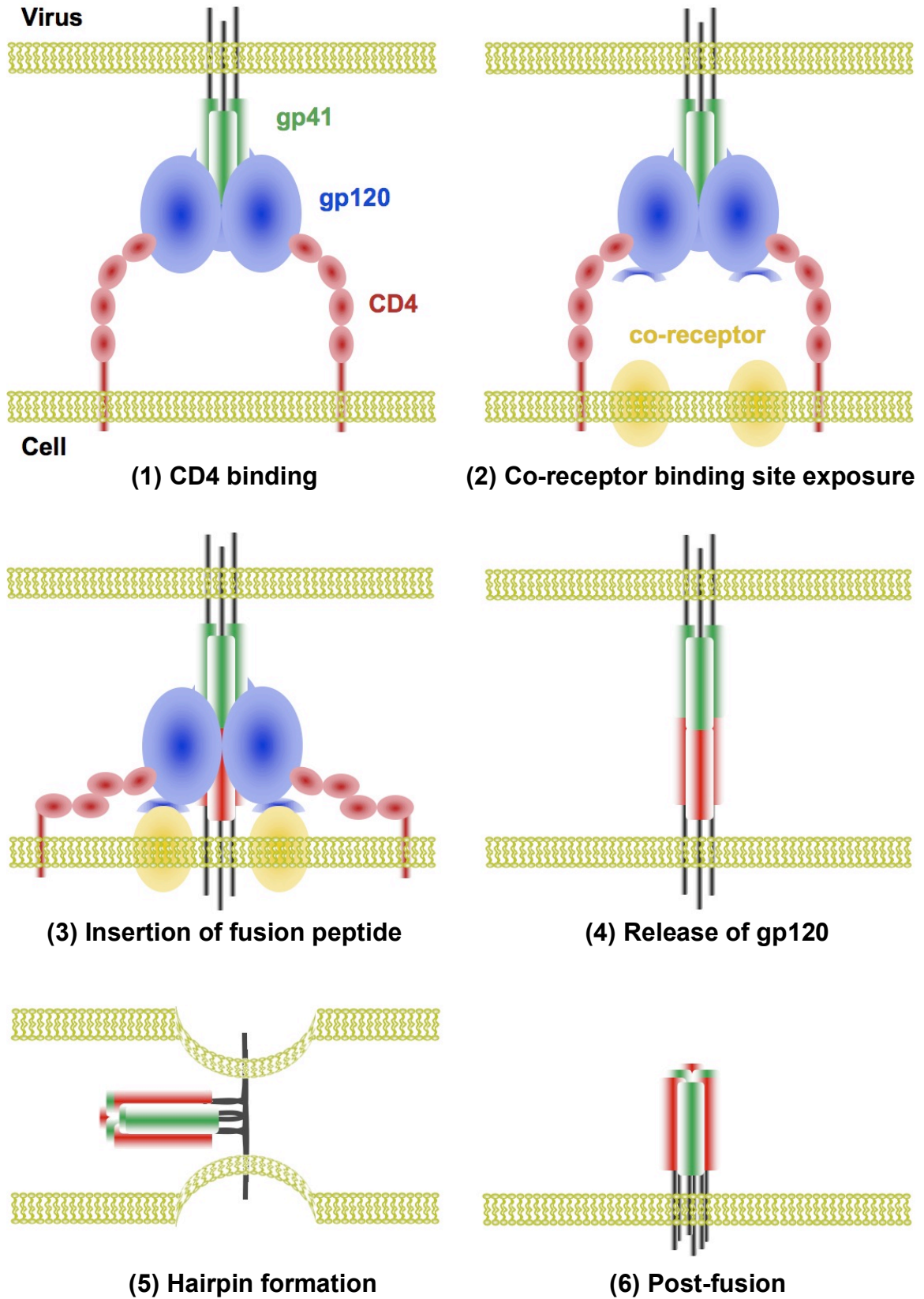


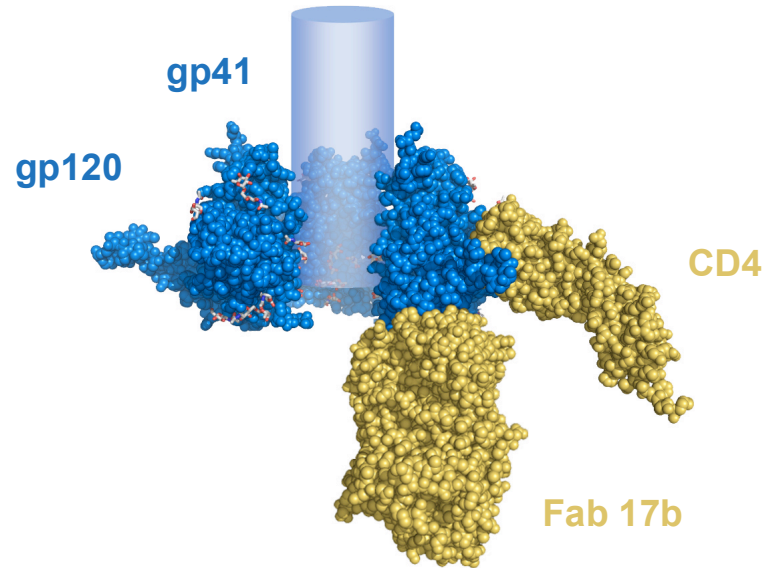
Figure 2. Schematic model of the HIV envelope spike fusion mechanism in 6 steps.

Isolation and characterization of broadly neutralizing monoclonal antibodies

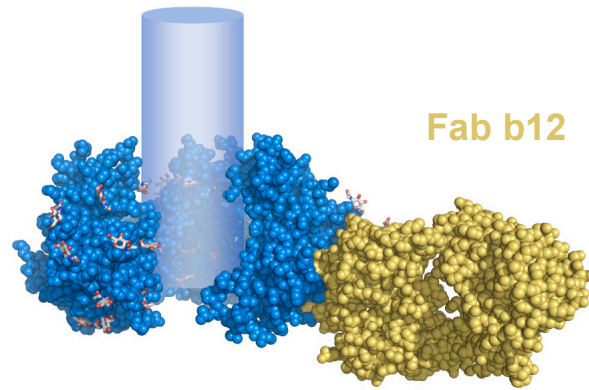
The envelope spike comprises the antigenic target on the virus and infected cells against which neutralizing antibodies are directed (Fig. 3). A limited group of anti-HIV human monoclonal NAbs that have been extensively characterized including 2G12, 4E10, and 2F5 were isolated from relatively healthy HIV infected patients in the early to mid 1990s (75, 93, 94). Three other NAbs belonging to this extensively characterized group are b12 and X5, which were selected by phage display libraries derived from the bone marrow of HIV-infected patients (95, 96). The epitopes recognized by these NAbs have been mapped to either gp120 or gp41 and can be classified as belonging to one of five distinct groups: (1) the MPER of the gp41 ectodomain (*e.g.*, 4E10, 2F5, and Z13); (2) the CD4-binding site (CDbs) of gp120 (*e.g.*, b12); (3) the CD4-induced (CD4i) coreceptor binding site of gp120 (*e.g.*, 17b and X5); (4) the heavily glycosylated and immunologically “silent” face of gp120 (*e.g.*, 2G12); (5) the V3 loop of gp120 (*e.g.*, 447-52D). Although initial experiments indicated that these antibodies bound their epitopes with low nanomolar (nM) affinities when tested against antigens derived from various CCR5- and CXCR4-specific laboratory-adapted HIV strains (74, 75, 96-102), subsequent characterization in pseudovirus neutralization assays demonstrated enormous variation in potency against an extensive panel of primary isolates (19). This finding highlighted the need to carefully examine the *in vitro* cross-reactivity of any potential therapeutic candidates.

Given that each of these broadly NAbs was isolated from a patient infected with a virus strain belonging to clade B, it is not surprising that these antibodies were found to effectively neutralize many if not most representatives of that clade (19). However,

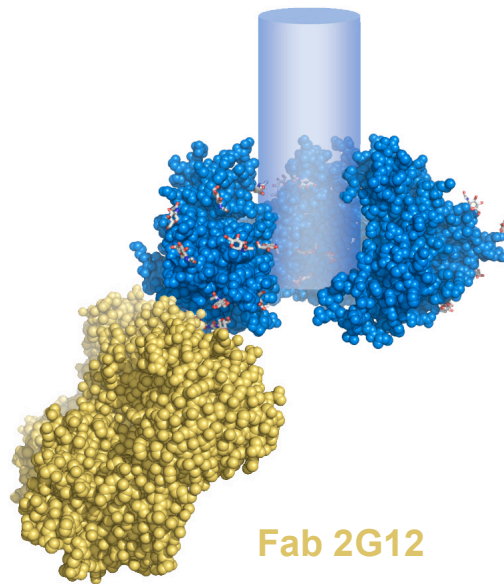
A



B



C



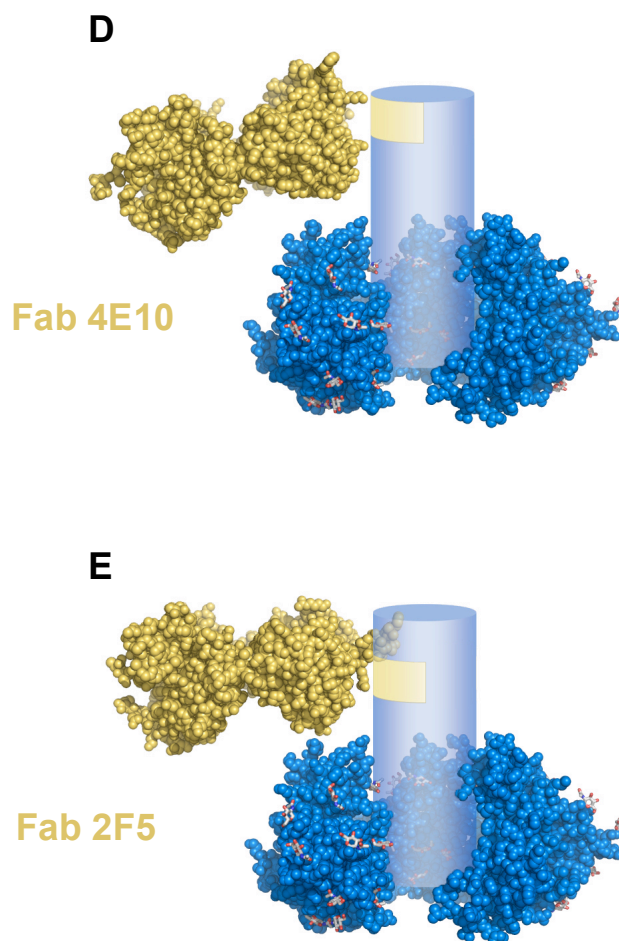


Figure 3. Models of the HIV envelope spike bound to CD4 and monoclonal antibodies. (A) CD4 (domains 1 and 2) and the CD4-induced mAb 17b (67), (B) the CD4-binding site mAb b12 (103), (C) the anti-carbohydrate antibody 2G12 (approximation of binding location) (104), (D) the anti-MPER antibody 4E10 (approximation of binding location) (105), and (E) the anti-MPER antibody 2F5 (approximation of binding location) (106). Trimeric gp120 and a schematic representation of gp41 are shown in blue.

unlike the anti-gp120 NAbs, which showed marked restrictions in clade-specific neutralization, the anti-gp41 NAbs, 4E10 and 2F5, were able to neutralize representatives from most clades with the exception of C and D for 2F5. The NAb X5 was exclusively restricted to clade B, 2G12 was primarily restricted to clades A and B, and b12 was primarily restricted to clades B, C, and D (19). The observation that anti-gp41 antibodies are by far the most cross-reactive may not be unexpected given that gp41 lacks the variable loops regions and the extensive glycosylation of gp120.

In spite of the limited success in identifying a small group of broadly reactive neutralizing antibodies (bNAbs), no one has yet been able to demonstrate a method to elicit them by vaccination, clear or effectively suppress a viral infection by their passive administration, or show that they can prevent infection using *in vivo* NHP models challenged with anything other than strains that are unusually sensitive to these antibodies. The following chapters include data that address the failure of these bNAbs to effectively suppress infection in humans and prevent infection in primates at realistically achievable concentrations. Findings related to steric occlusion of conserved binding sites and the impacts of antibody size and valency are presented in Chapter 2, limitations to epitope cross-linking are presented as a novel hypothesis called the “island effect” in Chapter 3, and deficiencies in the capacity of these bNAbs to trigger antibody-dependent cellular cytotoxicity (ADCC) are presented in Chapter 4.

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