Antibody function in neutralization and protection against HIV-1

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Antibody function in neutralization and protection against HIV-1

Antilichaam functie in neutralisatie en bescherming tegen HIV-1

(met een samenvatting in het Nederlands)

Proefschrift

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Almost all aspects of life are engineered at the molecular level, and without understanding molecules we can only have a very sketchy understanding of life iteself.

Francis Crick

Leadership is solving problems. The day soldiers stop bringing you their problems is the day you have stopped leading them. They have either lost confidence that you can help or concluded you do not care. Either case is a failure of leadership.

Coline Powell

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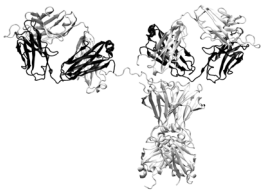

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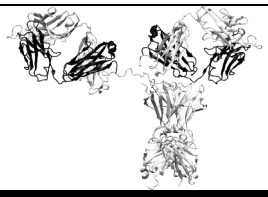

Introduction

HIV-1 background

AIDS, arguably the worst pandemic to afflict humans, was first identified in 1981. Patients were found to suffer from an increased susceptibility to opportunistic pathogens accompanied by a profound decrease in immune system cells known as CD4 T lymphocytes. Transmission seemed to be associated with bodily fluid contact and the cause was suspected to be a new virus. The virus, the cause of AIDS, human immunodeficiency virus type 1 (HIV-1), was first identified in 1983. Research has shown that HIV-1 evolved from the simian immunodeficiency virus found in a subspecies of chimpanzee (SIVcpz). The later discovered human immunodeficiency virus, type 2 (HIV-2) originated from simian immunodeficiency virus found in sooty mangabeys (SIVsm) (33). Transmission of both HIV-1 and HIV-2 from apes to humans most likely resulted from either the slaughter and/or the human consumption of bush meat infected with SIV. HIV is a lentivirus, i.e., a retrovirus causing illness but characteristically symptoms are delayed until some time after chronic infection has established. Recent evidence suggests the SIV/HIV lentiviruses first jumped the species barrier to humans as early as 1908 (30). HIV-1 group M (see below), the virus predominant in North America and Europe, arose in central Africa sometime around 1930 (30). However, those early zoonotic events did not result in sustainable human-to-human HIV transfers (30, 69). Matters changed drastically in about 1966 when a person, likely Haitian, unknowingly carried an easily transmissible variant of HIV-1 group M subtype B to Haiti (30). Approximately three years later HIV-1 subtype B arrived in the United States via another migration and the pandemic was underway (30). The United Nations estimates that 33 million people, including 2.2 million children, are infected worldwide (December 2008; http://www.unaids.org).

HIV-1 is characterized by its rapid genome evolution resulting from both mutation and recombination (106). The jump from SIV to an sustainable HIV-1 isolate appears to have required a complex series of mutations in the virus (33). Phylogenetic studies show that SIVcpz evolved into HIV-1 on at least three separate occasions giving rise to the three distinct genetically identifiable groups labeled M, N, and O (33, 69). Group M continues to evolve and has at least eleven clades (a group considered to share a common ancestor) referred to as A through K (33). The recombination rate of HIV-1 is one of the highest of all mammalian-related systems, with an estimated 2.8 recombination events occurring per genome per replication cycle (106). The genetic diversity among group M is further compounded by at least fifteen prominently circulating recombinant forms (69). Recombination may occur during reverse transcription in cells infected with two or more different strains of HIV-1 (69, 106). The resulting increase in number and complexity of HIV-1 variants presents a unique and difficult challenge in the quest for an effective vaccine (106).

Human transmission of HIV occurs most commonly by sexual intercourse and intravenous drug use with contaminated needles. More rarely, although unfortunately still common in the developed world, infected blood or blood products used as therapeutics mediate HIV transmission. Mother-to-child transmission forms another route of infection that most often occurs when an infected mother infects her baby either at birth during delivery, or through breast milk during feeding. The transmission rate of HIV by a newly infected mother can be up to 40%, but is lower if the mother has begun to produce antibodies against HIV. Although treatment of

pregnant women with the nucleoside analog zidovudine (AZT) can prevent the transmission of HIV to an unborn child, the perinatal transmission rate in Africa continues at about 25%. Overall, HIV-1 transmission could be significantly reduced through widespread education about safe sexual practices and the necessity for using clean needles. However, religious organizations and world politics play a critical role in curbing the use of these important tools. Most AIDS worldwide is caused by HIV-1, the more virulent of the two types of HIV, although HIV-2 is endemic to West Africa and is now spreading into India. The HIV-AIDS pandemic continues to expand its prevalence worldwide. Every day more than 10,000 people become infected, of whom 50% are women and 40% are young people between the ages of 15 and 24. 95% of the new infections each day occur in low and middle-income countries.

Many viruses cause an acute, brief infection that induces protective immunity. Other viruses can cause latent infection that can be controlled by an adaptive immune response. HIV infection, however, rarely leads to an immune response that controls or prevents viral replication. An HIV infection results in a loss of CD4 T lymphocytes and acute viremia, but the infection cannot be eliminated by the development of cytotoxic T-cell responses. In fact, along with cytopathic destruction, killing of CD4 T cells by CD8 cytotoxic cells in the lymphoid tissue of the gut is the main effect of an HIV infection. This counter offensive by the immune system fails and as a result, a persistent infection is established and continued virus replication occurs in newly infected cells.

A simplified schematic of a virion structure demonstrates that the HIV virus particle **(Figure 1)** is an enveloped retrovirus whose surface is decorated with protein spikes that are essential for viral entry into target cells. Each virion contains two copies of the viral RNA genome. The virus initiates the delivery of its genome through the interaction of envelope spikes with CD4 as a primary receptor and the chemokine receptors, CCR5 or CXCR4 as co-receptors. The viral cycle is subsequently carried out by transcription factors in activated T cells. The RNA genome is reversed transcribed and integrated into the host chromosomes. Copies of this viral genome (or provirus) can remain dormant for a very long time, indeed years. However, once activated, the proviral DNA is transcribed and generates new virus particles aided by host cell metabolism and viral accessory proteins.

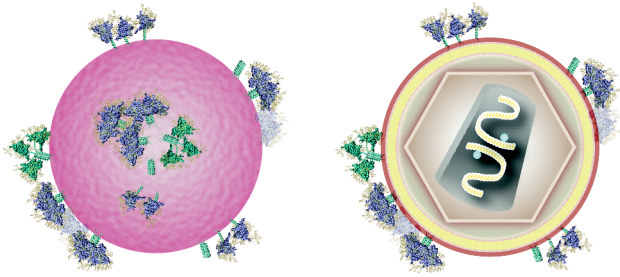

Figure 1. HIV-1 virion. Formal taxonomy describes HIV-1 as a member of the family of viruses called Retroviridae because of its ability to reverse transcribe its double-stranded RNA genome. It belongs to the genus called lentiviruses. The image on the left is a depiction an HIV-1 virion displaying various forms of viral surface glycoproteins. On the right, the virion is sliced to reveal the double-stranded RNA packaged within the viral capsid. For simplicity, not all enzymes and viral structural proteins are depicted. In reality, many molecules of these proteins are present in each virion.

Human antibodies

Antibodies are immunoglobulins (Ig) that are developed in the body to recognize and destroy invading pathogens that cause disease. The recognition and binding to foreign material, or antigen, requires that antibodies discern between molecular structures on the surface of invaders, called antigenic determinants, and surface structures of the host. For bacteria, antigenic determinants are usually exposed proteins or carbohydrates. For enveloped viruses the antigenic determinants relevant for virus inactivation (neutralization) are usually represented in the form of (glycosylated) protein spikes protruding from the surface. In order to provide broad and continued protection, the human body is capable of producing antibodies against billions of different specific epitopes. Antibodies are encoded by recombining multiple segments of germline gene segments during an individual's lifetime in B cells that are expanded upon antigen recognition. This antibody diversity and resulting repertoire is generated through somatic recombination events from a relatively small set of human gene segments after which additional diversity (and antibody specificity) is generated by somatic mutation. Somatic alteration infers that the newly recombined genes are not inherited and as a result, each individual creates a unique primary immunoglobulin repertoire that is continually modified during their lifetime.

The basic structure of antibodies consists of four polypeptide (protein) chains made from two identical heavy (~50 kD) chains and two identical light (~25 kD) chains that form three functional parts. The heavy and light chains are joined by disulfide bonds that allow each heavy chain to be linked to a light chain while also linking the two heavy chains together (10, 38, 81). From a simple two-dimensional perspective, antibodies are, in general, shaped either like the letter Y or T and are flexible due to a hinge region that links the upper and lower portions. It is this flexible Y shape that enables the antibody to carry out two principal functions in immune defense: (1) recognize and bind to foreign material (antigen) and (2) trigger complex elimination mechanisms. In essence, antibodies are extremely good adaptor molecules that link antigen and effector in a wide variety of situations. Hence, it is imperative to envision the interaction of antibodies with antigen and effector in a three dimensional setting (8, 11). The crystal structure of the anti-HIV-1 human antibody IgG1 b12 revealed a 'snapshot' representative of the many conformations of antibodies available in solution **(Figure 2)**. The structure was determined to be highly asymmetric and the overall shape between a Y and a T with a 143^{o} angle separating the two Fab arms (75).

Two identical fragments, each containing a light chain and a portion of the heavy chain, recognize antigens and are thus designated as an antigen binding fragment or Fab (**F**ragment: **a**ntigen **b**inding). The Fabs recognize antigen via complementary-determining regions (CDR) and because of the vast diversity of possible antigens, this region of the antibody must be the most variable and, therefore, the antigen-binding region is known as the variable or V region. Once the antibody is attached to antigen, the stem, or Fc fragment, becomes a flag to cells or proteins of the innate immune system that an invader has been recognized. The Fc fragment contains no antigen-binding activity but because it was originally observed

to crystallize easily, it was named Fc fragment, for **F**ragment **c**rystallizable. Antibody coated onto an infected cell can trigger the elimination of foreign material either through activation of complement or through the interaction of the Fc fragment and receptor proteins on the surface of immune cells called effector molecules. The interaction causes host immune cells known as effector cells, to be activated and to destroy the invading pathogens and infected cells. Alternatively, antibody-antigen complexes signal for the activation of the complement cascade resulting in pathogen destruction and elimination. The effector molecules recognized by the Fc region are mostly constant or conserved in sequence and structure that allows for the Fc antibody region to also be constant. Thus, it is known as the constant or C region.

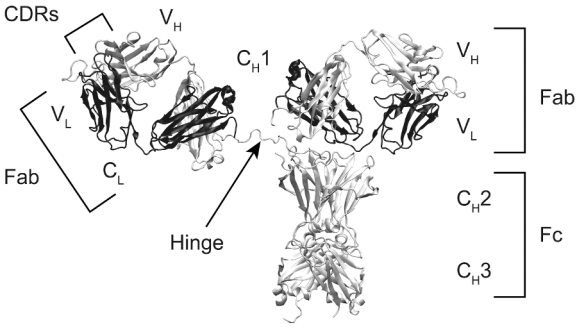

Figure 2. Human Immunoglobulin G (IgG) (IgG1 b12). Human IgG antibodies are comprised of 4 polypeptide chains, 2 light chains and 2 heavy chains. Each light chain contains a variable region and a constant region. Each heavy chain consists of a variable region and 3 constant regions. The hinge region allows for flexibility between the Fab arms and the Fc region.

There are five major classes of human immunoglobulins: IgA, IgD, IgE, IgG, and IgM. All five classes have the same basic structure, but differ in the sequences of their heavy chains which are termed α, δ, ε, γ, and μ, respectively (although it is noted that IgM and IgA primarily occur in multimers). The differences, primarily in the Fc region, define the effector functions of each class. Subclasses have been recognized for human IgG (IgG1, IgG2, IgG3, and IgG4), and for IgA (IgA1, IgA2). The most observable difference among the Ig classes and IgG subclasses is due to the nature and length of the hinge regions, characteristics that can be associated with function. For example, an extended length can be a function of flexibility that can allow for reaching variably spaced antigenic sites. In contrast, shorter hinge regions are generally associated with resistance to degradation by proteolysis resulting in greater serum stability as seen in IgA2 (94). It has furthermore recently been recognized that conformational changes in the hinge region may strongly affect antibody function. Thus, IgG4 antibodies may engage in dynamic Fab arm exchange that causes IgG4 molecules to lose their ability to cross-link antigen and form large immune complexes *in vivo* (87). A further example is the recent discovery of disulfide isoforms in IgG2 (96) that may impact both effector functions and antigen-binding affinity and avidity. These phenomena thereby provide yet another level of *in vivo* control of antibody and another dimension of antibody diversity.

Structural studies

The remainder of Chapter 1 of this Thesis will introduce conclusions deduced from the relevant structural studies that have defined the most important aspects of each of the major broadly neutralizing monoclonal antibodies (bnMAbs). Structural studies of the envelope glycoproteins, gp120 and gp41, and electron tomography images of HIV-1 Env trimer will be summarized to provide a basis for understanding the interactions of bnMAbs with infectious virus spikes. These reviews are intended to provide a visual background for later chapters describing molecular studies and protection experiments conducted with the bnMAbs.

The HIV Envelope (Env) spike

HIV is extremely successful in evading humoral immune mechanisms and this success is mainly attributable to the characteristics of the Env spikes that decorate the viral surface. It provides the sole target for neutralizing antibodies (63), and has been shown to reveal extreme diversity in both structure and sequence. The diversity among clades is achieved by multiple overlapping mechanisms that evade humoral immunity, including host-derived glycan coating and conformational masking, and by displaying a variety of immunodominant loops (39, 83, 91). Traditional vaccine development has been deterred by this diversity inherent to HIV.

Initially produced as a single chain glycoprotein precursor, gp160 is cleaved and assembled to yield a trimer of heterodimers formed by two glycoproteins, gp120 and gp41 (65, 95). These glycoproteins shape an oligomer of three gp120 molecules noncovalently associated with the ectodomain of a gp41 trimer ($gp120_3gp141_3$). The resulting compact structure, elegantly evolved to avoid neutralizing antibody responses, is expressed on an HIV-infected cell surface and subsequent viral budding on the virion surface (45).

The gp120 glycoprotein trimer that comprises much of the exposed region of the spike is heavily camouflaged by carbohydrates that account for about 40% to 50% of the molecular weight of a single gp120 molecule (43, 83). The conserved regions, the best potential target for broadly neutralizing antibodies, are either buried or very difficult for antibodies to access and sometimes are only accessible after conformational changes once the spike has bound to a target cell. Much of the gp41 trimer is inside the Env spike (76, 77, 82, 112), exposing only a short region between the virus membrane and the gp120 trimer. These characteristics of the envelope spike are further complicated by the instability of the structure that tends to dissociate or shed virion debris (60). Indeed, primary HIV-1 isolates have been shown to display a heterogeneous array of envelope molecules of two types designated as functional and nonfunctional (63). Infectious virions may express functional Env spikes generally considered to be the heterotrimeric structure comprised of $gp120_3gp41_3$ (65) as described above. The same virion may also display Env spikes that vary structurally as a result of gp120 degradation or shedding resulting in Env variants that expose nonneutralizing epitopes. It is also possible that the glycoslyation of gp120 may be inconsistent causing an increased accessibility to recognition by nonneutralizing antibodies. In fact, it is likely that HIV-1 virions bear mostly nonfunctional spikes and only a few functional spikes may mediate virus entry. Functional assay studies of

virus like particles (VLPs) derived from virus cultures have indicated that along with other potential forms, the primary nonfunctional form of HIV-1 Env may be a gp120/gp41 monomer (52). **Figure 3** summarizes the various putative forms of the Env spike on HIV-1.

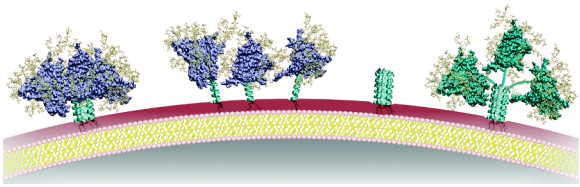

Figure 3. Variations in Env spike formation. Potential forms of Env include functional Env trimer, monomers of gp120/gp41, gp41 stumps, and uncleaved gp160. An HIV-1 virion likely displays a small number of spikes unevenly spaced or clustered. (See Fig.1.)

Until recently it has been unclear how many spikes exist on each virion and how those spikes may be arranged. In addition, the complexity and instability of the HIV-1 Env trimer have kept the structure somewhat of a mystery because of the difficulties in generating a three-dimensional model of an individual spike. To further complicate structural studies, intact viruses display irregular morphology (5). However, recent advances in cryo-electron tomography and image processing have increased the capabilities to study such irregular native membrane viruses. Two studies in particular have revealed images of viral spikes (Reviewed in 9, 103, 105). Zhu and colleagues compared Env spikes from intact, chemically fixed Simian Immunodeficiency Virus (SIV) to HIV-1 virions due to the high degree of sequence and structural similarity between the two viruses. While the Zanetti images are derived solely from SIV virions. The resulting images from the two studies propose an Env spike, similar in height and width, but differing greatly in overall shapes, especially in the depiction of the gp41 stem region. The differences in the arrangement of the transmembrane regions is unclear, but could be due to the existence of different conformations of the structure resulting from the imaging process (103). Also, it is important to note when viewing the proposed images that these imaging techniques cannot distinguish between infectious and non-infectious particles (9).

Notably, it was determined that an HIV-1 Env is coated with a sparse number of spikes and are believed to be unevenly spaced and tends to form clusters (105). The observed clustering may implicate yet another way in which HIV has evolved to evade immune detection because antibody responses are less likely to be elicited by irregular displays of spikes. Further, the interaction of clustered spikes may assist in pore formation in the target cell membrane providing a mechanism for host cell entry (9), although evidence exists that even a single HIV spike may be sufficient for infection (99).

In general, most *in vitro* cultured HIV particles are not infectious making the conclusions drawn from the averaged data of a few thousand virions in these studies open to interpretation. Perhaps more relevant, images recently reported by Liu, et al. of trimeric HIV Env using cryo-electron tomography combined with three-

dimensional structures have deduced major reorganizations of Env trimer upon binding with CD4. The study also depicts conformational changes relevant to antibody neutralization and attachment to target cells (44). The molecular models from all three studies, reproduced in **Figure 4**, are a good start towards a complete high-resolution structure of an infectious, functional HIV Env spike - the most sought after goal in current HIV vaccine research. This structure, it is anticipated, will provide crucial insight on viral evasion of neutralizing antibody recognition and should provide direction for vaccine design.

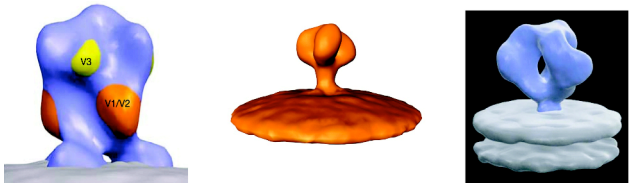

Figure 4. Proposed molecular structures of SIV and HIV Env spikes. **From left to right, Zhu and colleagues used cryo-electron tomography to compare Env spikes of SIV to HIV-1 revealing an image depicting the gp41 trimer separated at the viral membrane. In contrast, the Zanetti image (middle), derived solely from SIV virions, depicts a narrow stalk formation of gp41. The image on the right reported by Liu et al. is a three-dimensional structure of of trimeric Env displayed on HIV-1 using cryo-tomography and three-dimensional imaging (44, 102, 104).**

gp120

From the sequence analyses of different HIV isolates, it has been determined that gp120 consists of both variable (V1-V5) and conserved (C1-C5) regions. It has two receptor sites, one for the CD4 molecule and the second site dedicated for binding to chemokine receptors (CCR5 and CXCR4). The CD4 binding site is recessed suggesting difficulty for antibody access. The site is mostly inaccessible for antibody on monomeric gp120 unless conformational changes are triggered by CD4 binding. A crystal structure of the gp120 core with trimmed carbohydrates and mutated to remove most of the variable loops revealed that the protein consists of an inner domain and an outer domain linked by a bridging sheet **(Figure 5)** (41).

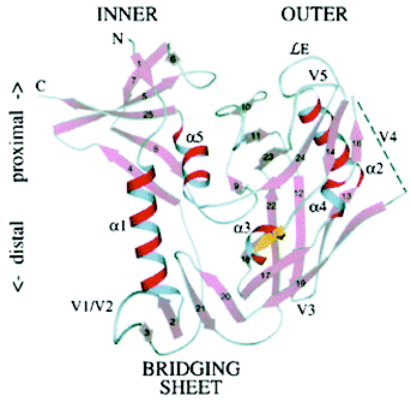

Figure 5. Structure of core gp120. Ribbon diagram of the structure of core gp120 from Clade B, strain HXBc2 HIV-1. In this orientation of gp120, the viral membrane would be oriented above, the target membrane below, and the C-terminal tail of bound CD4 would be coming out of the page (41).

The primary role of gp120 is to form an attachment of the virus particle to the target cell and thereby to bring the virus and target cell membranes within close proximity. Target cell entry by HIV-1 is orchestrated by the formation of a complex between the Env oligomer (Env), a receptor molecule (CD4) and a coreceptor (either CCR5 or CXCR4). After that has been achieved, the transmembrane protein gp41 mediates the fusion of the viral and the target cell membranes. Subsequently, this results in the injection of the virus genetic information into the target cell (92). The interaction surface on gp120 consists of two large cavities, one shallow containing hydrophilic and less conserved residues, and another containing hydrophobic and highly conserved residues deeply buried within the core of gp120 (65). Since nonfunctional Env is not believed to be involved in viral fusion or entry, nonneutralizing antibodies that may be present could play a different role in viral inhibition. For example, recent studies have shown that nonneutralizing antibodies can inhibit HIV-1 infection of macrophages, probably due to Fc receptor-mediated phagocytosis (35). Immune complexes formed by bound nonneutralizing antibodies could explain this. This observation also points to early reports of high concentrations of immune complexes in HIV-1 patients, yet typically low levels of neutralizing antibodies in their sera were detected (28, 53).

Coreceptors

Traditionally, the role of chemokine receptors in the human immune system is to promote chemotaxis, thereby allowing cells to move toward sites of inflammation. However, during an HIV-1 infection event, the chemokine receptor molecules CCR5 and CXCR4 become coreceptors assisting in virus and cell membrane fusion (51). Once the virus has engaged with the CD4 receptor expressed on the surface of the host target cell, either a T lymphocyte, macrophage or dendritic cell, conformational changes result to allow for coreceptor binding. CCR5 or CXCR4 coreceptor usage has become the preferred method by which to functionally group HIV-1 viruses, i.e., R5 viruses use CCR5 and X4 viruses use CXCR4. Some HIV-1 viruses use both CCR5 and CXCR4 and are termed dual tropic (4). A panel of gp120 mutants analyzed according to their ability to bind CCR5, revealed that the CCR5 binding site is more conserved than the CD4 binding site (70). Located in close proximity to the CD4 binding site, the critical residues of the CCR5 binding site are found near or within the bridging sheet on the gp120 core and some residues probably come into direct contact with the coreceptor molecule. The highly basic surface of the CCR5 binding site probably facilitates the interaction with the acidic N-terminal portion of the coreceptor (25, 26, 68). Understanding the nature of the Env conformation changes during viral entry may provide important clues for developing fusion inhibitors and vaccine design.

V3 loop

As previously noted, the conserved regions of gp120 have been generally accepted as the best targets to pursue for HIV vaccine design. However, a debate ensues over whether to target the variable loops, in particular the V3 loop, which is a site of extraordinary sequence variation in the HIV-1 clade B strains. However, the center of the V3 loop contains a relatively conserved sequence (i.e. a GPGR motif in many clade B viruses and a GPGQ sequence appears in nearly all other clades) that has been studied in some detail. It is believed that the conservation of the V3 center is required to maintain structural conformation to allow for effective interaction with

coreceptor molecules on the target cell membrane. The crystal structure of the V3 loop as determined from a complex with a V3-containing gp120 core, bound CD4 and an antibody, reveals 3 structural domains: the base, the stem, and the tip or crown (36). Indeed, a preservation of certain structural elements was observed in the V3 crown despite sequence variability in other regions of V3. However, sequence variability in the regions flanking the V3 crown may reduce stability or alter accessibility for antibody recognition. The hypervariable V3 loop has long been recognized as a key decoy of HIV because antibodies that recognize the V3 loop are type specific and easily evaded by the virus. Antibodies to the V3 loop were found to have some neutralizing activity against laboratory strains or T-cell line adapted viruses, but most anti-V3 loop antibodies do not show breadth or strength of neutralization against primary isolates. These characteristics have greatly reduced the interest in the V3 loop as a possible vaccine target. Nonetheless, through targeted antigen engineering, a few anti-V3 loop antibodies are providing insights into which conserved segments of V3 are accessible to antibody and that may elicit a greater breadth of neutralization.

gp41

More conserved overall than gp120, most of the surface of gp41 is occluded from antibody recognition before attachment and fusion because it is inside the functional Env spike (76, 77, 82, 112). The only exposure is at the membrane-proximal external region (MPER) near the C-terminus of the gp41 ectodomain, a region that is conceivably involved in viral fusion (**Figure 6**). Keeping the conserved epitopes of the fusion machinery hidden is of particular advantage to HIV-1 for two reasons: (1) masking the sites may make the Env spike less immunogenic, and (2) prevention of antibody binding to the conserved epitopes making it less antigenic (112). The bnMAbs, 2F5 and 4E10, are directed against linear epitopes mapped to the MPER, making the region an important potential vaccine target.

The foundation for understanding HIV-1 viral entry and insights on mechanisms to block host and viral cell membrane fusion were gained from the first x-ray crystal structures of gp41 (18, 85, 93). The structures provided the foundations for understanding the viral and host cell fusion events that occur in only minutes, thereby limiting access to briefly exposed epitopes on gp41 (112). Several recent studies have provided a better understanding of the gp41 MPER both structurally and functionally increasing the interest in vaccine design efforts (6, 17, 24, 37, 42, 73, 107). One of the main reasons for the invigorated interest in the gp41 MPER as a target for vaccine design is the appreciation for the tremendous breadth of HIV-1 primary isolate neutralization by 2F5 and 4E10. Another characteristic of the MPER that makes it an appealing template for vaccine design is that antibodies can access the region prior to Env interaction with CD4 and before prefusion conformational changes occur (23, 24, 52, 107). Of further note is that 2F5 and 4E10 can remain bound through late stages of fusion and thereby act similarly to fusion inhibitors (24, 31). Even though the advantages are clear, there are also potential obstacles to overcome in designing an immunogen based on the MPER of gp41. First, the proximity of the region to the viral membrane poses challenges due to the high degree of hydrophobicity. Consequently, the antibody binding sites of 2F5 and 4E10 are somewhat hydrophobic and under some circumstances will bind non-specifically (1, 32, 56, 73, 79). 2F5 and 4E10–like antibodies are extremely rare in the sera of HIV-1-infected individuals indicating that the MPER is probably poorly immunogenic in natural infection (22, 102). For these reasons, immunogens based on the MPER of gp41 must structurally recreate the

proper molecular environment that allows for the presentation of the 2F5 and 4E10 epitopes on the virus (109).

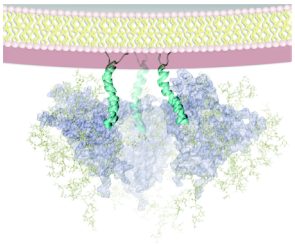

Figure 6. "gp41: HIV's shy protein" (112). Most of gp41 is hidden within the Env spike with only a small portion exposed near the viral membrane (known as the membrane proximal external region, or MPER). Extreme conformational changes occur after coreceptor binding, leading to virus and host cell membrane fusion and pore formation in preparation for insertion of virus RNA.

Neutralizing antibodies and immunogenicity

The major significance in recognizing the existence of multiple forms of Env and Env spikes for vaccine design is to establish their impact on HIV-1 infection and neutralization by antibody. Nonfunctional Env permits capture but does not lead to neutralization, which probably contributes to the poor neutralizing antibody response in natural infections. This is because the debris generally consists both of monomeric forms of gp120 and the resulting exposed gp41 stalks, which provide prominent targets for antibodies generated during natural infection (40). Consequently, serum antibodies of seroconverted HIV-1 infected individuals have been shown to have a much greater affinity for unprocessed gp160 or monomeric subunits of oligomeric Env and to have poor affinities for functional spikes as expressed on the surface of virions or infected cells (61). These antibodies have been shown to rarely neutralize primary HIV-1 isolates *in vivo* (21, 49, 66) but are capable of binding gp120 and gp41 *in vitro*.

Conversely, antibody binding to functional Env spikes can be correlated to neutralization (70), which is supported in the Env heterogeneity model wherein only neutralizing antibodies bind functional spikes while both neutralizing and nonneutralizing antibodies can bind nonfunctional spikes (63). The consequences of this evolutionary deception by HIV may include the slow development of autologous neutralizing antibodies in natural infection. It is suggested that accessibility and not quantity drives the relative immunogenicity of monomers and trimers making trimers a suboptimal target for antibodies (52). Thus, nonfunctional Env displayed on a virion is likely to be more immunogenic than soluble gp120 monomers generated by shedding during infection.

Many efforts have been made to improve the immunogenicity of those forms of HIV-1 Env glycoproteins that are recognized by neutralizing antibodies, such as for example, by the production of homogenous, stable soluble trimers expressed as gp140 fusion proteins (97, 98, 100, 101). However, animals immunized with soluble trimers were found to only yield antibodies with modest improvements in neutralization breadth or titer as assessed with primary HIV-1 isolates. Other approaches have focused efforts to reengineer gp120 by identifying the amino acid residues crucial for

gp120 recognition by both neutralizing and nonneutralizing antibodies and by hyperglycosylation of mutated forms of monomeric gp120 to shield nonneutralizing but not neutralizing sites. Such a panel of glycoengineered gp120s is currently being evaluated for their ability to elicit broadly neutralizing antibodies (57-59, 80).

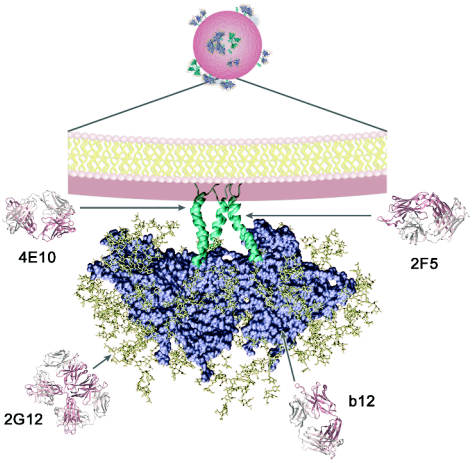

Figure 7. the bnMAbs binding sites on Env spike. The Fab structures of each of the 4 major bnMAbs are shown in relationship to their binding target on the putative HIV-1 Env spike. The anti-gp41 antibodies, 4E10 and 2F5, bind to the MPER region nearest the viral membrane. The conserved CD4 binding site is targeted by b12 and 2G12 binds to a cluster of oligomannoses near the top of the spike.

Broadly neutralizing antibodies against HIV-1 (bnMAbs)

After decades of research only four antibodies have been isolated and characterized that carry potent breadth of neutralization against HIV-1: namely b12, 2G12, 2F5, and 4E10 (**Figure 7**). The rarity of their existence may be explained not only by the difficult target they face, but also by the complex evolution of sequence and gene rearrangements required to develop antibody structures capable of accessing the conserved regions of the HIV Env. Indeed, sequence and structural analyses indicate extensive mutational maturation and the requirement of specific and, sometimes, unusual antibody features and adaptations such as long CDRs and Fab arm dimerization.

gp120 antibodies: b12 and 2G12

Only two broadly neutralizing antibodies against gp120 have been isolated. Both were isolated from the immune repertoire of seropositive individuals although the majority of naturally elicited antibodies against gp120 fail to neutralize primary isolates of HIV-1.

Antibody b12 was isolated from a combinatorial phage display library derived from bone marrow of a seropositive male who had been without symptoms for six years (13). Passive protection studies in hu-PBL-SCID mice (29) and macaques (34, 62, 88, 89) have shown that b12 protects against viral challenge. Because b12 recognizes a highly conserved epitope that overlaps the CD4 binding site on gp120, it recognizes and neutralizes more than half of the clade B primary isolates (3). The b12

epitope represents a site of vulnerability for the HIV-1 Env because despite its complexity, it must retain conserved determinants to mediate CD4 binding (104). The crystal structure of the antibody, solved in the absence of antigen by Saphire, et al (**Figure 8**), revealed that the long (18 amino acids) third complementarity-determining region in the heavy chain (CDR H3) projects 15 angstroms above the surface of the antigen-binding site. The extended finger-like loop formed by the CDR H3 is apexed by a tryptophan residue with acidic residues along one face that may, through charge repulsion, maintain the vertical projection of the loop (75).

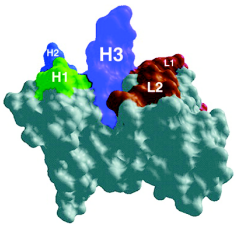

Figure 8. The extended CDR H3 of b12. The space-filled structure of the antigen-binding region of b12 reveals an 18 amino acid complementarity-determining region in the heavy chain (CDR H3) that extends 15 angstroms above the surface. The acidic residues may provide charge repulsion to maintain the vertical projection of the loop (75).

A conclusion from the structure, and suggested by *in silico* docking, is that the CDR H3 of b12 could probe the recessed CD4 binding site of gp120. Indeed, a more recent crystal structure of b12 in complex with a stabilized gp120 molecule revealed that the interaction is heavy chain dominated **(Figure 9)**. Three b12 residues (Asn 31, Tyr 53 and Trp 100), one amino acid from each of the heavy chain CDRs, combine to form about 40% of the contact surface and grip the CD4 binding loop (104). In that study (included as **Chapter 2** of this thesis) it was deemed that, unlike CD4 binding, b12 does not require or induce conformational changes as it binds with high affinity to gp120 at essentially the same exposed surface used by CD4 for initial contact. This would allow b12 to bind and neutralize primary isolates that would otherwise use conformational masking to avoid neutralization (104). However, in recent electron tomograph images, it was deduced that Env trimer binding to b12 results in a partial opening of the spike accompanied by some degree of rotation around an axis perpendicular to the viral membrane. Tomography therefore indicates that CD4 binding, results in a rotation twice as large in addition to an out-of-plane rotation and vertical displacement of gp120 (44).

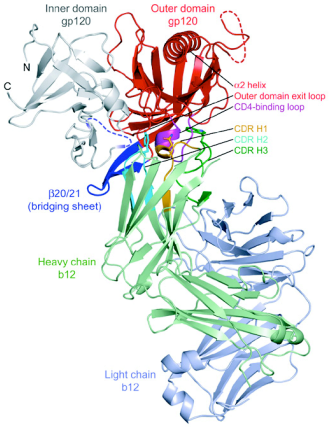

Figure 9. Fab b12 in complex with gp120 core. The interaction between b12 and its epitope overlapping the CD4 binding site on gp120 is achieved through the heavy chain alone. Each of the CDRs make contact with the CD4 binding loop with one amino acid each and combine to form about 40% of the contact surface (103).

Monoclonal human IgG1 2G12 recognizes a unique cluster of oligomannose sugars on the outer domain of gp120 (16). The heavily glycosylated region was designated the "silent face" of gp120 because it was expected to be nonimmunogenic and therefore would not elicit an antibody response. 2G12 has been shown to bind gp120 with high affinity via a unique structure of dimerized Fab arms containing interlocked VH domains (**Figure 10**). The domain-exchanged structure results in a tightly formed antibody combining site that works well for the recognition of the closely grouped sugar residues on the glycan shield of HIV (15, 16, 74, 78, 86). It is broadly neutralizing, particularly against clade B HIV-1 isolates (3). Unlike the other broadly neutralizing human monoclonal antibodies, which recognize epitopes on the Env spike that are sterically restricted, 2G12 appears to recognize a well-displayed epitope located on an area that may be described as the "top" of the spike (71, 90, 103).

Despite the unique qualities of 2G12, early protection studies to define the antibody's ability to protect against infection were conducted in combination with other antibodies, which in retrospect used insufficient concentrations of 2G12 to allow conclusions to be drawn. Therefore, the magnitude of a protective dose of 2G12 has remained unclear. The mucosal SHIV protection study described in **Chapter 7** is the first report of the individual contribution of 2G12 in preventing the establishment of infection.

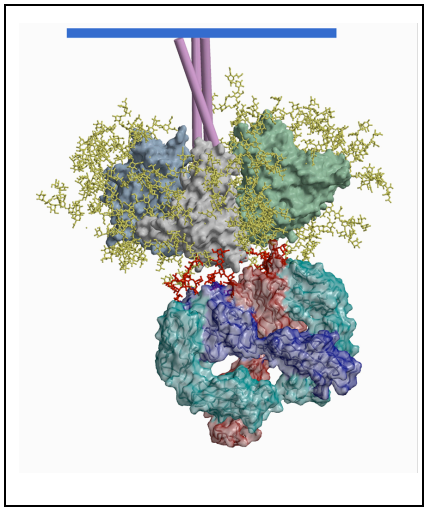

Figure 10. Domain-swapped F(ab')$_2$ 2G12 with Env. A model of mAb 2G12 F(ab')$_2$ bound to the HIV-1 Env spike. The heavy chains of 2G12 are shown in dark blue and light red, and the light chains are shown in azure (14).

gp41 antibodies: 4E10 and 2F5

Two bnMAbs, 2F5 and 4E10, have been identified that recognize neighboring epitopes on the membrane proximal region of gp41, a region of highly conserved mostly linear epitopes (7, 55, 56, 84, 111). Both 2F5 and 4E10 were originally obtained as IgG3 antibodies in hybridomas derived from peripheral blood mononuclear blood

lymphocytes (PBMCs) of HIV-1 seropositive non-symptomatic patients and were later class-switched to IgG1 to enable large scale manufacturing and to prolong *in vivo* half-life (7, 19, 67). Most studies have been performed with 2F5 and 4E10 in their IgG1 format. In a comprehensive cross-clade neutralization study by Binley, et al., 2F5 neutralized 67% and 4E10 neutralized 100% of a diverse panel of 90 primary isolates (3). Similar broad neutralization was seen against cloned isolates from acutely infected patients (50). The remarkable pan reactivity of 4E10, however, is tempered by the modest potency of the neutralization.

The core epitope of 2F5 is the short linear sequence, ELDKWA, located at the extreme C-terminal end of the ectodomain of gp41 (55). Following the trend of broadly neutralizing antibodies against HIV-1, the third complementarity-determining region of the heavy chain (CDR H3) of 2F5 is unusually long, comprised of 22 amino acid residues (110). Adjacent to the highly conserved 2F5 epitope, 4E10 recognizes the sequence NWFDIT (108, 109) in a tryptophan-rich region. The five highly conserved tryptophan residues have been shown to be critical for Env-mediated membrane fusion and virus infectivity (54, 72). **Figure 11** is a model of mAbs 2F5 and 4E10 Fabs bound to their epitopes close to the virus membrane. While the neutralizing epitopes of the MPER region are primarily linear, it has been shown that (unlike 2F5) 4E10 binds with less affinity to denatured gp41 (2, 111). More recently, a study has shown the stable interactions of 2F5 and 4E10 with lipid membrane-inserted epitopes and suggests how the antibodies may interfere with the function of the MPER during gp41-induced fusion (37).

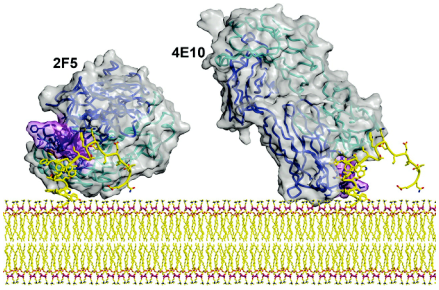

Figure 11. 2F5 and 4E10 at the viral membrane. Both 2F5 and 4E10 recognize highly conserved linear epitopes at the membrane proximal region (MPER) of gp41. Interactions of 2F5 and 4E10 at the lipid membrane may interfere with the function of the MPER during gp41-induced fusion with a host cell membrane (37).

As mentioned above, despite the importance of the MPER as a vaccine immunogen, to date there has not been a clear demonstration of the ability of anti-MPER antibodies 2F5 and 4E10 to protect or to generally act anti-virally *in vivo*. Most protection studies with 2F5 and 4E10 have been in combinations with other antibodies leaving the individual contribution of these antibodies not fully assessed (20, 27, 46-48, 64). For 2F5 alone, protection studies using rhesus macaques have been performed but yielded little confidence that 2F5 offered protection against intravenous $SHIV_{89.6PD}$ challenge (47). Our SHIV mucosal protection study (**Chapter 8**) is the first significant evidence of the individual contribution by 2F5 and 4E10 *in vivo* in protection against infection by an R5 SHIV.

Objective of thesis

The objective of this thesis is to examine, through focused research, the molecular and cellular mechanisms of how antibodies against HIV-1 neutralize *in vitro* and provide protection against infection *in vivo*. Traditional and empirical vaccine strategies using the proteins exposed on the viral surface to induce effective antibodies from the immune system seem simple and direct, however, the instability and highly mutable HIV Env spike prevents traditional vaccine efforts. Fortunately, although extremely rare and difficult to elicit, a few bnMAbs derived from natural infection have been identified and have been produced in large quantities to aid in protection and antibody function studies. Antibody passive protection studies have demonstrated that bnMAbs can provide protection against monkeys intravaginally and intrarectally challenged with SHIV, currently the best model for human infection (47, 48, 62). Because the transferred antibodies used in these studies provide protection and sterile immunity, it follows that a naturally elicited potent neutralizing antibody response remains a clear goal for an HIV vaccine candidate. However, this goal remains elusive and represents one of the most important obstacles in developing an effective vaccine (12).

The research presented in this thesis traces the search for the qualitative and quantitative requirements of neutralizing antibody to protect against HIV infection through studies using the 4 most recognized and best-characterized broadly neutralizing antibodies against HIV-1: b12, 2G12, 2F5 and 4E10. Before definitive conclusions can be drawn as to how neutralizing anti-HIV antibodies can be used in understanding the required natural adaptive immune response in humans, a closer look at each of these bnAbs in structure and function was required. The studies presented in this thesis address the protective abilities of each of these antibodies in animal studies and *in vitro* experiments uncovering either structural, molecular or protein biochemical properties of each antibody. Additionally, the interaction by neutralizing antibodies with the HIV-1 Env trimer (the virus spike) should be explored and the crucial determinants of the viral surface proteins that must be presented to the human immune system to elicit a potent and protective response must be understood. As a foundation for understanding the molecular determinants of antibody b12, the structural information in ***Chapter 2*** details the binding epitope and provides a sound basis for appreciating the antibody in context with HIV-1 Env trimer. The study was conducted by first constructing gp120 molecules that were constrained to stay in the CD4-bound conformation. A crystal structure of b12 in complex with the constrained gp120 provides details of the CD4 binding site and a depiction of the b12 epitope at an atomic level. ***Chapter 3*** details a study in which the protective dose of b12 was established in the macaque mucosal challenge model. The vaginal challenge model with macaques is one of the best models for studying the natural heterosexual human transmission of HIV. Simian/human immunodeficiency virus (SHIV) is a chimera containing an SIV core coated with an HIV Env. The resulting virus allows for infection to be established in the macaque with a disease progression similar to HIV-AIDS. The HIV coat makes it possible to study human antibody protection against infection in the macaque. The virus used in the study has the R5 phenotype that means the virus uses CCR5 as a coreceptor. Viruses that use the CCR5 coreceptor account for the majority of viruses isolated from acutely infected

individuals. The study established that a serum neutralizing titer could be correlated to protection against mucosal viral challenge. In follow-up to the protection study, we were curious about the way in which b12 was able to prevent infection. In particular, we wanted to determine if effector functions played a role in protection by b12. ***Chapter 4***, describes experiments to develop and evaluate a panel of Fc variants of b12 designed to ablate or greatly diminish effector functions. Point mutations were introduced in the FcγR receptor and C1q binding sites of the antibody located in the second domain of the heavy chain constant region (CH2). Two variants (K322A, and L234A, L235A) identified in the study were selected for *in vivo* studies conducted and presented in ***Chapter 5***. The variants, more simply referred to as KA and LALA, were used in conjunction with wild type b12 in a SHIV/macaque mucosal challenge study. The variants were shown to be equal to wild type b12 in neutralization and antigen binding but differed in their abilities to mediate effector function. Specifically, KA maintained a negligible ability to bind C1q or to initiate complement activation. The double mutation of the LALA variant greatly reduced binding to Fcγ receptors as well as C1q and therefore neither induced antibody dependent cellular cytotoxicity (ADCC) nor complement dependent cytotoxicity (CDC). Treatment with either wild-type b12 or KA protected 8 of 9 macaques, but treatment with LALA left 4 of 9 macaques unprotected. In addition, in infected animals differences in the onset and magnitude of viral loads were noted. The study demonstrated that effector functions are not necessary for neutralizing antibody to prevent infection. However, the likelihood of infection is greatly increased without the ability of antibody to bind Fcγ receptors and to initiate effector cell-mediated killing of infected cells.

Drawing upon our dose-dependent protection by b12 shown in Chapter 3 using a single high-dose mucosal challenge, we aimed to determine whether a lower amount of b12 would provide protection in a low-dose repeated mucosal challenge. Single high-dose challenges contain much more virus than is contained in a human sexual encounter even if either partner is acutely infected and the risk factors are high such as, for example, in the presence of sexually transmitted diseases (STDs). The study described in ***Chapter 6*** reveals that indeed, when a more physiologically relevant mucosal viral challenge is made, b12 can protect even at serum concentrations that did not prevent infection following high dose challenge. We included a LALA variant-treated group to compare protection in the absence of effector function activities. The outcome of the protection experiment revealed that significantly more challenges were required to infect b12 than non-antibody treated controls. Overall, somewhat fewer challenges resulted in infection of LALA-treated animals, supporting the findings in Chapter 5 that effector function contributes to protection. More importantly, the serum neutralizing titers of the protected animals were determined to be low enough to reasonably expect a similar titer could be achieved by vaccination.

As discussed earlier, the antibody 2G12 addresses an epitope of mannose residues on gp120 and neutralizes a broad range of primary isolates. Early protection studies with 2G12 hinted that the antibody might provide protection at relatively low serum neutralizing titers. In ***Chapter 7***, we explored protection by 2G12 against a SHIV mucosal challenge. The outcome of the protection experiment was that 3 of 5 macaques were completely protected at surprisingly low serum neutralizing titers. The unusual protective ability of 2G12 underscores the realization that the gp120 glycan shield should not be excluded as a target for vaccine design. A similar

conclusion regarding a vaccine target can be made from the study described in ***Chapter 8***. Here, we look at protection by the bnMAbs 2F5 and 4E10 that are directed against nearly adjacent epitopes in the highly conserved membrane proximal external region (MPER) of gp41. All previous protection studies with 2F5 and 4E10 have been conducted in combination with other antibodies leaving the individual contribution of these antibodies not fully understood. Based on observations from these earlier studies and *in vitro* neutralization titers, we designed an intrarectal mucosal challenge study using the R5 $SHIV_{Ba-L}$. Complete protection (6 of 6) of all animals in each 2F5 and 4E10 treatment groups was seen. Unlike b12 and 2G12 that provide steric hindrance prior to viral and host cell engagement, the MPER antibodies 2F5 and 4E10 prevent fusion between the viral membrane and the target cell. Of note, is the recognition that the MPER should remain as an important possible target for vaccine design. Finally, in ***Chapter 9*** we investigate the efficacy of an isotype-switched version of b12. In this study the light chain and variable heavy chain of b12 were re-engineered into an IgA2 antibody, after which we evaluated the neutralization, antigen binding, and epithelial cell uptake properties of the new variant of b12. Our study established that IgA2 as well as IgG1 b12 interferes with the earliest steps of HIV-1 transmission across mucosal surfaces. The availability of IgG1 and IgA2 HIV-1 bnMAb will be used to investigate the impact of these isotypes in topical and systemic protection studies and to evaluate their relative importance for a HIV-1 vaccine in the future.

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Figure acknowledgements (Chapter 1)

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Fig. 4. Liu, J, et al**,** 2008. Nature **455:**109-13 (No. 44 above)
Zanetti, G., et al**,** 2006. PLoS Pathogens **2:**e83 (No. 103 above)
Zhu, P., et al, 2006. Nature **441:**847-852 (No. 105 above)
Fig. 5. Kwong, P. D., et al**,** 1998. Nature **393:**648-659 (No. 41 above)
Fig. 8. Saphire, E. O., et al, 2001. Science **293:**1155-1159 (No 75 above)
Fig. 9. Zhou, T., et al, 2007. Nature **445:**732-737 (No 104 above)
Figs. 10 and 11.
Burton, D. R., R. L. Stanfield, and I. A. Wilson. 2005.
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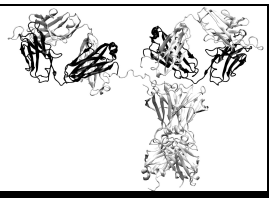
Abbreviations (from Chapter 1)

Ab	antibody
Ag	antigen
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired Immunodeficiency Disease Syndrome
AZT	Azidothymidine
bNMAbs	broadly Neutralizing Monoclonal Antibodies
CCR5	chemokine receptor 5 of the CR ligand-binding family normally functioning in chemoattraction
CD4	clusters of differentiation cell-surface molecule 4 (primary chemokine receptor molecule used by HIV-1 expressed on the surface of T lymphocytes, macrophages and dendritic cells)
CDC	complement-dependent cytotoxicity
CDR	complementarity determining regions
CXCR4	chemokine receptor 4 of the CXC ligand-binding family normally functioning in chemoattraction
ELISA	enzyme-linked immunosorbent assay
Env	envelope glycoproteins
Fab	**F**ragment: **a**ntigen **b**inding
Fc	**F**ragment **c**rystallizable
FcγR	Fcγ receptor
gp120	HIV-1 surface glycoprotein (120kD)
gp41	HIV-1 transmembrane glycoprotein (41kD)
gp160	HIV-1 precursor glycoprotein (160kD)
hu-PBL-SCID	human-peripheral blood lymphocyte–severe combined immune deficiency
Ig	Immunoglobulin
kD	kilo Dalton
MPER	Membrane Proximal External Region
SHIV	Simian-Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SIVcpz	Simian Immunodeficiency Virus found in chimpanzee
SIVsm	Simian Immunodeficiency Virus found in sooty mangabeys
VLP	Virus-like particles

Chapter 2



Structural Definition of a Conserved Neutralization Epitope on HIV-1 gp120

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ARTICLES

Structural definition of a conserved neutralization epitope on HIV-1 gp120

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The remarkable diversity, glycosylation and conformational flexibility of the human immunodeficiency virus type 1 (HIV-1) envelope (Env), including substantial rearrangement of the gp120 glycoprotein upon binding the CD4 receptor, allow it to evade antibody-mediated neutralization. Despite this complexity, the HIV-1 Env must retain conserved determinants that mediate CD4 binding. To evaluate how these determinants might provide opportunities for antibody recognition, we created variants of gp120 stabilized in the CD4-bound state, assessed binding of CD4 and of receptor-binding-site antibodies, and determined the structure at 2.3 Å resolution of the broadly neutralizing antibody b12 in complex with gp120. b12 binds to a conformationally invariant surface that overlaps a distinct subset of the CD4-binding site. This surface is involved in the metastable attachment of CD4, before the gp120 rearrangement required for stable engagement. A site of vulnerability, related to a functional requirement for efficient association with CD4, can therefore be targeted by antibody to neutralize HIV-1.

The human immunodeficiency virus type 1 (HIV-1) crossed from chimpanzees to humans early in the twentieth century and has since infected ~1% of the world's adult population[1,2]. This spread and the absence of an effective vaccine are to a large degree a consequence of the ability of HIV-1 to evade antibody-mediated neutralization[3–5]. On HIV-1, the only viral target available for neutralizing antibodies is the envelope spike, which is composed of three copies of the gp120 exterior envelope glycoprotein and three gp41 transmembrane glycoprotein molecules[6,7]. Genetic, immunological and structural studies of the HIV-1 envelope glycoproteins have revealed extraordinary diversity, manifest in a variety of immunodominant loops, as well as multiple overlapping mechanisms of humoral evasion, including self-masquerading glycan and conformational masking[8–11]. These evolutionarily honed barriers of diversity and evasion have confounded traditional vaccine development.

Two strategies have been proposed to surmount these barriers: examination of known broadly neutralizing antibodies (2F5, 2G12, 4E10 and b12) to identify susceptible targets of neutralization[12], and analysis of functional constraints to identify potential sites of vulnerability[13]. To facilitate viral entry, the gp120 glycoprotein must bind to cell-surface CD4 (ref. 14), alter its conformation to reveal a site for co-receptor attachment[15], and trigger conformational rearrangements in the gp41 glycoprotein to mediate fusion of viral and host cell membranes[16,17]. Constraints on envelope (Env) variation and exposure, associated with required functions of viral entry, provide potential footholds for broad, antibody-mediated neutralization.

Here we combine analysis of function with clues from antibody. We constructed stabilized gp120 molecules, constrained to stay in the CD4-bound conformation even in the absence of CD4, and tested the effect of this stabilization on the binding kinetics of CD4 and on antibodies reactive with sites of receptor binding. We then determined the crystal structure of the broadly neutralizing antibody b12 in complex with one of the stabilized gp120 molecules. Analysis of this structure, combined with detailed antigenic analyses of gp120 molecules stabilized to various extents in the CD4-bound conformation, not only reveals the functionally conserved surface that allows for initial CD4 attachment, but also provides an atomic-level description of the b12 epitope, which serves as a key target for humoral neutralization of HIV-1.

Recognition of the CD4-binding site

Conformational flexibility of HIV-1 gp120 complicated analysis of antibody recognition. To circumvent this complication, we used an iterative structure-based scheme to stabilize gp120 in its CD4-bound state (Table 1, Supplementary Fig. 1 and Supplementary Tables 1 and 2). The CD4-bound state of gp120 comprises an inner domain, outer domain and four-stranded bridging sheet mini-domain[18]. Five disulphides and four cavity-altering substitutions were created to restrict interdomain movements and to stabilize bridging sheet formation. Crystallographic analysis of gp120 variants with these substitutions in complex with CD4 and antibody 17b at 2.0–2.2 Å resolution showed that four of five disulphides formed and that disulphide and cavity-altering substitutions induced minimal structural perturbation. We also analysed a two-disulphide variant at 2.5 Å resolution as well as a three-disulphide variant at 2.8 Å resolution (Table 1). In both structures, all potential disulphides formed.

To measure the degree of conformational fixation, we used isothermal titration calorimetry to assess the entropy of interaction between the different stabilized gp120 cores and CD4 (Table 1 and Supplementary Table 3). Disulphides had a more substantial effect than cavity-altering substitutions. Tethering the centre two strands of the bridging sheet (by linking residues 123–431) or the termini-proximal ends of the domains (96–275 or 231–267) reduced the entropy of interaction by 15–30%. A more substantial effect (a 60% reduction) was gained by tethering the bridging sheet to the inner domain (109–428). Thermodynamic analysis thus quantified

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Table 1 | Characterization of stabilized gp120 cores.

gp120*	Resolution (Å)	Entropy of CD4 interaction (−TΔS) (kcal mol^{-1})	Ligands															
			CD4		CD4-induced antibodies				CD4-binding-site antibodies									
					17b		m6		b12	b3	b6	b11	b13	m14	m18	F91	F105	15e
			On† (×10^4)	K_d (nM)	On† (×10^4)	K_d (nM)	On† (×10^4)	K_d (nM)	K_d(WT)/K_d(mutant)									
Wild type (WT)	2.00	39.7	2.5	52	0.69	560	2.2	2,400	1	1	1	1	1	1	1	1	1	1
F2	2.05	39.1	4.7	3.5	2.9	530	14	680	0.28	1.0	4.6×10^{-5}	1.6×10^{-3}	0.73	5.2×10^{-3}	0.29	9.0×10^{-3}	$<10^{-5}$	$<10^{-5}$
Ds1 F123	2.20	28.3	3.5	14	3.4	230	12	490	0.43	0.29	1.9×10^{-5}	9.0×10^{-4}	0.95	0.059	0.21	4.2×10^{-3}	$<10^{-5}$	$<10^{-5}$
Ds2 F2	2.00	17.2	5.3	4.8	10	200	32	400	0.085	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	0.029	$<10^{-5}$	1.5×10^{-4}	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
Ds3 F2	2.00	26.9	3.8	1.9	9.5	120	25	220	0.42	0.72	3.3×10^{-5}	2.5×10^{-3}	0.67	3.3×10^{-3}	0.28	2.7×10^{-3}	$<10^{-5}$	$<10^{-5}$
Ds4 F2	2.00	34.2	4.5	2.8	2.3	820	8.4	2,200	0.30	0.97	0.49	1.6×10^{-3}	0.56	2.4×10^{-3}	0.34	6.2×10^{-3}	$<10^{-5}$	$<10^{-5}$
Ds12 F123	2.50	18.5	4.9	10	3.7	320	19	710	0.60	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	7.6×10^{-3}	$<10^{-5}$	3.2×10^{-3}	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
Ds123 F12	2.80	18.7	3.0	1.9	66	19	140	32	1.1	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	1.6×10^{-3}	$<10^{-5}$	2.6×10^{-3}	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$

Stabilized gp120 cores were characterized by X-ray crystallography, isothermal titration calorimetry and surface-plasmon resonance. K_d, dissociation constant.
* gp120 core variants as defined by cavity-altering substitutions: F1, M95W; F2, T257S/S375W; F3, A433M; and also by disulphide (Ds)-bond-forming cysteine substitutions: Ds1, W96C/V275C; Ds2, I109C/Q428C; Ds3, T123C/G431C; Ds4, K231C/E267C.
† On-rate of ligand binding to gp120 (units in $M^{-1}s^{-1}$).

the degree of variant gp120 stabilization and demonstrated substantial remaining flexibility, despite the presence of up to three stabilizing disulphides.

Because the epitope for CD4-induced antibodies is only formed in the CD4-bound state[10,19,20], we could use the increase in antibody on-rate to assess the degree to which stabilization 'preformed' the variant gp120 molecules in the CD4-bound state. Reasonable correlations were observed between the on-rate for CD4-induced antibodies and the entropy of CD4 binding ($r = 0.74$, $P < 0.036$ for antibody 17b (ref. 19); $r = 0.76$, $P < 0.029$ for antibody m6 (ref. 21)), with the most extreme change for the three-disulphide variant (a 95-fold increase in on-rate for antibody 17b and a 65-fold increase for antibody m6) (Table 1 and Supplementary Fig. 1c).

Notably, almost no change in CD4 on-rate was observed with the stabilized gp120 molecules (Table 1, Supplementary Table 4 and Supplementary Fig. 1c), suggesting that the initial contact surface recognized by CD4 is present in unliganded gp120 before the CD4-induced conformational change. Similarly invariant on-rates have recently been reported for CD4 binding to gp120 molecules restrained from achieving a CD4-bound conformation by removing a flexible loop[22]. Thus, in contrast to the CD4-induced antibodies, a conformational change is not required to expose an initial site of contact for CD4. Rather, it serves to lock CD4 into place once contact has been made.

To determine the degree to which fixation of gp120 in its CD4-bound conformation restricts antigenic recognition, we tested binding of a panel of CD4-binding-site antibodies to the stabilized gp120 variants (Table 1). The average overall difference in free energy of binding for CD4-binding-site antibodies to wild type and stabilized variants correlated well with the change in entropy of variant gp120 binding to CD4 ($r = 0.89$, $P < 0.0035$) (Supplementary Fig. 1c). Conformational constraint thus effectively hides the CD4-bound conformation of gp120 from recognition by antibodies that target the site of CD4 binding. Indeed, of all the CD4-binding-site antibodies tested, only the unique, broadly neutralizing antibody b12 was able to bind well to stabilized gp120.

Structure of a b12–gp120 complex

Although gp120 is the primary target of neutralizing antibodies elicited during natural infection[23], most gp120-reactive antibodies are ineffective at neutralizing primary HIV-1 isolates (reviewed in refs 4, 24). Only two gp120-reactive antibodies (b12, 2G12) with effective neutralization activity against diverse primary HIV-1 isolates have thus far been identified[25–27]. *In vivo*, b12 can protect macaques against vaginal challenge from pathogenic simian–human immunodeficiency virus (SHIV)[28]. Although the structure of the entire, unbound b12 immunoglobulin (IgG1) has been determined[29], a molecular description of its interaction with gp120 remained elusive.

To facilitate a structural understanding of b12 neutralization of HIV-1, we screened crystallizations of b12 in complex with various forms of gp120. The lack of b12 conformational fixation of gp120 (ref. 10), coupled to inherent gp120 flexibility, complicated crystallization of a b12–gp120 complex; to overcome this complication, we

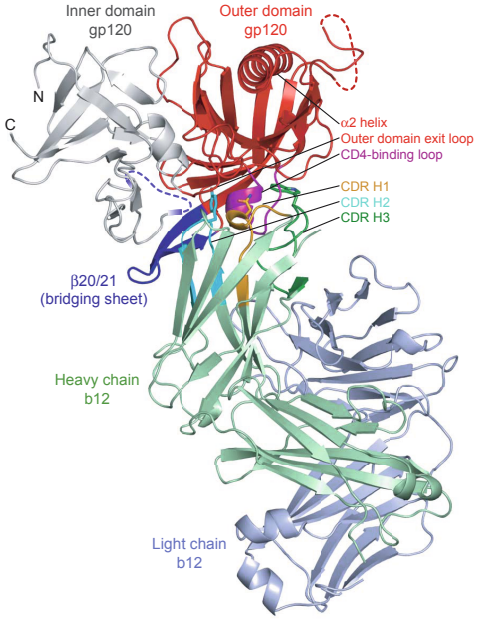

Figure 1 | Structure of b12 in complex with an HIV-1 gp120 core. Polypeptide chains are depicted in ribbon representation, with disordered regions as dashed lines. The gp120 inner domain is grey, and the outer domain is red, except for the CD4-binding loop, which is purple. The strands and associated loops, which in the CD4-bound conformation correspond to the bridging sheet, are blue. The b12 light chain is blue–grey and the b12 heavy chain is green, with associated CDRs highlighted in orange (H1), cyan (H2) and dark green (H3). The heavy-chain dominance of the binding interaction is apparent, with the nearest light-chain approach separated by ~10 Å from gp120. Heavy-chain-only interactions are rare, although heavy-chain interactions predominate in a number of viral Env–antibody complexes, including those from SARS coronavirus[49] and influenza virus haemagglutinin[50]. Three b12 residues (Asn 31, Tyr 53 and Trp 100) from each of the heavy-chain CDRs are depicted in stick representation. Together, these three residues combine to form ~40% of the b12 contact surface. They can be seen gripping the CD4-binding loop, the central focus of the b12 interaction with gp120.

used the mutationally stabilized gp120 molecules. Complexes with wild-type core or with single-disulphide variants (96–275 or 123–431) failed to produce crystals suitable for structural analysis. However, diffraction to 2.3 Å was obtained from hexagonal crystals of the antigen-binding fragment (Fab) of b12 in complex with a two-disulphide-stabilized gp120 core. We solved the complex structure by molecular replacement and refined it to an *R*-value of 19.3% (R_{free} = 25.5%) (Supplementary Table 5).

Notably, only the heavy chain of b12 interacted with gp120, with each of the three heavy-chain complementarity-determining regions (CDRs) making extensive contact (Fig. 1, Supplementary Table 6 and Supplementary Figs 2 and 3). Despite this unusual heavy-chain-only usage, the surface areas of interaction were in the range typical for antibody–protein interfaces (reviewed in ref. 30), with a total of 1,480 Å^2 buried[31] in the interaction (737 Å^2 on gp120 and 743 Å^2 on b12).

The gp120 surface bound by b12 was confined largely to the gp120 outer domain, consistent with predictions from alanine substitutions (Supplementary Table 7 and ref. 32). We examined this surface for features that might make it amenable to antibody recognition. The outer domain is composed of two barrels, stacked end to end (Fig. 2). The termini-distal barrel comprises seven anti-parallel β-strands; the other contains six β-strands embracing the α2 helix. b12 binds at the barrel–barrel juncture to the gp120 face opposite the α2 helix. Although most of the gp120 backbone is involved in secondary structure interactions, the barrel juncture contains a number of structural elements with unpaired backbone, often used by antibodies in recognition. Principal among these are two parallel loops—the CD4-binding loop and the outer domain exit loop—that extend from the only two parallel β-strands in the entire gp120 core. b12 takes advantage of this backbone reactivity, forming six direct and four water-mediated hydrogen bonds with the main-chain atoms of these two loops (Supplementary Fig. 2). Overall, the outer domain comprises 82% of the gp120 contact surface with b12, with the CD4-binding loop forming a little over one-half of this surface.

Comparison of b12- and CD4-binding

Both CD4 and b12 bind primarily to the outer domain of gp120, which is remarkably well preserved between unliganded, b12- and CD4-bound states (Figs 2 and 3). Their entropies of interaction, however, are very different, with CD4 inducing a 40–50 kcal mol^{-1} change, and b12 inducing only a 6 kcal mol^{-1} change[10]. This difference in conformational fixation of gp120 is seen in the divergent atomic-mobility values of the domains (Fig. 3b).

The angles of approach of CD4 and b12 to gp120 are similar, although not precisely the same (Fig. 3c). If the gp120 outer domains of b12- and CD4-bound structures were superimposed to orient equivalently b12 and CD4, about one-half of CD4 domain 1, which makes all of the contacts with gp120, is not encompassed by the b12 Fab (despite the volume of the Fab variable domains being twice as large as that of domain 1 of CD4). The projection of CD4 outside of the angle of b12 approach suggests that the parameters that sterically restrict b12 binding to the functional spike are not overly stringent.

The contact surfaces of CD4 and b12 on gp120 have considerable overlap (Fig. 3a and Supplementary Fig. 4). Most of this overlap is on the outer domain, where the CD4-binding loop is a central focus of binding for both CD4 and b12. CD4 and b12, however, interact with the CD4-binding loop quite differently (Fig. 3d). b12 uses all three of its CDR heavy-chain loops to grasp virtually all surface-exposed portions of the loop. In contrast, CD4 only binds to one side of the loop, making anti-parallel hydrogen bonds between CD4- and gp120-main-chain atoms.

The primary difference between b12 and CD4 interactions with gp120 involves the conformationally mobile β20/21. For b12, these interactions are peripheral to the binding surface, with alanine substitution of the primary b12 contact with β20/21 (at b12 residue Asn 56) having little impact on overall b12 binding[33]. In contrast, CD4 interactions with β20/21 form an integral part of the binding surface, burying 160 Å^2 of surface area and forming a topologically contiguous contact surface with the CD4-binding loop.

To delineate further the differences in binding between b12 and CD4, we characterized binding interactions with an HIV-1 gp120 fragment, termed OD1 (ref. 34). This fragment comprises residues 252–482 and encompasses the entire outer domain including V3 as well as the β20/21 excursion. Binding of b12 to OD1 showed nearly identical rates of association compared to binding of b12 to HXBc2 core gp120, although the dissociation rate was about 15-fold more rapid (Supplementary Fig. 5). We were unable to detect binding of CD4 to OD1 (Supplementary Fig. 5). Because CD4 demonstrated virtually no change in on-rate when tested on conformationally stabilized gp120 molecules, we turned to a dodecameric variant of CD4 (D1D2-Igαtp (ref. 35)), as avidity from multivalent binding provides an effective means by which to reduce off-rate. We observed that D1D2-Igαtp binds with virtually identical rates of association to both OD1 and core gp120 (Supplementary Fig. 5).

The results suggest the following series of molecular interactions for b12 and CD4 binding to gp120 (Supplementary Fig. 6). Initial contact by CD4 occurs with the structurally invariant outer domain, to a surface constitutively exposed on the envelope spike. In primary isolates, which are generally resistant to neutralization by soluble CD4, this interaction is not stable and CD4 readily 'falls off'. However, at the cell surface (or with dodecameric CD4), multiple CD4 molecules can bind simultaneously to the viral spike and use avidity to enhance stability. The avidity-enhanced outer domain–CD4 complex provides a receptive contact surface for the bridging sheet. A highly coordinated rearrangement of the inner domain allows for formation of the bridging sheet, which welds CD4 into place.

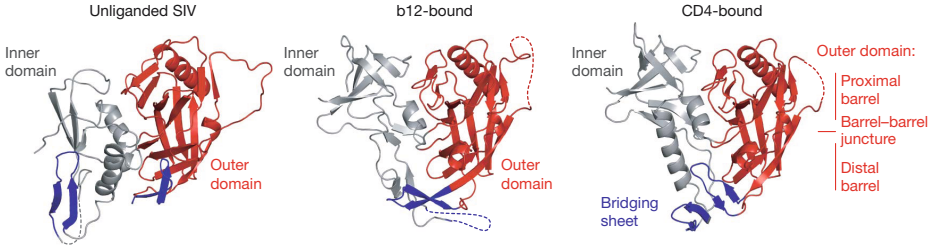

Figure 2 | Conformational states of gp120. The unliganded, b12- and CD4-bound conformations of gp120 are depicted, with polypeptide in ribbon representation and disordered regions as dashed lines. Inner domains are grey, outer domains are red and regions that in the CD4-bound state correspond to the bridging sheet are blue. Both b12- and CD4-bound conformations are of the Ds12 F123 variant of HIV-1, whereas the unliganded structure is of simian immunodeficiency virus (SIV). Comparison of these three gp120 conformations highlights not only the structural plasticity of the inner domain and bridging sheet, but also the conformational stability of the outer domain.

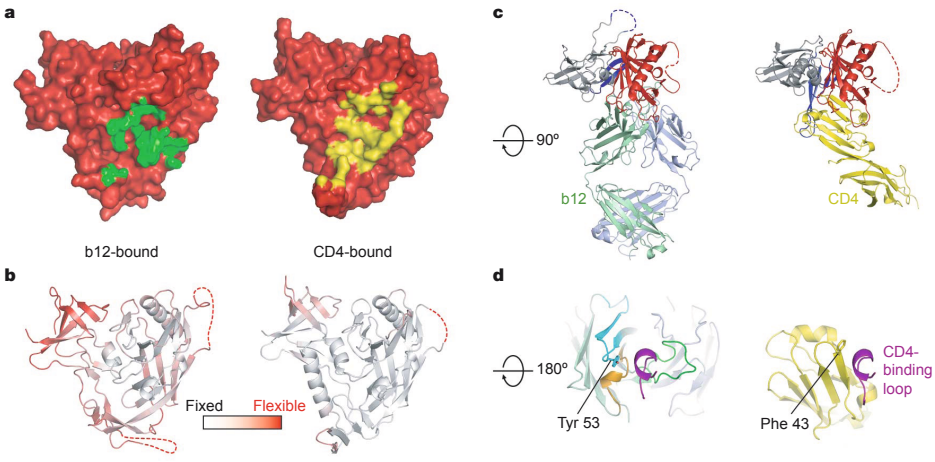

Figure 3 | b12 and CD4 recognition of gp120. The orientation of gp120 in **a** and **b** is the same as in Fig. 2, with **c** and **d** rotated about a horizontal axis by 90° and 180°, respectively. **a**, Molecular surface of gp120 in red, with the b12-contact surface in green (left) and the CD4-contact surface in yellow (right). **b**, Ribbon diagram of the b12- and CD4-bound gp120 coloured according to the atomic mobility of the polypeptide, with white for fixed and red for flexible. In the b12-induced conformation, only the outer domain is fixed by interaction with antibody, with the average atomic mobility of the outer domain about one-half that of the inner domain. By contrast, CD4 fixes the entire core, resulting in outer and inner domains of similar overall atomic mobility. **c**, Comparison of b12 and CD4 angles of attachment. The polypeptide chains are depicted in ribbon representation, and coloured according to Fig. 1, with CD4 in yellow. The similarity in angle of attachment as well as the binding focus of b12 on the outer domain (red) are evident in this orientation. **d**, The CD4-binding loop of gp120 (purple) is shown with b12 (left) and CD4 (right). There are parallels between several key CD4 contact residues and those of b12. For example, Phe 43 of CD4 (shown in stick model) inserts into a critical juncture at the nexus of the inner, outer and bridging sheet regions of gp120, whereas Tyr 53 of b12 (also shown in stick model) inserts at a similar position, although displaced by ~3 Å.

Contact by b12 occurs at the same constitutively exposed surface initially recognized by CD4. However, b12 is able to latch onto this outer domain surface with high affinity, without additional gp120 conformational change. This absence of conformational constraint allows b12 to bind and neutralize primary isolates that otherwise would be protected by conformational masking. In this manner, b12 uses the functionally conserved initial contact site for CD4 on gp120 to neutralize effectively HIV-1 (Fig. 4).

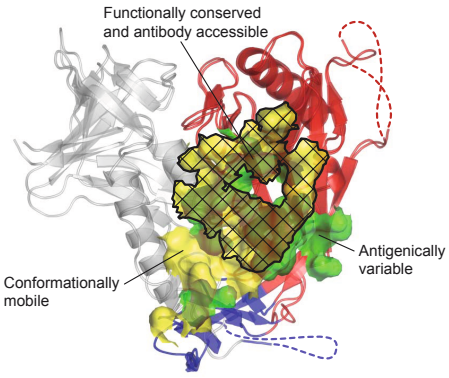

Figure 4 | Structural definition of a conformationally invariant, antibody-accessible portion of the CD4-binding site. The b12- and CD4-bound conformations of gp120 are shown in ribbon representation, after superposition of outer domains (red). A semitransparent molecular surface shows the contact surfaces of b12 (green) and CD4 (yellow). Subsets of these surfaces, corresponding to regions of conformational flexibility (for example, of the inner domain (grey) or bridging sheet (blue)), are delineated, as are regions of b12 contact outside of the conserved CD4-binding site. As can be seen, functional analysis serves to transcend the particulars of b12 binding, whereas antibody defines accessibility. Although we have formally shown only the b12 contact surface to be accessible in the context of a functional viral spike, the highly effective neutralization of D1D2-Igαtp and the kinetics of its association with both core and OD1 variants of gp120 suggest that the CD4-binding surface on the outer domain is accessible.

A site of HIV-1 vulnerability

Wedged between the glycan-shielded silent face and the conformationally flexible inner domain/bridging sheet, with its projecting variable loops, the identified site is recessed, although it must be accessible to a molecule as large as CD4 to serve its function. Otherwise, its size and reactive loops make it suitable for recognition by antibody. Recent serological analysis of long-term non-progressors attributes the broad and potent neutralizing properties of some of these sera to antibodies directed against this site[36]. The potential of receptor-binding-site antibodies to effect broad neutralization has been recognized since the first viral structures revealed conserved sites of receptor binding[37,38]. Here we show how an unanticipated requirement for the maintenance of a substantial receptor on-rate allows b12 to access and disable this most difficult of targets, the highly protected HIV-1 Env.

METHODS

Structure-based stabilization of gp120 in its CD4-bound conformation. Cavity-altering substitutions were identified by examination of highly conserved residues that bordered interdomain cavities, whereas all Cβ−Cβ distances between 3–7 Å were examined to identify potential stabilizing disulphides. A high-throughput expression system, coupling transient expression with swainsonine, endoglycosidase H and concanavalin A, was devised from related procedures[39] to produce gp120 suitable for crystallization. Briefly, codon-optimized variants were constructed in the context of core gp120 (HXBc2) and expressed transiently in 293 cells under control of the CMV/R promoter[40]. Swainsonine (20 mg l^{-1}; Biomol) was added to the cell-culture medium 2 h before DNA transfection, and supernatants were collected after 48 and 96 h. Secreted gp120 molecules were purified by 17b-affinity chromatography and deglycosylated with endoglycosidase H (Endo Hf; New England Biolab) at pH 5.9, 37 °C, with gp120 containing residual uncleaved glycan moieties removed by passage over concanavalin A Sepharose (Sigma-Aldrich). Ternary complexes of deglycosy-

lated core gp120, d1d2 of CD4 and 17b Fab were prepared as described previously[41], with $P222_1$ crystals grown (Supplementary Table 1) around conditions identified for the *Drosophila*-produced wild-type core[18]. Because crystals were small (rods, typical diameter 40 μm) and subject to high radiation dosages during data collection, difference Fourier maps comparing the initial and final swatches of data were inspected to identify radiation-induced disulphide breakage[42], and the refined models (Supplementary Table 1) adjusted to reflect the initial, radiation-damage-free structure.

Surface-plasmon resonance. A Biacore 3000 surface-plasmon resonance spectrometer was used to measure kinetic constants (see Supplementary Table 4 and Supplementary Fig. 5 for details).

Isothermal titration calorimetry (ITC). Wild-type core and variant gp120 molecules were dialysed in PBS buffer and titrated with d1d2 of CD4 on a MicroCal VP-ITC at 37 °C. Resultant data were examined using Origin software (MicroCal).

Structural determination of a b12–gp120 complex. The antigen-binding fragment (Fab) of b12 was produced by papain digestion, and purified with Superdex S200 chromatography (0.35 M NaCl, 2.5 mM Tris pH 7.1, 0.02% NaN_3). The Fab peak was pooled and mixed with deglycosylated gp120, which was produced by transient transfection as described above, and the resultant complexes purified by S200 chromatography. Crystals of Fab b12 and a two-disulphide variant (Ds12 F123) were grown by mixing 0.5 μl of complex (4 mg ml^{-1}) in S200 buffer with 0.5 μl of droplet mix (10.5% PEG 8,000, 0.2 M glycine, 105 mM Mg-acetate, 52.5 mM Na-cacodylate pH 6.5) and equilibrating in hanging droplets over reservoirs (droplet mix without glycine) at 20 °C. Hexagonal bi-pyramids (200 μm in length by 90 μm in diameter) were crosslinked[43], transferred to 15% PEG 8,000, 150 mM Mg-acetate, 100 mM Na-cacodylate pH 6.5, 30% ethylene glycol, 2.5% 2*R*,3*R*-butandiol, 2.5% trehalose, and flash-frozen in a nitrogen-cryostat stream. Data were collected at 100 K and processed with HKL2000 (ref. 44). Molecular replacement (AMoRe[45]) identified a 5.2σ peak (15–3 Å data) for the Fab portion (chains H and L) of the b12 IgG (Protein Data Bank 1HZH)[29], and phases from the rigid-body refined molecule allowed unambiguous placement of the outer domain in $F_o - F_c$ density. Iterative model building (XtalView[46]), combined with refinement (CNS[47], Refmac[48]), were used to define the remaining ordered parts of gp120.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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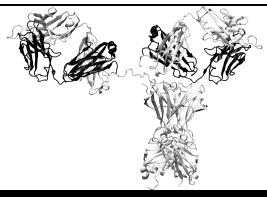
Author Contributions T.Z. and P.D.K. carried out structure-based stabilization, SPR analyses and structural determinations; L.X. and G.J.N. constructed gp120 substitutions and developed and implemented a high-throughput gp120-production system suitable for crystallization; B.D. and R.W. carried out ITC characterizations; A.J.H., M.B.Z. and D.R.B. provided b12, b3, b6, b11 and b13, and mutant b12 binding; D.V.R. and J.A. provided D1D2-Igαtp and associated SPR analyses; S.-H.X., X.Y. and J.S. provided OD1 and preliminary design and antigenic analyses; and M.-Y.Z. and D.S.D. provided m6, m14 and m18. All authors contributed to the manuscript preparation.

Author Information Coordinates and structure factors have been deposited in the Protein Data Bank and may be obtained from the authors (accession codes 2nxy–2ny6 for the nine variant gp120 molecules with CD4 and 17b; accession code 2ny7 for the b12–gp120 complex). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.D.K. (pdkwong@nih.gov).

Chapter 3



Antibody Protects Macaques against Vaginal Challenge With a Pathogenic R5 Simian/Human Immunodeficiency Virus at Serum Levels Giving Complete Neutralization In Vitro

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A major unknown in human immunodeficiency virus (HIV-1) vaccine design is the efficacy of antibodies in preventing mucosal transmission of R5 viruses. These viruses, which use CCR5 as a coreceptor, appear to have a selective advantage in transmission of HIV-1 in humans. Hence R5 viruses predominate during primary infection and persist throughout the course of disease in most infected people. Vaginal challenge of macaques with chimeric simian/human immunodeficiency viruses (SHIV) is perhaps one of the best available animal models for human HIV-1 infection. Passive transfer studies are widely used to establish the conditions for antibody protection against viral challenge. Here we show that passive intravenous transfer of the human neutralizing monoclonal antibody b12 provides dose-dependent protection to macaques vaginally challenged with the R5 virus $SHIV_{162P4}$. Four of four monkeys given 25 mg of b12 per kg of body weight 6 h prior to challenge showed no evidence of viral infection (sterile protection). Two of four monkeys given 5 mg of b12/kg were similarly protected, whereas the other two showed significantly reduced and delayed plasma viremia compared to control animals. In contrast, all four monkeys treated with a dose of 1 mg/kg became infected with viremia levels close to those for control animals. Antibody b12 serum concentrations at the time of virus challenge corresponded to approximately 400 (25 mg/kg), 80 (5 mg/kg), and 16 (1 mg/kg) times the in vitro (90%) neutralization titers. Therefore, complete protection against mucosal challenge with an R5 SHIV required essentially complete neutralization of the infecting virus. This suggests that a vaccine based on antibody alone would need to sustain serum neutralizing antibody titers (90%) of the order of 1:400 to achieve sterile protection but that lower titers, around 1:100, could provide a significant benefit. The significance of such substerilizing neutralizing antibody titers in the context of a potent cellular immune response is an important area for further study.

Increasingly it is apparent that eliciting a T-cell response through vaccination is highly beneficial in terms of being able to control human immunodeficiency virus type 1 (HIV-1) replication following infection (2). Nevertheless, there is still great interest in eliciting a neutralizing antibody response that may synergize with the T-cell response or possibly even provide sterile protection on its own. Interestingly, studies of vaccination against a murine retrovirus show that the best protection is provided by a combination of specific B, $CD4^+$ T, and $CD8^+$ T cells (7). It was suggested that persistent infection with the retrovirus could be prevented only when antibody-producing cells were present (8).

Classically, antibody protection against viral challenge is investigated through passive transfer studies. In the case of HIV-1, this has been difficult for polyclonal antibody preparations because of the generally low titers of neutralizing antibody in serum elicited by natural infection or immunization. A few monoclonal antibodies have been generated that do effectively neutralize primary HIV-1 isolates, and these have been used in passive transfer studies. Thus, the neutralizing human monoclonal antibody b12 was shown to protect hu-PBL-SCID mice against challenge with two primary HIV-1 viruses (JR-CSF and AD6) (10). In each case, protection required concentrations of antibody in serum at the time of challenge that were sufficient to neutralize essentially all of the virus inoculum. A similar requirement for complete neutralization of the challenge virus was made in a study using macaques (38). Here the intravenous challenge virus was a simian/human immunodeficiency virus (SHIV) derived from a primary virus ($SHIV_{DH12}$), and the infused antibody was derived from the plasma of chimpanzees infected with HIV_{DH12}. The polyclonal antibody preparation had a very high neutralizing titer which was completely specific for the challenge virus (5). The neutralizing antibodies 2G12 and 2F5 in combination with a polyclonal human anti-HIV preparation (HIVIG) showed partial protection against intravenous challenge with the pathogenic $SHIV_{89.6PD}$ virus (24). When a similar study was performed using a vaginal challenge with $SHIV_{89.6PD}$, there was some indication that protection was easier to achieve. For example, in contrast to the intravenous challenge study, partial protection was also observed with a single antibody (2G12) with only modest neu-

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tralizing activity against this challenge virus (26). Overall, however, most of the macaque data indicated that sterile protection required complete antibody neutralization of challenge virus. Similar conclusions were reached for HIV-1 challenge of hu-PBL-SCID mice (11, 29) or SHIV challenge of macaques (1) using viruses containing the *env* genes of T-cell-line-adapted viruses.

Vaginal challenge of macaques with a SHIV is probably one of the best animal models available for natural HIV-1 infection of humans. This is particularly the case for heterosexual transmission of HIV-1, which is the primary route of infection worldwide. The studies of Mascola and colleagues described above are therefore of particular value (26). One limitation of those studies is that they use a virus, $SHIV_{89.6PD}$, that differs in its coreceptor usage from those viruses that are usually involved in human transmission or early systemic spread in the body. Thus, $SHIV_{89.6PD}$ expresses an envelope showing dual R5X4 tropism with a strong bias toward X4 usage. This is known since $HIV\text{-}1_{89.6}$ entry can be completely blocked by X4 inhibitors but is insensitive to a CCR5-specific inhibitor (12). However, viruses isolated during acute infection of both women and men usually (>90% of the time) have the R5 phenotype. Similarly, R5 viruses are generally involved in mother-to-infant transmission. Furthermore, CCR5-Δ32 homozygous individuals, who lack CCR5 expression, are strongly protected against HIV-1 infection (15, 20). Therefore it is clearly of considerable importance to understand the efficacy of antibody against mucosal challenge with an R5 virus in the macaque model. A further consideration is that the studies described above using $SHIV_{89.6PD}$ in macaques did not provide a complete titration of protection with antibody concentration. To address these issues, we investigated the ability of the neutralizing antibody b12 at various doses to protect against vaginal challenge with the R5 virus $SHIV_{162P4}$.

$SHIV_{162P4}$ is based on molecular clones of SIV_{mac239} and the well-established R5 HIV-1 primary isolate SF162 and was isolated following three sequential in vivo passages in rhesus macaques as described elsewhere (13, 22, 40). For the purposes of the antibody protection studies described here, the important features of $SHIV_{162P4}$ are that it is an R5 virus and that it replicates to high titers following infection. However, the virus does display pathogenic behavior in that a proportion of animals show depressed CD4 counts and progress to AIDS-like symptoms.

The antibody chosen for the protection studies was the human monoclonal antibody immunoglobulin G1 (IgG1) b12 (4). This antibody shows broad neutralization of primary HIV-1 isolates and is directed to an epitope on gp120 overlapping the CD4 binding site (32, 34, 41). The antibody effectively neutralizes $SHIV_{162P4}$ in vitro, with 90% neutralization occurring at a concentration of b12 of 1 to 2 μg/ml. Since concentrations in serum of up to the order of 500 to 1,000 μg/ml can be achieved in macaques via passive immunization, this potency ensures that a wide range of effective antibody concentrations can be studied in vivo.

The results show that the antibody b12 can completely protect macaques against vaginal challenge with an R5 virus at the highest dose used (25 mg/kg of body weight). The dose titration shows partial protection at an intermediate dose (5 mg/kg) and no protection at the lowest dose (1 mg/kg) used. Together with other protection experiments, the data emphasize that antibody alone provides sterile protection with complete virus neutralization independent of virus isolate, neutralizing antibody or a combination of antibodies, challenge route, or animal model used. However, the benefit in terms of a lower viremia may be apparent at lower effective antibody concentrations.

MATERIALS AND METHODS

Macaques. Protocols for animals (female macaques, 4.5 to 8.5 kg) were reviewed and approved by the relevant Institutional Animal Care and Use Committees. The animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care standards. For all procedures, animals were lightly anesthetized with 10 mg of ketamine HCl/kg. All macaques were experimentally naive and were negative for antibodies against HIV-1, SIV, and type D retrovirus at the start of the experiments.

Thirty days prior to virus challenge, the animals received 30 mg of medroxyprogesterone acetate (Depo-Provera; Upjohn, Kalamazoo, Mich.) by intramuscular injection (26). Antibody preparations were administered by intravenous injection 6 h prior to virus challenge. The challenge virus diluted in 1 ml of phosphate-buffered saline (PBS) was introduced atraumatically into the vagina with an 8 French pediatric feeding tube attached to a syringe barrel. Macaques were maintained in an immobilized state, with the perineum slightly elevated, for approximately 15 min post-viral challenge. The animals were monitored by assessment of routine hematology, CD4 and CD8 lymphocyte subset counts, blood chemistry, and plasma viral loads at regular intervals. Inguinal lymph nodes were biopsied at 7 and 21 days postchallenge. Vaginal fluids were collected weekly as described below.

Challenge virus. The virus used in this study was $SHIV_{162P}$ passage 4, which has been described elsewhere (13, 40). Briefly, $SHIV_{SF162}$ was constructed by replacing the *tat*, *rev,* and *env* genes of the pathogenic molecular clone SIV_{mac239} with corresponding regions from the R5, HIV-1 molecular clone SF162 (40). $SHIV_{162P4}$ was then derived from $SHIV_{SF162}$ by serial passaging of the latter virus in vivo; this involved three sequential blood-bone marrow transfusions into naive macaques, followed by virus reisolation (13). $SHIV_{162P4}$ retains the R5 phenotype of $HIV\text{-}1_{SF162}$ (13).

Neutralization assays. Neutralization assays were performed using phytohemagglutinin (PHA)-activated rhesus peripheral blood mononuclear cells (PBMC) as target cells. All assays were performed with cells from a single rhesus macaque (no. 355); cells from this animal replicated $SHIV_{162P}$ efficiently. PBMC were isolated on Histopaque-1077 (Sigma, St. Louis, Mo.) and stimulated overnight with 8 μg of PHA (Sigma)/ml and 100 U of interleukin 2 (provided by Maurice Gately via the NIH AIDS Research and Reference Reagent Program)/ml. Antibody was incubated with 100 50% tissue culture infective doses ($TCID_{50}$s) of SHIV for 1 h at 37°C in a volume of 100 μl in round-bottom microtiter plates. An equal volume of PHA-stimulated rhesus PBMC in medium (RPMI 1640 supplemented with 10% fetal bovine serum [FBS], 100 U of interleukin 2/ml, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml) was then added. The plates were incubated for 3 days at 37°C, washed three times with RPMI 1640, and incubated in culture medium for an additional 4 days. The amount of SHIV present in each well was quantified using a p27 antigen enzyme-linked immunosorbent assay (ELISA) (Retro-tek; Zeptometrix, Buffalo, N.Y.) as recommended by the manufacturer.

Plasma viral loads. Quantitative assays for the measurement of SIV RNA were performed at Bayer Diagnostics (Berkeley, Calif.) using a branched DNA signal amplification assay for SIV (23). The lower limit of the assay was 400 SHIV RNA copies per ml.

Antibodies. IgG1 b12 is a human antibody (IgG1, κ) recognizing an epitope overlapping the CD4 binding site of gp120 (3, 4). Recombinant IgG1 was expressed in Chinese hamster ovary (CHO-K1) cells in glutamine-free Glasgow minimum essential medium supplemented with 10% dialyzed fetal bovine serum (Tissue Culture Biologicals, Tulare, Calif.), minimal essential medium nonessential amino acids (Gibco-BRL, Grand Island, N.Y.), 1 mM minimal essential medium sodium pyruvate (Gibco-BRL), 500 μM L-glutamic acid, 500 μM L-asparagine, 30 μM adenosine, 30 μM guanosine, 30 μM cytidine, 30 μM uridine, 10 μM thymidine (Sigma), 100-U/ml penicillin, 100-μg/ml streptomycin, and 50 μM L-methionine sulfoximine (Sigma) in a 3-liter spinner flask, purified using protein A affinity chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden), and then dialyzed against PBS. Care was taken to minimize contamination with endotoxin, which was monitored using a quantitative chromagenic Limulus Amoebecyte Lysate assay (BioWhittaker, Walkersville, Md.) performed

TABLE 1. Plasma viral loads after vaginal challenge with various doses of $SHIV_{162P4}$

Animal	Challenge dose[a]	Log viral load in plasma on day:									Day of death
		7	11	14	21	28	60	90	321	376	
J440	600	<2.6	4.0	5.3	6.6	6.2	6.0	6.5			Day 174[b]
N083	600	<2.6	4.8	5.9	6.3	5.7	4.1	3.0	<2.6	<2.6	Day 434[c]
I806	600	<2.6	3.6	5.1	6.6	5.2	<2.6	<2.6	<2.6	<2.6	
N462	300	<2.6	2.8	4.7	6.4	5.7	Not done	<2.6	<2.6	<2.6	
N321	300	<2.6	6.2	7.1	6.9	6.4	5.5	7.2			Day 117[d]
I624	300	<2.6	4.2	5.6	5.7	5.3	<2.6	<2.6	<2.6	<2.6	
M290	100	<2.6	<2.6	3.7	5.0	6.3	<2.6	6.0	<2.6	<2.6	
L492	100	2.8	5.4	6.8	6.6	4.7	3.0	3.0	4.8	5.0	
H658	100	<2.6	4.6	6.3	7.6	6.4	<2.6	3.5	6.4	6.4	Day 439[e]

[a] Challenge dose in $TCID_{50}$s.
[b] Found dead. Autopsy findings: thymic atrophy, amyloidosis, emaciated, pneumonia.
[c] Found dead. Autopsy normal, suspect anesthetic death.
[d] Euthanized because of severe weight loss. Autopsy findings: emaciated, pneumonia, colitis.
[e] Euthanized. Autopsy findings: thymic atrophy, amyloidosis.

according to the manufacturer's recommendations. When detected, endotoxin was removed using polymyxin affinity column chromatography (Bio-Rad, Hercules, Calif.). Antibody used for the passive transfer experiments contained <1 IU of endotoxin/ml. The control IgG was purified from sera from normal human donors, and endotoxin levels were also below 1 IU/ml.

Vaginal antibody measurements. The accurate determination of the actual concentrations of antibody in mucosal secretions was performed as described by Kozlowski et al. (16). Vaginal secretions were absorbed to cellulose wicks (1 by 15 mm) (Solan Weck-Cel surgical spears; Xomed Surgical Products, Jacksonville, Fla.). Prior to sample collection, each wick was placed in a sterile test tube and weighed. After insertion of a vaginal speculum, wicks were grasped with a forceps and gently placed into the posterior vaginal fornix. After 5 min, wicks were collected, returned to the test tube, weighed again, and frozen at −70°C. The weight of the absorbed vaginal fluid was calculated from the weight increase, assuming that the density of vaginal fluid is similar to that of water. This volume was used to determine the dilution factor for each sample in the following extraction procedure. The vaginal fluid was extracted from the wicks by adding 200 μl of PBS containing 1% fetal bovine serum and a protease inhibitor cocktail [200 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 160 μM aprotonin, 10 μM bestatin, 3 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, 4 μM leupeptin, 2 μM pepstatin A (Calbiochem, La Jolla, Calif.)]. After incubation on ice for 30 min, the diluted vaginal fluid was collected by spinning it through a 0.45-μm-pore-size filter (Spin-X; Corning, Corning, N.Y.) for 10 min at 14,000 × *g* at 4°C. The clarified supernatant was then tested for antibody content, using an ELISA. Actual antibody concentrations in vaginal fluids were calculated by multiplying by the dilution factor as determined above. Relatively small amounts of vaginal fluid were collected (typically 50 to 200 μl). The samples were taken at 6 h, 7 days, and 14 days post-b12 infusion.

Antibody assays by ELISA. We used two different ELISAs to determine b12 concentrations in serum and vaginal fluid. First we used a gp120 ELISA. Recombinant $gp120_{JR\text{-}FL}$ (kindly provided by Paul Maddon, Progenics Pharmaceuticals, Tarrytown, N.Y.) was coated to the wells of a microtiter plate (Corning) at a concentration of 2 μg/ml by incubation overnight at 4°C. The plates were washed four times with PBS–0.05% Tween 20 and blocked with 3% bovine serum albumin. Following washing, serial dilutions of serum or vaginal fluid samples were applied to the plate and incubated for 2 h at 37°C. A b12 antibody standard curve was run on each plate. After washing, goat anti-human IgG $F(ab')_2$ fragments coupled to alkaline phosphatase (Pierce, Rockville, Ill.) were added and incubated for 1 h at 37°C. The plates were washed, and bound conjugate was detected with *p*-nitrophenyl phosphate substrate (Sigma).

The second assay was an ELISA based on the B2.1 peptide. B2.1 is a homodimer of the peptide HERSYMFSDLENRCI-(biotinylated Orn)-KK (dimer molecular weight, 5,572.2; >95% pure and >90% dimer; synthesized by AnaSpec, San Jose, Calif.). This peptide, isolated by a random peptide library approach, binds the b12 antigen binding site with high specificity as described in detail elsewhere (43). B2.1 was coated to the wells of a microtiter plate at a concentration of 5 μg/ml by incubation overnight at 4°C. After four washes with PBS–0.05% Tween-20, the plates were blocked with 3% bovine serum albumin. Serial dilutions of serum or vaginal fluid samples were then applied to the plate and incubated for 4 h at 4°C. After washing, goat anti-human IgG $F(ab')_2$ fragments coupled to alkaline phosphatase (Pierce) were added and incubated for 1 h at room temperature before detection of bound antibody as described above.

RESULTS

Infection of rhesus macaques by $SHIV_{162P4}$ by the vaginal route. $SHIV_{162P4}$ is an HIV/SIV chimera containing the *env*, *tat*, *rev,* and *vpu* genes from the prototypical R5 primary isolate $HIV\text{-}1_{SF162}$ in the context of the molecular clone of SIV_{mac239} (13, 40). Pathogenic variants of $SHIV_{162P}$ were isolated by inoculation of the molecular clone into two rhesus macaques followed by three sequential blood-bone marrow transfusions. The virus used in this study was obtained after the third passage and is designated $SHIV_{162P4}$. This virus retains the R5 phenotype of the parental $HIV\text{-}1_{SF162}$.

To establish the dose of $SHIV_{162P4}$ required for infecting female rhesus macaques by the vaginal route, we titrated the virus in a challenge experiment. Three groups of three monkeys were pretreated with medroxyprogesterone acetate and challenged 30 days later with 600, 300, or 100 $TCID_{50}$s of $SHIV_{162P4}$. All animals became infected and had detectable plasma viremias two weeks after challenge (Table 1). Moreover, lymph node biopsies from all animals on day 18 tested positive for p27 antigen in a coculture assay (data not shown). All animals experienced high viral loads ranging from 5.1 to 7.1 logs between 2 and 4 weeks postexposure, which in three animals decreased to undetectable levels (<400 RNA copies per ml) at 2 to 3 months postexposure but which were sustained in others. There was no correlation between the challenge dose and the maintenance of an elevated viral load, which appears to be a phenomenon that is inherently variable from animal to animal. Three animals with high viral loads (one from each group) died on days 117, 174, and 439, respectively. Each of these animals had pathological lesions consistent with simian AIDS (Table 1). A fourth animal with an undetectable viral load died after 434 days of an unrelated cause and without pathological abnormalities. In the following experiments we used a $TCID_{50}$ dose of 300 to ensure that the challenge was robust and that infection was guaranteed in the absence of antibody intervention.

Protection against $SHIV_{162P4}$ vaginal challenge by IgG1 b12. Previous experiments, discussed above, have generally found

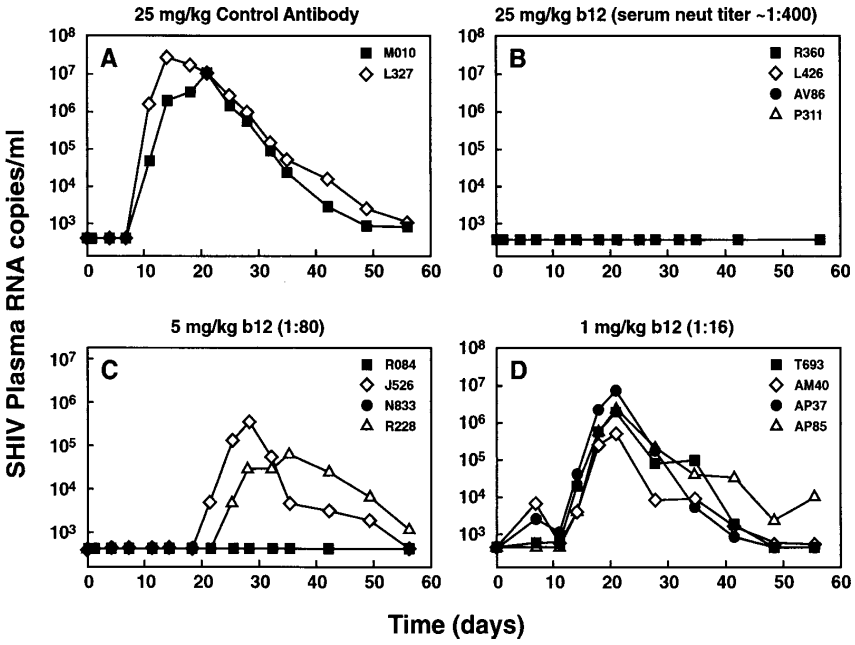

FIG. 1. Temporal analysis of $SHIV_{162P4}$ plasma viremia in macaques pretreated with different doses of antibody b12. Plasma viral loads are shown for animals treated with 25 mg of control antibody/kg (A), 25 mg of b12/kg (B), 5 mg of b12/kg (C), and 1 mg of b12/kg (D) following vaginal challenge with $SHIV_{162P4}$. Each curve depicts an individual animal. The curves for R360, L426, AV86, and P311 are coincident. The curves for R084 and N833 are coincident. Neut, neutralizing.

that relatively high titers of neutralizing antibody in serum are required to protect animals against HIV-1 challenge. With these data in mind, we designed a study to determine the titers of neutralizing antibody required to protect rhesus macaques from vaginal challenge with an R5 virus.

IgG1 b12 is a broadly neutralizing antibody directed against an epitope overlapping the CD4 binding site on gp120. The primary isolate $HIV\text{-}1_{SF162}$ is neutralized (concentration at which 90% of the organisms are inhibited [IC_{90}]) by b12 at a concentration of 2 μg/ml in PHA-activated human PBMC-based neutralization assays. $SHIV_{162P4}$, derived from $HIV\text{-}1_{SF162}$, was neutralized by 90% at antibody concentrations of 6 and 2 μg/ml in assays using PHA-activated PBMC from humans and rhesus macaques, respectively. This indicates that cloning of the SF162 envelope into the SIV genetic background and in vivo passage of the virus to create $SHIV_{162P4}$ did not significantly change the neutralization sensitivity to b12. Based on knowledge of the in vitro neutralization sensitivity of $SHIV_{162P4}$ to b12 and what was learned from previous passive immunization studies, we hypothesized that complete protection of a macaque would require a concentration of b12 in serum in excess of 200 μg/ml. We therefore administered IgG1 b12 intravenously to groups of four monkeys in doses of 25, 5, and 1 mg/kg. A control group of two monkeys received a 25-mg/kg dose of normal human polyclonal IgG that lacked any antiviral activity in in vitro neutralization assays. The monkeys were challenged 6 h later with 300 $TCID_{50}$s of $SHIV_{162P4}$.

The two monkeys pretreated with the control antibody developed high levels of viremia starting on day 11, peaking at 1×10^7 to 2×10^7 RNA copies per ml of plasma between days 14 and 21 (Fig. 1), a profile very similar to that of the

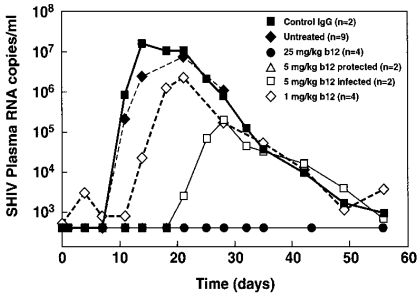

FIG. 2. Comparison of averaged plasma viral loads for macaques in different treatment groups. The mean viral loads were calculated for all the animals in each of the groups described in the legend to Fig. 1. In addition, we calculated the mean viral load of the nine untreated animals from the challenge virus titration experiment. Although the viral challenge dose in this experiment varied, calculating the mean was warranted since the onset as well as the peak viremias fell into a relatively narrow range (Table 1). The 5-mg/kg group was split into two subgroups corresponding to the two completely protected monkeys (curve is coincident with the 25-mg/kg curve) and the two monkeys in which viremia was delayed.

TABLE 2. Antibody concentration and neutralization titers achieved in plasma and vaginal fluids[a]

Antibody	Dose (mg/kg)	Animal ID	b12 concn in plasma at time of challenge (μg/ml)		Neutralization titer in plasma at time of challenge (IC_{90})[b]	b12 concn in vaginal fluid at time of challenge (μg/ml)	
			gp120 ELISA	B2.1 ELISA		gp120 ELISA	B2.1 ELISA
b12	25	R360	710	840	1:400	30	13.5
		L426	725	600	1:400	20	25
		AV86	695	910	1:400	18.6	27.9
		P311	690	940	1:400	17.4	21.8
b12	5	R084	180	210	1:40	1.0	1.5
		J526	175	230	1:80	3.1	2.5
		N833	170	360	1:80	3.2	7.8
		R228	170	290	1:80	22.2	37
b12	1	T693	12	12	1:16	ND	ND
		AM40	20	20	1:16	ND	ND
		AP37	15	15	1:16	ND	ND
		AP85	15	18	1:32	ND	ND
Control IgG	25	M010	0	0	<1:8	0	0
		L327	0	0	≤1:8	0	0

[a] Neutralization of $SHIV_{162P4}$ by b12 (IC_{90}) was achieved at a b12 concentration of 2 μg/ml. ND, not done.
[b] All neutralization assays were performed with $SHIV_{162P4}$ challenge virus in PHA-activated rhesus PBMC and determination of p27 antigen by ELISA.

$SHIV_{162P4}$-challenged monkeys in the virus titration experiment described above (Table 1; Fig. 2). In contrast, SHIV plasma RNA remained undetectable in all four monkeys pretreated with b12 at 25 mg/kg and also in two of the four monkeys pretreated with 5 mg of b12/kg. However, two of the four monkeys in the 5-mg/kg group did become infected, although the viral loads in these animals were strongly reduced compared to those for control animals and the peak viremia was delayed (Fig. 2). The monkeys pretreated with the lowest amount of b12 (1 mg/kg) all became infected with viral loads that were of similar magnitude to those of the control monkeys yet appeared slightly delayed in their onset (Fig. 2).

Coculture assays with PHA-activated rhesus PBMC were performed with PBMC from all monkeys. Results of the assays were in agreement with the plasma viral load data described above. All the cocultures with PBMC from the monkeys that developed detectable plasma RNA viral loads were positive for p27 antigen on day 14, except for the culture involving cells from animal R228 from the 5-mg/kg b12 group; this animal was the slowest to develop plasma viremia postinfection. PBMC cocultures for this monkey tested positive at a later time point (32 days postinfection). Cocultures with PBMC from the monkeys with undetectable viral loads in contrast remained negative for p27 antigen in ELISA during a 1-month culture period (data not shown). Finally, we tested the sera from the protected monkeys for anti-p27 antibody in ELISA. All four monkeys from the 25-mg/kg group and the two protected monkeys from the 5-mg/kg group tested negative for p27 antibody on day 42 and day 84 postinfection (data not shown). The absence of detectable SHIV RNA in plasma, p27 antigen in PBMC coculture assays, and anti-p27 in serum indicate a sterile protection in these monkeys.

Antibody and serum neutralization titers for b12-treated monkeys. To verify the b12 concentrations and neutralization titers achieved at the time of challenge, we determined b12 concentrations by ELISA and performed rhesus PBMC-based neutralization assays with the $SHIV_{162P4}$ challenge stock (Table 2). ELISAs were performed using recombinant $gp120_{JR\text{-}FL}$ and the peptide B2.1. The B2.1 peptide is a mimotope derived from a random-peptide library and is a highly specific probe for IgG1 b12 (43). The IgG1 b12 concentrations determined with both the gp120 and B2.1 ELISAs are in good agreement with each other and the neutralization data. The 90% neutralization titers achieved in the three groups of animals were approximately 1:400, 1:80, and 1:16 for the 25-, 5-, and 1-mg/kg doses, respectively (Table 2). The half-life of IgG1 b12 in plasma was about 1 week (data not shown).

Concentrations of antibody in vaginal fluid. Concentration of IgG1 b12 in vaginal fluid were determined by gp120 and B2.1 ELISAs as shown in Table 2. The values shown are actual IgG1 b12 concentrations as we corrected for the dilution factor during sample preparation. The concentrations of IgG1 b12 in the vaginal fluids at the time of challenge of the animals treated with the two highest doses of b12 are 25- to 100-fold lower than the concentrations achieved in the plasma and range from 1 to 10 times the b12 IC_{90} against the challenge virus. Notably, a neutralization titer of about 1:10 in vaginal fluid in combination with a plasma neutralization titer of 1:80 may be insufficient for providing sterile immunity as shown by the infection of animal R228.

It is of interest that b12 concentrations in the vaginal fluid did not peak immediately postinfusion, as approximately 2- and 1.5-fold higher (mean) b12 concentrations were detected 7 and 14 days later. In contrast, b12 plasma concentrations, as expected, peaked immediately after (intravenous) administration (not shown). If vaginal antibody levels contribute to protection, then our studies may therefore slightly underestimate the protective effect corresponding to a given titer of neutralizing antibody in serum.

Absence of neutralization escape in the infected animals in the 5-mg/kg b12 treatment group. Among the monkeys treated with 5 mg of b12/kg, two were completely protected whereas two others became infected with a strongly delayed and reduced plasma viremia. It is formally possible that these break-

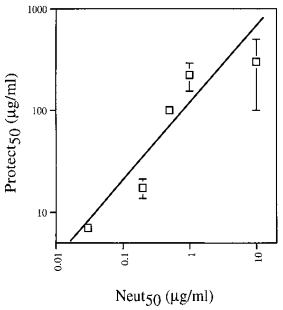

FIG. 3. The concentration of b12 resulting in half-maximal protection ($Protect_{50}$) in animals as a function of the concentration resulting in half-maximal neutralization ($Neut_{50}$) in vitro. The concentrations of b12 required to neutralize 50% of the challenge virus in vitro and completely protect 50% of animals against infection with the same virus were derived from this study and previous studies using hu-PBL-SCID mice (10, 29). The data points shown (left to right) correspond to HIV-1_{LAI} (hu-PBL-SCID), HIV-1_{SF2} (hu-PBL-SCID), HIV-1_{JR-CSF} (hu-PBL-SCID), $SHIV_{162P4}$ (macaques), and HIV-1_{AD6} (hu-PBL-SCID). For experiments in which half-maximal protection was not determined exactly, an error bar is shown with a range indicating the lower and upper limits of the antibody concentration required.

through infections occurred through the presence of b12 neutralization-resistant SHIV variants in the challenge stock or the generation of neutralization escape variants in vivo. Accordingly, virus was rescued and expanded by PBMC coculture from one infected monkey treated with 5 mg of b12/kg (R228) and one infected monkey from the control antibody group (M010). The PBMC used were isolated from blood drawn at the peak of viremia, i.e., day 14 for M010 and day 32 for R228. Neutralization assays demonstrated that sensitivities of both the M010- and the R228-rescued SHIV to neutralization by b12 remained equal to that of the challenge virus $SHIV_{162P4}$ (data not shown).

DISCUSSION

The principal goal of this study was to evaluate the ability of neutralizing antibody to protect against mucosal challenge by an R5 HIV-1. Using vaginal challenge in an SHIV/macaque model, the results strongly suggest that neutralizing antibody titers in the serum correlate with protection against HIV-1 infection via mucosal exposure. At serum neutralizing (90%) antibody titers on the order of 1:400, the human monoclonal antibody b12 provided sterile protection against vaginal challenge with the R5 chimeric virus $SHIV_{162P4}$. At titers of 1:80, 50% of the animals were protected, whereas at titers of 1:16, all the animals became infected. Thus, sterile protection corresponds to antibody concentrations in serum that neutralize essentially all the virus in corresponding in vitro neutralization assays. This observation, initially made with the hu-PBL-SCID mouse model (10, 30), has now been made for b12 protection against HIV-1 in a number of systems, including different animal models, challenge viruses (including both primary and laboratory-adapted viruses), and routes of challenge. In Fig. 3, the relationship between b12 neutralization in vitro (antibody concentration at half-maximal neutralization) and protection in vivo (antibody serum concentration at half-maximal protection) is plotted. The linearity of the curve, with a slope of approximately 200, illustrates the predictive value of the neutralization titer for protection. The relatively high value of the slope indicates the stringent requirement for protection, i.e., antibody concentrations required for protection are 2 orders of magnitude greater than those required for neutralization. The graph therefore emphasizes a strong correlation between neutralization and protection independent of the animal model, challenge route, or HIV-1 isolate used.

Passive transfer studies using other neutralizing anti-HIV antibodies have generally conformed to our observations with b12 (1, 24, 26, 38). Mascola et al. (26), however, did report that the antibody 2G12 and combinations of this antibody with the antibody 2F5 and polyclonal immune IgG (HIVIG) were somewhat more effective in sterile protection against mucosal challenge with the chimeric virus $SHIV_{89.6PD}$ than against intravenous challenge. In particular, those studies showed a surprising efficacy of the antibody 2G12 in mucosal protection. The reasons for this efficacy are unclear, but understanding it is clearly important.

Generally, examination of the literature on passive transfer to naive animals for a wide variety of different viruses and model systems shows that titers of neutralizing antibody in serum around or greater than 1:100 are required for sterile protection (28). For instance, serum neutralizing antibody titers of 1:380 are required to sterilely protect cotton rats against respiratory syncytial virus challenge (33). Therefore, HIV-1 does not seem to be unusual in this regard, and indeed one could anticipate that in naive animals in the absence of other protective immunity, such high neutralizing antibody titers would be required. One distinguishing feature of HIV-1 is that protective neutralizing antibody titers using monoclonal antibodies correspond to relatively high antibody concentrations. This is because the anti-HIV monoclonal antibodies available are relatively modest in their neutralizing potency compared to those against some other viruses. Primary isolate neutralizing antibodies, such as b12, 2G12, and 2F5, typically show 90% neutralization at concentrations of 1 to 10 μg/ml, so that complete neutralization, which may require 100 times 90% neutralization values, corresponds to 100 μg/ml to 1 mg/ml. In contrast, neutralizing monoclonal antibodies against other viruses can show 90% neutralization at 0.1 μg/ml, corresponding to only about 10 μg/ml for complete protection (28). Since neutralization is correlated with affinity for the HIV-1 envelope trimer, at least for T-cell-line-adapted viruses (31, 34, 36), the modest neutralizing activity of anti-HIV-1 antibodies likely reflects a modest affinity for the trimer. In turn this may reflect the fact that the primary isolate-neutralizing anti-HIV-1 antibodies are cross-reactive and able to recognize a variety of closely related molecular shapes (i.e., envelope epitopes from a range of HIV-1 isolates) rather than a single uniquely defined molecular shape.

The vaginal model employed in the earlier studies of Mascola et al. (26) was very similar to the one employed here. A caveat of the model may be that a progesterone analog was used to thin the vaginal epithelium. HIV-1 and SIV infection can occur by direct transmission through the vaginal mucosa (14, 23, 27). However, the intact vaginal mucosa represents a formidable barrier against infection, since 100- to 1,000-fold-

greater doses of SIV_{mac251} are required to establish infection compared to intravenous injection of virus (27, 39). The incidence of vaginal transmission of SIV can be enhanced by progesterone treatment, which produces a thinning of the vaginal epithelium (23). The increased infection rates after progesterone treatment then allow challenge of animals with a viral inoculum which is comparable to the dose of virus likely to be present in seminal fluid of HIV-1-infected individuals (10 to 1,000 $TCID_{50}$ per ejaculate) (6, 42). However, the vaginal thinning may create conditions that are significantly different from those present during human transmission. For example, it has been pointed out that thinning may allow greater transudation of IgG into the vaginal fluid than normal (35) and that the hormonal treatment itself may influence the amount of antibody in the vaginal secretion (17–19, 21, 37). If protection were mediated by vaginal IgG this could lead to an overestimation of the efficacy of antibody under normal conditions. However, the concordance of the results reported here with those of intravenous challenge experiments using HIV-1 and of studies with other viruses suggests that such an overestimation is probably not occurring.

The early events involved in transmission of HIV-1 across mucosal surfaces are still a subject of great uncertainty. Dendritic cells may be among the first cells infected after virus penetration of the mucosal epithelium (14, 25). Interestingly, it has been shown previously that neutralizing antibodies can block the entry of HIV-1 into dendritic cells and, in addition, effectively interrupt the transmission of HIV-1 from dendritic cells to T cells (9). This may be crucial, although again we note that the data are consistent with protection occurring by neutralization of free virus by systemic antibody.

The major focus of our study was the ability of antibody to provide sterile protection from viral challenge. However, antibody may provide benefits at doses below those required for sterile protection. In the study of Mascola et al. (26) on $SHIV_{89.6PD}$ vaginal challenge, such doses produced lower viremias and a lesser depletion of CD4 cells. In this study, we also noted that an intermediate dose produced a lower and delayed viremia. At the lowest dose, however, which nevertheless produced a neutralizing titer in serum of about 1:16, viremia was approximately equal in magnitude and only slightly delayed relative to results with control animals. We have not investigated the longer-term effects of antibody on the potential disease course in this study.

In $SHIV_{89.6PD}$ infection, CD4 depletion is extremely rapid and pronounced in most animals; in some respects the infection is more aptly described as acute than persistent. In $SHIV_{162P4}$ infection, on the other hand, CD4 depletion is observed most prominently in intestinal lymphocytes and is not well observed in circulating T cells (13). Disease can take a long time to develop, as illustrated in the cohort of animals used in the virus titration arm of this study. It will be interesting to see if any of the animals that were treated with 5 mg of b12/kg but became infected subsequently develop disease.

Finally, in a vaccinated individual, the immune system can call upon specific T and B cells as well as preexisting antibody to combat a pathogen. With a murine retrovirus infection, it has been shown that cellular and humoral responses in combination are required for viral clearance (7). Passive transfer studies have shown that antibody alone can protect against HIV-1/SHIV challenge but at levels that are probably not attainable by vaccination. A major question now, in our opinion, is whether antibody at lower levels can act in concert with cellular responses to prevent HIV-1 infection.

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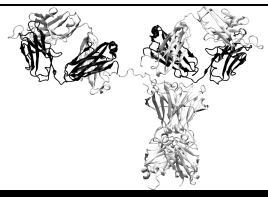
Chapter 4



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The human antibody immunoglobulin G1 (IgG1) b12 neutralizes a broad range of human immunodeficiency virus-type 1 (HIV-1) isolates in vitro and is able to protect against viral challenge in animal models. Neutralization of free virus, which is an antiviral activity of antibody that generally does not require the antibody Fc fragment, likely plays an important role in the protection observed. The role of Fc-mediated effector functions, which may reduce infection by inducing phagocytosis and lysis of virions and infected cells, however, is less clear. To investigate this role, we constructed a panel of IgG1 b12 mutants with point mutations in the second domain of the antibody heavy chain constant region (CH2). These mutations, as expected, did not affect gp120 binding or HIV-1 neutralization. IgG1 b12 mediated strong antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) of HIV-1-infected cells, but these activities were reduced or abrogated for the antibody mutants. Two mutants were of particular interest. K322A showed a twofold reduction in FcγR binding affinity and ADCC, while C1q binding and CDC were abolished. A double mutant (L234A, L235A) did not bind either FcγR or C1q, and both ADCC and CDC functions were abolished. In this study, we confirmed that K322 forms part of the C1q binding site in human IgG1 and plays an important role in the molecular interactions leading to complement activation. Less expectedly, we demonstrate that the lower hinge region in human IgG1 has a strong modulating effect on C1q binding and CDC. The b12 mutants K322A and L234A, L235A are useful tools for dissecting the in vivo roles of ADCC and CDC in the anti-HIV-1 activity of neutralizing antibodies.

The broadly neutralizing antibody immunoglobulin G1 (IgG1) b12, directed to an epitope overlapping the CD4 binding site of gp120, was originally isolated from a human immunodeficiency virus type 1 (HIV-1)-infected individual by means of phage-display library cloning (11). This antibody neutralizes T-cell-line-adapted (TCLA) viruses and a broad range of primary viruses in various in vitro assays (13, 30, 41, 52). Several studies demonstrated that IgG1 b12 completely protects severe combined immunodeficiency (SCID) mice populated with human peripheral blood lymphocytes (hu-PBL-SCID mice) from infection with both TCLA and primary viruses (21, 41). The protection against primary viruses is apparent even if the antibody is given several hours after viral challenge (21). Furthermore, IgG1 b12 also protected against vaginal challenge with a pathogenic R5 SHIV (simian immunodeficiency virus [SIV]/HIV chimera expressing HIV-1 envelope) in rhesus macaques (43). In addition to IgG1 b12, a second broadly neutralizing monoclonal antibody (MAb) to gp120 (2G12 [53]) and three broadly neutralizing MAbs to gp41 (2F5, Z13, and 4E10 [9, 39, 60]) have been described. Recent studies demonstrated that 2G12 and 2F5, alone or in combination with one another, can protect against intravenous and/or mucosal SHIV challenge in macaques (3, 35, 36). Sterile protection typically requires that high antibody serum concentrations be achieved (e.g., in vitro neutralization titers of 1:100 or greater) (40, 42, 43), although some exceptions have been noted. In vaginal challenge studies with $SHIV_{89.6PD}$ in rhesus macaques, for example, MAb 2G12 protected at antibody serum concentrations close to the 90% virus neutralization titer (36).

The antiviral activity of antibodies can be mediated by the neutralization of free virions or by binding to virus-specific proteins expressed on the surface of infected cells and the recruitment of Fc-mediated effector function (40).The importance of Fc-mediated effector function in protection against HIV-1 infection, however, is unclear. In a recent study, Binley and colleagues infused serum immunoglobulins purified from SIV_{mac251}-infected macaques (SIVIG) into other SIV_{mac251}-infected macaques and measured the impact on plasma viremia of infected animals. The effects on viral load observed were very modest and transient, with kinetics which seemed inconsistent with the neutralization of free viruses as the mechanism driving the effect, and a role of Fc-mediated effector mechanisms was therefore suggested. An experiment using SIVIG $F(ab')_2$ fragments to address this hypothesis, however, was inconclusive (7).

Fc receptors expressed on human peripheral blood cells play an important role in stimulating a variety of cytotoxic, phagocytic, and inflammatory functions. Once a virus-infected cell is opsonized by IgG, it may cross-link FcγR on the cell surface of an effector cell and mediate a cytotoxic response (antibody-dependent cellular cytotoxicity [ADCC]). Arrays of antibody

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Fc's presented on the surface of an infected cell may also activate the classical pathway of complement activation ultimately leading to cell lysis (complement-dependent cytotoxicity [CDC]).

Multiple sites on IgG have been proposed to interact with FcγR. Mutagenesis studies have shown that the lower hinge region (234-LLGGPS-239) of IgG plays an important role in the binding of IgG Fc receptors (14, 19, 28, 33, 34, 38, 47, 58, 59). High-affinity binding to FcγRI is most notably affected by mutation of L235. Mouse (m)IgG2b, which does not bind FcγRI, has a glutamic acid at this position, and substitution of this residue by leucine was shown to restore the binding affinity of mIgG2b to be comparable to that of hIgG1 (19). The binding affinity of IgG for FcγRII, in contrast, seems more sensitive to mutations of L234 than L235, indicating that the FcγR interaction sites are overlapping but not identical (33). Residues in the lower part of the hinge region itself and the lower CH2 domain in addition may have a modulating effect on FcγR affinity (15, 33).

Complement activation via the classical pathway is activated through binding of C1q to the Fc domain of IgG or IgM, complexed with antigens (12, 25). Duncan and Winter (18) showed alanine substitutions in mIgG2b at positions E318, K320, K322, and N297, the last leading to the removal of carbohydrate, resulting in mutants in which binding to human C1q was strongly reduced compared to the wild type and the ability to mediate CDC was abrogated. Since E318, K320, and K322 are conserved residues in human IgG and IgG of several other species, they were designated the binding site for C1q (18). However, this binding motif has been conserved in all four human IgG subclasses which, in apparent conflict, exhibit large differences in their C1q binding abilities. Several studies have indeed implicated additional residues in C1q binding and have suggested that the binding sites for C1q on mIgG2b and hIgG are not completely identical (8, 27, 38, 51, 59). In a recent study on rituxan, a chimeric MAb with hIgG1 constant domains used in the therapy of non-Hodgkin's B-cell lymphomas, Idusogie and colleagues demonstrated that alanine substitution at positions D270, K322, P329, and P331 but not at positions E318 and K320 significantly reduced the ability of the chimeric MAb to bind C1q and activate complement, suggesting that E318 and K320 are only of minor importance for complement activation by hIgG1 (27). Some studies have furthermore suggested that C1q binding and complement activation may be modulated by residues in the lower hinge region (38). Thus, there are species differences in C1q binding. Finally, it has been shown that C1q binding alone is not sufficient for complement activation and complement-mediated cell lysis (51). Intrinsic factors, such as segmental flexibility of the hinge region (10, 50) but also extrinsic factors such as antigen specificity and density (5, 6), may play an important modulating role.

In the present study, we introduced point mutations in the heavy chain constant domain of IgG1 b12. Amino acid mutations were chosen on the basis of the studies discussed above. However, because of subtle species differences in C1q and FcγR binding and the possible modulation of ADCC and CDC by antigen specificity and density, the impact of these mutations on the biological activity of IgG against HIV-1-infected cells was not immediately clear. The mutants were compared for their ability to bind FcγR and C1q and to mediate ADCC and CDC. These mutants can now be used to examine the role of Fc-mediated effector function in protection against HIV-1 infection in vivo.

MATERIALS AND METHODS

Cell lines, viruses, and MAbs. Uninfected CEM-NKr and CEM-NKr cells chronically infected with HIV-1_{MN} were obtained from Shermaine Tilley (Public Health Research Institute, New York, N.Y.) (1). To obtain clones of the infected CEM-NKr cells with an increased level of envelope expression, we performed a limiting dilution and characterized for Env expression by flow cytometry. A clone with a higher level of Env expression (75% of cells expressing Env) was selected for our experiments. Adult human elutriated monocytes were obtained from Advanced Biotechnologies, Columbia, Md. HIV-1 primary isolates HIV-$1_{JR\text{-}CSF}$, HIV-$1_{JR\text{-}FL}$ (contributed by Irvin Chen) (31), and HIV-$1_{89.6}$ (contributed by Ronald Collman) (16) were obtained from the National Institutes of Health (NIH) AIDS Research and Reagent Reference Program (ARRRP). IgG1-CLB was a purified paraprotein obtained from the CLB, Amsterdam, The Netherlands. Anti-FcγRI MAb 10.1 was provided by Nancy Hogg (Leukocyte Adhesion Laboratory, London, United Kingdom). Fab 10.1 was prepared by papain digestion. Fab fragments of anti-FcγRII MAb IV.3 and $F(ab')_2$ fragments of anti-FcγRIII MAb 3G8 were provided by Medarex (Anandale, N.J.). Humanized OKT3 (IgG1 and IgG4) antibody was provided by Robert A. Zivin (R.W. Johnson Pharmaceutical Research Institute, Raritan, N.J.).

Mutagenesis of heavy-chain constant domain. Mutatagenesis was performed on the heavy-chain constant region derived from the IgG1 b12-expression plasmid pDR12 (see below) (13). A *Sac*I-*Sal*I endonuclease restriction fragment from pDR12, containing the CH2 fragment, was subcloned into M13 mp18. Site-directed mutagenesis (32) was performed using the Muta-gene M13 in vitro mutagenesis kit (Bio-Rad, Hercules, Calif.). Five clones encoding the desired changes (K322A, L234A, L235E, G237A, and L234A, L235A) were identified by automated DNA sequencing. The mutated *Sac*I-*Sal*I fragments were then cloned back into in the pDR12 expression vector.

Expression and purification of antibodies. Recombinant antibody was expressed in the vector pDR12 (provided by Raju Koduri and Dean Sauer). It contains a b12 light chain and heavy-chain expression cassette in which transcription is driven from a human cytomegalovirus promoter. The heavy-chain expression cassette contains the genomic human IgG1 gene. Selection and amplification of the plasmid was done on the basis of expression of the gene for glutamine synthetase (4).

IgG1 b12 mutant DNAs, prepared as described above, were cut with *Sal*I and transfected into Chinese hamster ovary cells (CHO-K1 cells; American Type Culture Collection, Manassas, Va.) using lipofectin reagent per the manufacturer's recommendations (Life Technologies, Grand Island, N.Y.). Cells were distributed in six-well tissue culture plates, and clones were selected with L-methionine sulfoximine ranging in concentration from 40 to 100 μM (Sigma, St Louis, Mo.). Wells containing discrete colonies were assayed by enzyme-linked immunosorbent assay (ELISA) for antibody production. The highest producers were cloned by limiting dilution, expanded, and grown in 3-liter spinner flasks.

Recombinant IgG1 was expressed in CHO-K1 cells in glutamine-free Glasgow minimum essential medium (GMEM supplemented with 10% dialyzed fetal bovine serum [FBS]) (Tissue Culture Biologicals, Tulare, Calif.), MEM nonessential amino acids (Gibco-BRL, Grand Island, N.Y.), 1 mM MEM sodium pyruvate (Gibco-BRL), 500 μM L-glutamic acid, 500 μM L-asparagine, 30 μM adenosine, 30 μM guanosine, 30 μM cytidine, 30 μM uridine, 10 μM thymidine (Sigma), 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 50 μM L-methionine sulfoximine (Sigma) in a 3-liter spinner flask. The supernatants were sterile filtered and purified over protein A-Sepharose Fast Flow (Pharmacia, Arlington Heights, Ill.). The antibody was eluted in 0.1 M citric acid, pH 3.0. The pH of the antibody solution was immediately brought to neutrality by the addition of 1 M Tris (pH 9.0), and the antibody was dialyzed against phosphate-buffered saline (PBS). Antibody concentrations were determined by the absorbance at 280 nm and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibody yields using this method ranged from 5 to 25 mg/liter.

Recombinant gp120 ELISA. IgG1 b12 and the Fc mutants were tested for binding to gp120 in ELISA essentially as described previously (17). Briefly, recombinant monomeric gp$120_{JR\text{-}FL}$ (provided by Paul Maddon and Bill Olson, Progenics, Tarrytown, N.Y.) was coated to the wells of a microtiter plate by incubating overnight at 4°C. The plates were washed four times with PBS containing 0.05% (vol/vol) Tween 20 and blocked with 3% bovine serum albumin (BSA). The blocking solution was removed, and serial dilutions of the antibody set were added in duplicate (diluted in PBS–1% BSA–0.05% Tween 20) and

incubated for 1 h at 37°C. The wells were washed and incubated for 1 h at 37°C with alkaline phosphatase-labeled goat anti-human IgG $F(ab')_2$ fragments (Pierce, Rockford, Ill.) (1:500 dilution in PBS–1% BSA–0.05% Tween 20). The plates were washed and developed with nitrophenol substrate (Sigma), and the absorbance was read at 405 nm.

HIV-1 neutralization. Neutralization of HIV-1 primary isolates was assessed using a phytohemagglutinin (PHA)-activated peripheral blood mononuclear cell (PBMC)-based assay as described previously (60). PBMCs (from three CCR5 wild-type donors) were isolated and stimulated with PHA (5 μg/ml) (Sigma) for 48 h, followed by PHA and interleukin-2 (40 U/ml) (obtained from the ARRRP, contributed by Hoffman-La Roche) for 72 h in RPMI 1640 medium containing 10% heat-inactivated FBS, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine. The antibodies were diluted, and 50 μl per well was pipetted into round-bottom microtiter plates, after which an equal volume containing 100 50% tissue culture infective doses of HIV-1 stock was added. The antibody-virus mixture was incubated for 1 h at 37°C. Next, 100 μl of PHA-activated PBMCs (5×10^5/ml) was added to each well. After an overnight incubation, the cells were washed two times with tissue culture medium. On day 7, the cultures were collected and treated with 1% (vol/vol) Empigen (Calbiochem, La Jolla, Calif.). Triplicate samples were then tested for p24 content using an ELISA, as originally described by Moore et al. (37). In brief, sheep anti-p24 Ab D7320 (Aalo Bioreagents) was coated overnight on 96-well polystyrene enzyme immunoassay (EIA) plates (Costar) in 100 mM $NaHCO_3$, pH 8.5. The plates were washed in PBS, and p24 was captured from serial dilutions of the HIV-1-containing samples in PBS–0.1% Empigen. After a 3-h incubation, unbound p24 was washed away and bound p24 was detected with alkaline phosphatase-labeled antibody BC1071 (International Enzymes) diluted 1:3,000 in PBS containing 20% sheep serum and 2% nonfat dry milk. After a 1-h incubation, the plates were washed and developed with an AMPAK kit (Dako Diagnostics) as recommended by the manufacturer. Production of p24 antigen in the antibody-containing cultures was compared to p24 production in cultures without antibody run in the same assay, and the antibody concentrations resulting in a 90% reduction in p24 content were determined.

FcγR binding assays. FcγR binding assays were performed essentially as described by Parren et al. (45). Binding of antibody to FcγR was assessed using antibody monomers or dimers as indicated. Antibody dimers were prepared by incubating overnight with $F(ab')_2$ fragments of mouse anti-human κ-light chain MAb K35 in a molar ratio of 1:1, which resulted in stable tetrameric complexes as detailed by Huizinga et al. (26). FcγR-transfected cells (3×10^5 in 100 μl of PBS–1% BSA) were incubated with 25 μl of serial dilutions of antibody monomers or dimers for 45 min at 4°C, washed, and then incubated with fluorescein isothiocyanate-labeled $F(ab')_2$ fragments of goat anti-human IgG. After washing, cell-bound antibody was detected using flow cytometry. The assays were performed using IIA1.6 cells transfected with FcγRIA and γ-chain, or FcγRIIa (H131), and Jurkat cells transfected with FcγRIIIa (24, 45, 54, 55).

C1q ELISA. Antibody was diluted to 1.25 μg/ml in PBS and coated overnight at room temperature onto EIA ELISA plates (Costar, Corning, N.Y.). Plates were washed three times with PBS–0.05% Tween 20 and a titration of human C1q (Calbiochem) prepared in PTG (PBS–0.02% Tween–0.1% gelatin) was added. After a 4-h incubation at room temperature, the plates were washed four times with PBS–0.05% Tween 20. A mixture of goat anti-human C1q (Calbiochem) and rabbit anti-goat IgG alkaline phosphatase conjugate (Sigma), both diluted at 1/1,000 in PTG, was added, and the plates were incubated for 1 h at room temperature. The plates were washed four times and developed using nitrophenol substrate (Sigma). Absorbance was measured at 405 nm. All data are expressed as means of triplicates.

ADCC with TCLA HIV-1. ADCC was assessed in standard chromium release assays (56). Effector cells were either PBMCs or adult human elutriated monocytes, as noted in the text. The PBMCs were isolated by centrifugation over Histopaque-1077 (Sigma). Cells were washed in PBS and resuspended in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml) at a density of approximately 4×10^6 cells/ml and incubated overnight at 37°C prior to use as effector cells in ADCC. The following day, 10^6 target cells were labeled with $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, Ill.) for approximately 2 h in 50 μl of fetal calf serum (FCS) containing 10 mM HEPES. These target cells were either uninfected CEM-NKr cells or CEM-NKr cells chronically infected with $HIV\text{-}1_{MN}$. After labeling, target cells were washed four times with RPMI 1640–10 mM HEPES. In each well of a microtiter plate, 10^6 washed ^{51}Cr-labeled target cells were incubated with antibody for 30 min at 37°C in a total volume of 150 μl. Then, 10^6 PBMCs or 6×10^5 human elutriated monocytes (monocytes were thawed rapidly at 37°C and washed once with RPMI 1640 before addition to target cells) were added as a source of effector cells in 50 μl of assay medium, bringing the total volume to 200 μl. The plates were spun at 1,000 rpm for 5 min in a Beckman GS-6R centrifuge to pellet the cells prior to a 4-h incubation at 37°C. At the end of this incubation period, plates were spun another time as described above, 100 μl of supernatant was collected, and ^{51}Cr release was measured in a gamma counter (Packard, Meriden, Conn.). The percent specific lysis was calculated as follows: (experimental release − spontaneous release)/(total release − spontaneous release) × 100%. Total ^{51}Cr release was determined by substituting 50 μl of antibody for Empigen detergent (Calbiochem). All data are expressed as the means of triplicate determinations.

CDC assay. Target cells (CEM-NKr cells; uninfected or infected with $HIV\text{-}1_{MN}$) were labeled by $Na_2{}^{51}CrO4$ as described above. After three washes, the labeled cells were sensitized by adding wild-type or mutant IgG1 b12 to the cells at a final concentration of 10 μg/ml and were incubated at 4°C for 1 h. After three washes, 2×10^5 sensitized target cells were dispensed into 96-well U-bottom microtiter plates. Rabbit serum (Calbiochem) was used as a source of complement and was serially diluted with RPMI 1640. The latter was added to sensitized target cells at 100 μl/well, and the plates were incubated at 37°C for 1 h. At the end of this time, plates were spun and 100 μl of supernatant was used to measure the ^{51}Cr release as described above.

RESULTS

Antigen recognition and neutralization. Five IgG1 b12 mutants were constructed by introducing point mutations in the lower hinge (L234A, L235E, G237A, and double mutant L234A, L235A) and the N-terminal end of the CH2 domain (K322A). Although it was unlikely that these mutations would influence the affinity of the antibody for gp120, all five antibodies were tested for binding to recombinant $HIV\text{-}1_{JRFL}$ gp120 in ELISA. All mutants bound similarly to gp120 as expected (Fig. 1a). In addition, we tested all mutants in a PHA-activated PBMC-based neutralization assays with $HIV\text{-}1_{JR\text{-}FL}$ (Fig. 1b), $HIV\text{-}1_{JR\text{-}CSF}$, and $HIV\text{-}1_{89.6}$ (Table 1). All mutants neutralized these primary isolates similarly (Fig. 1b; Table 1).

Binding of wild-type and mutant IgG1 b12 to FcγRI, FcγRIIa, and FcγRIIIa. The ability of antibodies to mediate ADCC is dependent on the relative affinity of the antibody for FcγRI, -II, and -III. We first measured the ability of IgG1 b12 and the mutant antibodies described above to bind to FcγRI by flow cytometry using both monomeric and dimeric IgG1 (cross-linked with an anti-light chain MAb) (26). Binding of IgG by the high-affinity receptor FcγRI is usually studied using monomeric IgG. We, however, also included dimeric IgG which allowed us to also examine possible lower-affinity interactions. In these binding studies, we used FcγRIa- and γ-chain-transfected IIA1.6 cells (54). Binding of both dimeric and monomeric wild-type and mutant IgG1 b12 to IIA1.6 cells revealed that the mutation at position 234 reduced the affinity for FcγRI about fivefold, whereas the other two mutations in the lower hinge (L235E and G237A) reduced IgG1 b12 affinity for FcγRI about 40-fold. Combining the 234 and 235 mutations completely abolished FcγRI binding, and binding was reduced to undetectable levels even in the IgG dimer-binding assay. The FcγRI binding affinity of K322A was not affected. The rank order of FcγRI-binding by the IgG1 b12 variants therefore is as follows: b12 = K322A > L234A ≫ G237A, L235E, double-mutant L234A, L235A (Table 2).

Next, we tested the ability of our antibody panel to bind to the low-affinity receptors FcγRIIa and FcγRIIIa. The mutation in the CH2 domain (K322A) only slightly reduced the binding affinity to both FcγRIIa and FcγRIIIa compared to wild-type IgG1 b12 (Table 2). All mutations in the lower hinge region in contrast abolished binding to both FcγRIIa and FcγRIIIa (Table 2).

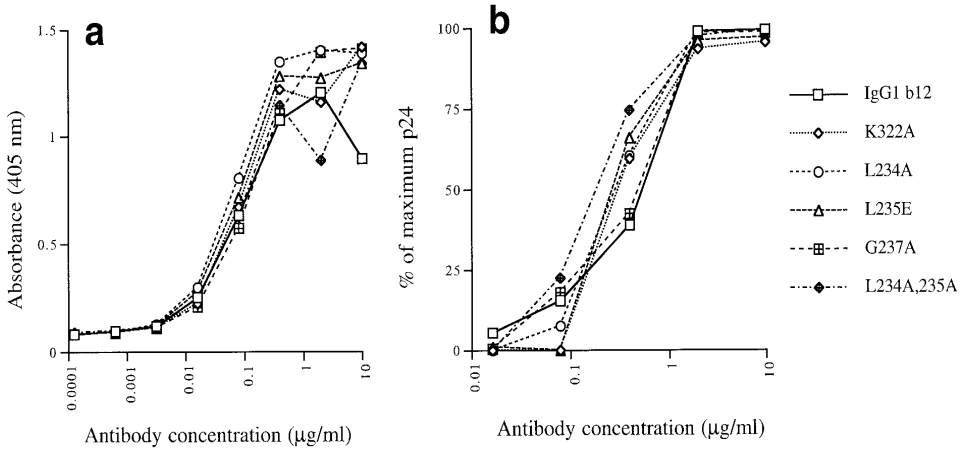

FIG. 1. Binding of wild-type and mutant IgG1 b12 to recombinant HIV-1_{JR-FL} gp120 in ELISA (a) and neutralization of HIV-1_{JR-FL} (b).

Therefore, the importance of L235 for FcγRI binding was confirmed, but binding could only be completely abrogated by introducing a double mutation at L234 and L235. In contrast to previous studies on hIgG3 (34), mutagenesis of L234 as well as L235 in hIgG1 abolished both FcγRII and FcγRIII binding.

ADCC. Antibody (IgG1 b12) bound to envelope expressed on the surface of HIV-1-infected cells may recruit effector cells, such as NK cells or monocytes, by interacting with specific Fc receptors and induce ADCC leading to lysis of the infected cells. In order to measure the ability of IgG1 b12 and the mutants described above to mediate ADCC of HIV-1-infected cells, we incubated serial dilutions of these antibodies with ^{51}Cr-labeled HIV_{MN}-infected CEM-Nkr cells in the presence of PBMCs and purified monocytes as effector cells. As shown in Fig. 2a and b, IgG1 b12 mediated specific cell lysis of HIV_{MN}-infected CEM-NKr cells in the presence of both human PBMCs and purified monocytes, whereas ADCC by mutant K322A was reduced. The ability of IgG1 b12 mutants L235E, L234A, G237A, and double mutant L234A, L235A to mediate ADCC of HIV-1_{MN}-infected CEM-NKr cells was strongly reduced with PBMCs and abolished with monocytes as effector cells (Fig. 2a and b). The positive control serum, FDA-2, is a potently neutralizing serum with a 90% HIV-1_{MN} neutralization titer of 1:4,000 (44); ADCC of IgG1 b12 on noninfected CEM-Nkr cells was used as a negative control.

To verify the studies with the IgG1 mutant antibodies, we examined the IgG1 b12-mediated ADCC of HIV_{MN}-infected CEM-NKr with PBMCs and monocytes in the presence and absence of anti-FcγR antibodies. $F(ab')_2$ fragments of an anti-FcγRIII antibody, 3G8, efficiently inhibited the PBMC-mediated ADCC of HIV_{MN}-infected CEM-NKr (Fig. 3a). No inhibition of ADCC was observed with Fab fragments of anti-FcγRI 10.1 or anti-FcγRII IV.3. Thus, ADCC of HIV-infected cells by PBMCs is mediated through FcγRIIIa. This is in agreement with the strong reduction of ADCC in mutants in which binding to FcγRIIIa was abolished (Fig. 2a). In addition, the ability of mutant K322E to mediate ADCC was reduced about twofold compared to IgG1 b12, which corresponds with its reduction of binding to FcγRIIIa (Fig. 2a; Table 2).

As shown in Fig. 3b, anti-FcγRI (Fab 10.1) but not anti-FcγRII (Fab IV.3) or anti-FcγRIII [$F(ab')_2$3G8], inhibited monocyte-mediated ADCC of HIV-1_{MN}-infected CEM-Nkr-MN cells. These results suggest that ADCC of HIV-infected cells by monocytes is mediated through FcγRI. A relatively small (fivefold) reduction in FcγRI binding affinity as observed for the mutant L234A is therefore sufficient to abrogate the monocyte-mediated ADCC of HIV-1-infected cells (Fig. 2b).

C1q binding and complement activation. The ability of b12 and the mutant antibodies to bind C1q and CDC of HIV-1-

TABLE 1. Neutralization of HIV-1 primary isolates by wild-type and mutant IgG1 b12

Antibody	90% Inhibitory concn (μg/ml)		
	HIV-1_{JR-FL}	HIV-1_{JR-CSF}	HIV-$1_{89.6}$
IgG1 b12	2	50	6.3
K322A	2	50	6.3
L234A	2	50	6.3
G237A	2	25	12.5
L235E	2	50	3.1
L234A, L235A	2	50	6.3

TABLE 2. Binding of IgG1 b12 and IgG1 b12 Fc mutants to FcγR

Antibodies	K_{50} (μg/ml)[a]			
	FcγRI (monomer binding)	FcγRI (dimer binding)	FcγRIIa	FcγRIIIa
b12	0.16	0.3	4.8	7
K322A	0.17	0.4	8.3	13
L234A	1.1	1.68	>50	>50
G237A	6	7.6	>50	>50
L235E	7	12	>50	>50
L234A, L235A	>50	>50	>50	>50
IgG1-CLB	0.12	0.2	1.8	10

[a] Data are expressed as the antibody concentration at which half maximal binding was achieved, K_{50} of >50 μg/ml indicate that this was not achieved.

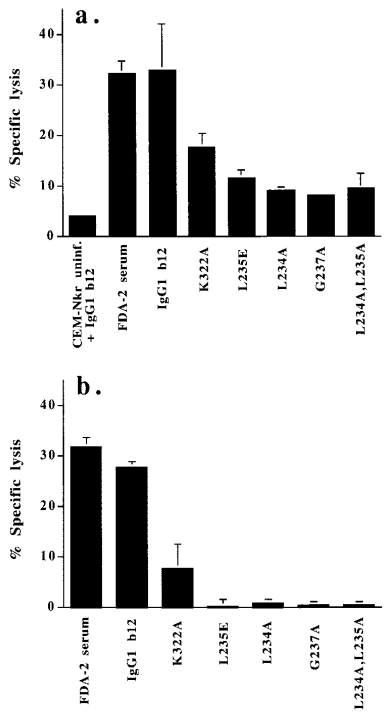

FIG. 2. ADCC of CEM-Nkr cells infected with HIV-1_{MN} by PBMC and purified human monocytes. Uninfected and HIV-1_{MN}-infected CEM-NKr cells were labeled with ^{51}Cr for 2 h at 37°C. The labeled target cells were incubated with wild-type or mutant IgG1 b12 before addition of cultured PBMCs (a) or purified human monocytes (b) as effector cells. Wild-type and mutant IgG1 b12 were used at 12.5 μg/ml. Serum from an HIV-1-seropositive patient (FDA-2 [44]) was used at a 1/4,000 dilution as a positive control. Uninfected CEM-NKr cells incubated with wild-type IgG1 b12 were included as a negative control. The assays were performed twice with similar results.

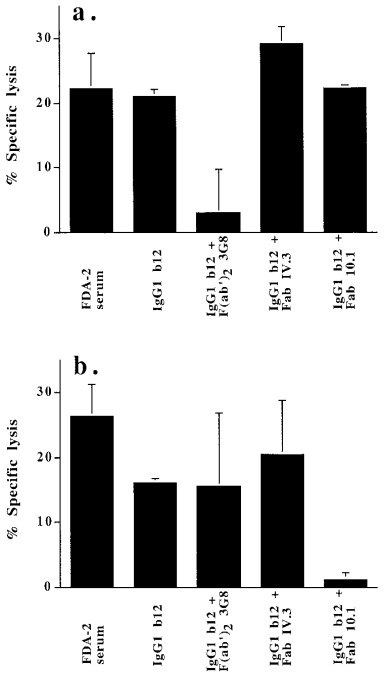

FIG. 3. Inhibition of ADCC by anti-FcγR antibodies. ADCC of HIV-1_{MN}-infected CEM-NKr cells by PBMC (a) or purified human monocytes (b) in the presence and absence of $F(ab')_2$ fragments of anti-FcγRIII MAb 3G8 (20 μg/ml), Fab fragments of anti-FcγRII MAb IV.3 (5 μg/ml), and Fab fragments of anti-FcγRI MAb 10.1 (20 μg/ml). IgG1 b12 was used at a concentration of 12.5 μg/ml. Serum from an HIV-1-seropositive patient (FDA-2 [44]) was used at a 1/4,000 dilution as a positive control. The assays were performed twice with similar results.

infected cells was investigated. As shown in Fig. 4a and b, wild-type IgG1b12 bound well to C1q and mediated a potent CDC of HIV-1_{MN}-infected CEM-Nkr cells in the presence of rabbit serum as a source for complement. The ability of the K322A mutant to bind C1q and mediate CDC was strongly reduced. All b12 variants with mutations in the lower hinge region (L235E, L234A, G237A and L234A, L235A) furthermore were reduced in their ability to mediate CDC of HIV-1-infected cells as well (Fig. 4a and b).

These results strongly suggest that for hIgG1, the residue at position 322 at the N-terminal end of the CH2 domain as well as residues in the lower hinge region are involved in C1q binding and consequent complement activation.

DISCUSSION

The role of effector function in the anti-HIV-1 activity of antibody is poorly understood. It has been shown in a number of passive antibody transfer studies that MAbs or polyclonal antisera capable of neutralizing the challenge virus can protect against HIV-1 infection (2, 3, 20–22, 35, 36, 40, 41, 46). Significantly, passive immunization studies using broadly neutralizing antibodies, including b12, have recently been used to protect rhesus macaques from intravenous and mucosal challenge with pathogenic primary HIV-1 isolate-derived SHIVs (35, 36, 43).

Neutralizing antibody may limit the dissemination of an enveloped virus, such as HIV-1, by at least two separate mechanisms. First, the antibody may interact with free virus and neutralize it by interfering with the attachment or fusion of its target cells, thereby protecting the cells from infection. Second, the neutralizing antibody may bind to envelope expressed on the surface of infected cells and induce cell lysis by the recruitment of effector functions, including ADCC and CDC (reviewed by Parren and Burton [40]). The relative roles of neutralization and Fc-mediated effects in HIV-1 infection have not yet been directly determined. In a recent study, Binley and colleagues (7) suggested that a transient effect on viral load by the infusion of anti-SIV antibodies was likely due to killing of SIV-infected cells, although this conclusion could only be drawn by inference.

We set out to directly study the role of Fc-mediated effector function in HIV-1 infection by the preparation of a panel of Fc

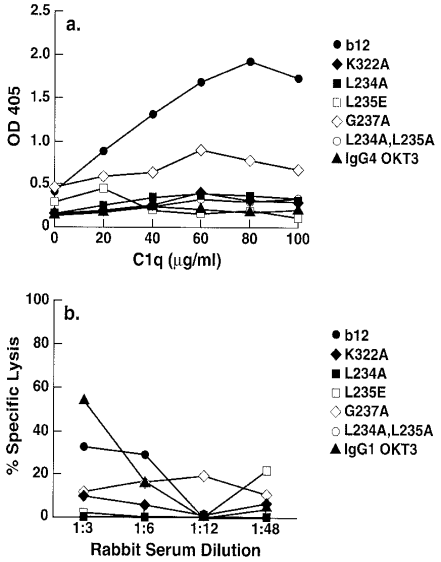

FIG. 4. Binding of wild-type and mutant IgG1 b12 to C1q (a) and complement-mediated lysis of HIV-1_{MN}-infected CEM-Nkr-MN cells by IgG1 b12 and IgG1 b12 mutants (b). Isotype variants of anti-CD3 MAb OKT3 (IgG1 and IgG4) were used as controls.

mutants of the broadly HIV-1-neutralizing MAb b12. The FcγR binding assays demonstrated that single mutations at residues 234 and 235 strongly reduced the binding to FcγRI and completely abolished binding to FcγRIIa and FcγRIIIa. Our results are in agreement with previous studies showing the importance of the lower hinge region of IgG1 in FcγRI, FcγRII, and FcγRIII binding (38, 48, 57, 59). We also showed that double mutation of amino acids 234 and 235 completely abolished binding to all FcγRs.

We examined the nature of the FcγRs involved in the ADCC of HIV-infected cells by using anti-FcγR antibodies. Our data indicate that ADCC by PBMCs is inhibited only in the presence of anti-FcγRIII antibody, while ADCC by monocytes is abolished in the presence of anti-FcγRI antibody. Alsmadi and Tilley (1) proposed previously that ADCC of HIV-infected cells is exclusively mediated through FcγRIII expressed on the surface of $CD56^+$ NK cells. In their study, they measured ADCC activity using cultured PBMCs as effector cells which are likely reduced in monocyte content due to the adherence of these cells to plastic. Similarly, we found that PBMC-dependent ADCC was primarily mediated through FcγRIII, even though monocytes represented up to 8% of total cells in our PBMC preparations, as measured by fluorescence-activated cell sorting (FACS) using a CD14 marker (data not shown). The effective ADCC by purified monocytes in vitro in our study, however, indicates that it is likely that monocytes or macrophages expressing FcγRI contribute to the ADCC of HIV-1-infected cells in vivo. Therefore, we suggest that ADCC of HIV-infected cells in vivo may be mediated by both FcγRI and FcγRIIIa.

Human IgG1 has the ability to bind C1q and lyse cells by activating complement through the classical pathway. The binding site for C1q on murine IgG2b was mapped to residues 318, 320, and 322 (18). We measured the ability of b12 and the mutants described to bind to C1q and to activate complement-mediated cell lysis. We used heterologous rather than homologous complement, as this resulted in more efficient lysis of HIV-1-infected (human) cells (data not shown). Changing lysine 322 to alanine completely abolished binding to C1q and complement activation, which confirms a previous report (27). Less expected was the reduction of C1q binding and complement activation by mutations in the lower hinge. In an earlier study, Morgan and colleagues had reported on the reduction of C1q binding and complement activation by changes in residues 235 and 237 for a chimeric mouse/human antibody (38). We show that in a fully human IgG1, L235, G237, and, in addition, L234 in the lower hinge region appear to play a significant direct or indirect role in C1q binding.

A concern may be that the null phenotype of the L234A, L235A double mutation for FcγR as well as C1q binding is a result of more-drastic rearrangements of the IgG Fc structure of this mutant. However, in a recent study, we have shown that the binding of the L234A, L235A double mutant to FcRn was only slightly reduced (<25%) compared to wild-type b12 (57). Protein A and G binding furthermore were unaffected (not shown). Proteins A, G, and FcRn all bind to the CH2-CH3 interface. The retention of FcRn binding, in particular, is significant as this receptor has two important functions, namely the cross-placental transport of maternal IgG to the fetus and the protection of IgG from normal serum protein catabolism (23, 29, 49). The serum half-life of the L234A, L235A mutant should therefore not be significantly affected.

In summary, we demonstrated that manipulation of residues 234, 235, and 237 in the lower hinge region of hIgG1 modulates binding to FcγRs. Our data also indicated that, in hIgG1, both the lower hinge and N-terminal end of the CH2 domain are involved in C1q binding and complement lysis. Furthermore, using both PBMCs and purified monocytes as effector cells, we found that ADCC of HIV-infected cells is mediated through both FcγRI and FcγRIIIa. As shown in our results, mutant K322A was only slightly, up to twofold, reduced in its ability to mediate ADCC and did not mediate CDC. On the other hand, the double mutant L234A, L235A mediated neither CDC nor ADCC. Both mutants were unchanged in their ability to neutralize primary HIV-1 isolates. Therefore, these mutants display specific valuable features that can be used in future in vivo studies. Our goal will be to use these mutants in in vivo passive antibody transfer-SHIV challenge studies in rhesus macaques to elucidate the relative roles of neutralization and of ADCC and complement activation in protection against HIV-1 infection and pathogenesis.

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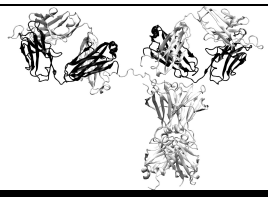
Chapter 5



Fc Receptor but not Complement Binding is Important in Antibody Protection Against HIV

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LETTERS

Fc receptor but not complement binding is important in antibody protection against HIV

Ann J. Hessell[1]*, Lars Hangartner[1]*, Meredith Hunter[2], Carin E. G. Havenith[3], Frank J. Beurskens[3], Joost M. Bakker[3], Caroline M. S. Lanigan[1], Gary Landucci[4], Donald N. Forthal[4], Paul W. H. I. Parren[3], Preston A. Marx[2] & Dennis R. Burton[1]

Most successful vaccines elicit neutralizing antibodies and this property is a high priority when developing an HIV vaccine[1,2]. Indeed, passively administered neutralizing antibodies have been shown to protect against HIV challenge in some of the best available animal models. For example, antibodies given intravenously can protect macaques against intravenous or mucosal SHIV (an HIV/SIV chimaera) challenge and topically applied antibodies can protect macaques against vaginal SHIV challenge[3,4]. However, the mechanism(s) by which neutralizing antibodies afford protection against HIV is not understood and, in particular, the role of antibody Fc-mediated effector functions is unclear. Here we report that there is a dramatic decrease in the ability of a broadly neutralizing antibody to protect macaques against SHIV challenge when Fc receptor and complement-binding activities are engineered out of the antibody. No loss of antibody protective activity is associated with the elimination of complement binding alone. Our *in vivo* results are consistent with *in vitro* assays indicating that interaction of Fc-receptor-bearing effector cells with antibody-complexed infected cells is important in reducing virus yield from infected cells. Overall, the data suggest the potential importance of activity against both infected cells and free virus for effective protection against HIV.

Neutralization is the most sought-after property of vaccine-induced antibodies and is a property that is measured *in vitro* as the ability of antibodies to inhibit viral entry into target cells in the absence of other anti-viral functions. Generally, neutralization results from inhibition of viral attachment, fusion events, or both[5,6]. *In vivo*, neutralizing antibodies can also exert anti-viral effects by other mechanisms, including effector functions mediated by the crystallizable fragment (Fc) part of the antibody molecule, such as complement activation and antibody-dependent cellular cytotoxicity (ADCC). These effector functions can act against both free virions and virus-infected cells[7].

The critical importance of effector functions for antibody-mediated protection against a number of viruses has been well documented[5,8,9], but there are few reports for HIV or SIV. In post-exposure studies of HIV-1-challenged hu-PBL SCID mice, it was reported[10] that treatment of mice with cobra-venom-factor to inactivate serum complement activity abrogated protection. In established SIV infection in macaques, polyclonal non-neutralizing anti-SIV immunoglobulin G was passively infused and it was suggested that ADCC might be responsible for producing a modest decline in levels of SIV-infected cells[11]. Serum ADCC activity measured *in vitro* has also been associated with protection[12,13], although other studies have not found a correlation[14]. ADCC can be triggered by non-neutralizing antibodies[15,16], presumably by binding to non-functional forms of envelope on the surface of infected cells.

Here we explore the role of antibody effector function in protection against HIV-1 in the SHIV/macaque model using a neutralizing human monoclonal antibody and variants of this antibody in which effector functions have been specifically disabled. The human monoclonal antibody b12 has been shown to neutralize a wide range of HIV isolates from different clades[17,18] and, when administered systemically or topically, to protect macaques against vaginal challenge with a SHIV that uses the chemokine receptor 5 (CCR5)[4,19]. The antibody recognizes a conserved epitope overlapping the CD4-binding site of gp120 (ref. 20). This epitope constitutes a major target of HIV vaccine design.

The generation and characterization of a series of Fc variants of antibody b12 has been described[21]. Here we focused on two antibody variants (Fig. 1a) from that study. The K322A (KA) variant, designed to abrogate complement activation, was shown to bind human complement C1q with greatly lowered affinity and to inefficiently activate human complement. The L234A,L235A (LALA) variant was shown to bind human Fcγ receptors weakly, to trigger ADCC with lowered efficiency and also to bind and activate human complement weakly. Figure 1b, c confirms, as predicted and as described in part in ref. 21, the antigen-binding and neutralization properties of the variant antibodies are equivalent to those of wild-type b12. Next we compared the abilities of the antibodies to interact with human and macaque effector-triggering molecules. Both variants show markedly decreased binding of human and macaque C1q (Fig. 2a) and complement activation as assessed by C3 fixation (Fig. 2b). Thus, neither variant activates the classical pathway of complement, and the absence of C3 fixation also rules out activation by the alternative or lectin pathways. Figure 2c shows that b12 and the KA variant interact similarly with recombinant human and macaque FcγRIa, FcγRIIa and FcγRIIIa, and with human FcγRIIb, but the LALA variant interacts with FcγRs considerably less well. Of note, the LALA variant is defective in binding to both FcRγIIa and FcRγIIb so major effects arising from disproportionate signalling by activating and inhibitory receptors[22] are not anticipated. In summary, wild-type b12 is complement and FcγR competent, the KA variant is disabled with respect to complement but competent with respect to FcγR binding, and the LALA variant is defective both in terms of complement and FcγR binding.

We next investigated the effect of Fc mutation on pharmacokinetics of the three b12 antibodies in macaques. None of the

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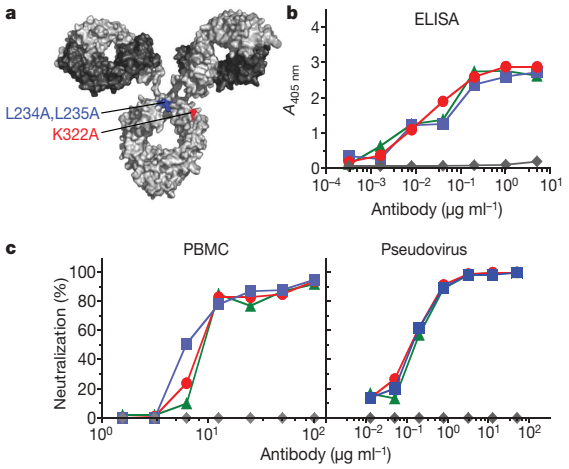

Figure 1 | Effector function variants of b12: location of substitutions, antigen binding and neutralization properties. **a**, The molecular surface representation of IgG1 b12 depicts the location of the substitutions K322A and L234A,L235A. **b**, Binding, by ELISA, of b12 and variants to $SHIV_{SF162P3}$ gp120 captured from a viral lysate. **c**, Two assay formats of $SHIV_{SF162P3}$ neutralization by b12 and variants: PBMC-based neutralization and a single-round replication luciferase reporter assay of pseudotyped virus. Controls for ELISA and PBMC-based neutralization are non-HIV human IgG1 mAbs; the control for the pseudovirus assay is serum from an HIV-1 seropositive patient (N16). Image provided by E. O. Saphire. Symbols as in Fig. 2.

mutations is close to the neonatal Fc receptor (FcRn)-binding site responsible for the maintenance of IgG half-lives, and indeed the half-lives of all three b12 species in macaques were similar (Supplementary Table 1).

A protection experiment (Fig. 3), comparing the abilities of the three b12 antibodies to prevent or modulate infection by a CCR5-using SHIV, was performed following Depo-Provera treatment of all animals to thin the vaginal epithelium[19]. One day before vaginal challenge with 300 50% tissue culture infectious doses ($TCID_{50}$) of $SHIV_{SF162P3}$, each animal was given an intravenous dose of 25 mg kg^{-1} of one of the b12 antibodies or an isotype control human IgG. The experiment included nine animals in each of the b12 and b12 variant groups and four animals in the control group. Blood was drawn frequently to monitor viral infection, transferred antibody levels and serum-neutralizing activity. All 4 controls became infected with a peak viraemia of 3×10^6–4×10^7 virus copies per ml between days 14 and 21. In the b12 group, 8 out of 9 animals were protected and showed no detectable virus at day 159 following challenge. The single infected animal showed a relatively low peak viraemia of 10^6 virus copies per ml at a time point (day 38) much delayed when compared to controls. Similarly, in the KA variant group, 8 out of 9 animals were protected, with a single infected animal showing a relatively low peak viraemia (8×10^5 virus copies per ml) occurring late (day 42) after challenge. The viral loads in the control group differed significantly from those in both the b12 and the KA variant groups from day 10 post challenge and onwards ($P < 0.01$, two-way analysis of variance (ANOVA) with Bonferroni post hoc test; see Supplementary Table 3 for detailed day-by-day analyses.)

In contrast, transfer of the LALA variant left 4 out of 9 animals unprotected with similar peak viraemia to that of control animals, in

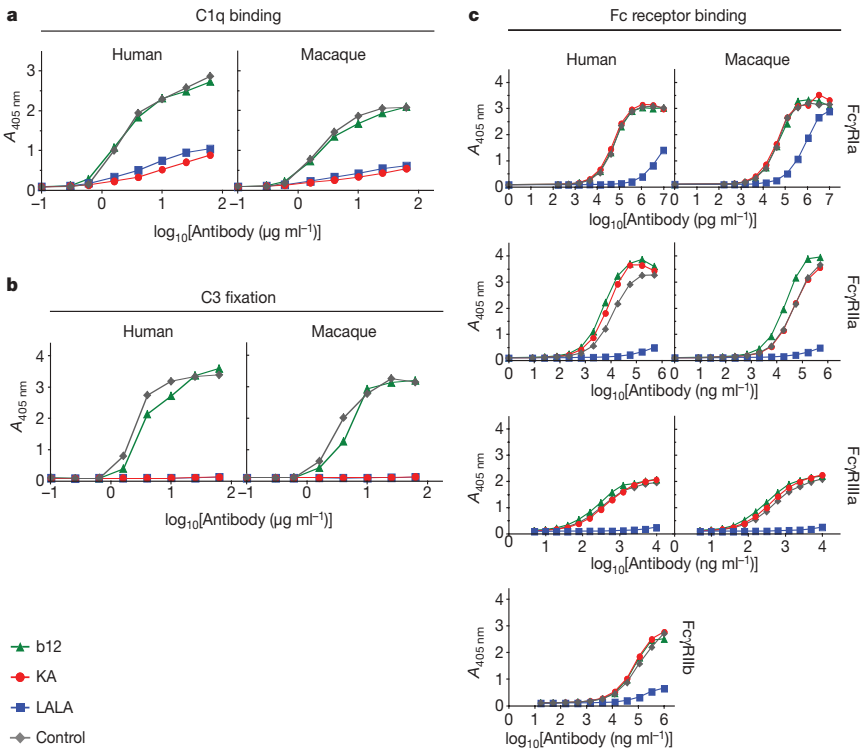

Figure 2 | Interaction of b12 and variants with effector molecules. **a**, Binding of b12 and variants to C1q from human and macaque serum. **b**, C3 fixation by b12 and variants in human and macaque serum. Wild-type b12 and IgG1 isotype control bind C1q and fix C3. KA and LALA variants show little C1q binding and little C3 fixation. **c**, Binding of b12 and variants to human and macaque activating FcγRs Ia, IIa and IIIa and to human inhibitory FcγRIIb. The KA variant shows similar FcγR binding, whereas the LALA variant shows weak or no binding to human or macaque FcγRs. (Macaque FcγRIIb is not available.) See Supplementary Table 4 for apparent affinities (IC_{50}).

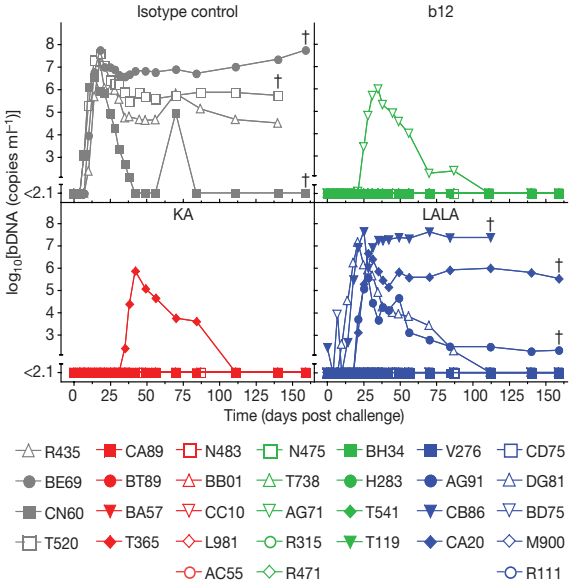

Figure 3 | Temporal analysis of plasma viral loads. All animals in the control group (4/4) experienced peak viraemia (3×10^6–4×10^7) between days 14 and 21. Only 1 of 9 animals in both the wild-type b12 and KA variant groups became infected, each with a much-delayed onset of infection and decreased peak viraemia. In contrast, 4 animals became infected in the LALA variant group with viraemia onset and peak similar to the control group. The assay minimum detection limit stated by the manufacturer is 125 bDNA copies per ml (2.1 $\log_{10}$ [bDNA (copies per ml)]) with a 95% confidence level. Protected animals showed no consistently measurable infection. Each curve depicts an individual animal. †, Euthanized animals as a result of virally induced pathology.

the range of 4×10^5–1×10^7 virus copies per ml, albeit somewhat delayed. Indeed, two-way ANOVA with Bonferroni post hoc test revealed a difference in viral loads between the control group and the LALA variant group during early infection (day 10, $P < 0.05$; days 14–21, $P < 0.001$; day 25, $P < 0.05$). After day 25, a difference between the control group and the LALA variant group could no longer be observed (except for a difference between both groups on day 70 ($P < 0.01$), which was due to an unusually high viral titre in the control group on that day; see Supplementary Table 3). Taken together, our statistical analyses indicate that the b12 and KA antibodies confer profound protection from viraemia, whereas the LALA antibody confers only partial protection in which infected animals develop peak viraemias similar to controls.

The neutralizing antibody titres (half-maximal inhibitory concentration, IC_{50}) in the sera of the animals at the time of challenge varied between 1:1,592 and 1:4,881 (Supplementary Table 2). Statistical analysis revealed no differences in the neutralization titres at the time of challenge among the groups ($P > 0.05$, see Supplementary Table 3), indicating that differences in the levels of serum-neutralizing activity were not responsible for the patterns of protection described.

To investigate further the status of the protected animals that showed no detectable virus by branched DNA (bDNA) measurements, one protected animal from each of the groups was depleted of $CD8^+$ T cells using a mouse–human chimaeric anti-CD8 antibody, a proven procedure to detect virus from monkeys that previously suppressed viral loads below the level of detection[23]. None of the animals showed detectable virus following $CD8^+$ T-cell depletion (Supplementary Fig. 1), indicating that sterilizing immunity was probably achieved in the protected animals.

Overall, the challenge experiments reveal that antibody can prevent HIV infection without the recruitment of effector functions. However, in the absence of Fcγ-receptor-binding function, the likelihood of infection is greatly increased. This raises the questions: (1) how does Fcγ-receptor-binding function influence antibody protection, and (2) are there implications for HIV vaccine design? Fcγ-receptor-binding may influence protection through clearance of viral particles. Thus, LALA-coated virions, lacking Fcγ-receptor-binding, may be less efficiently cleared, for example, by phagocytic cells, than b12-coated virions, and this may increase the chances of infection. Alternatively, the LALA variant may be unable to mediate activities such as ADCC against infected cells and this vulnerability may increase the probability of a productive infection. To investigate the latter possibility, we used an antibody-dependent cell-mediated virus inhibition (ADCVI) assay[24] to compare the ability of wild-type and variant b12 antibodies to inhibit virus yield from infected cells in the presence of Fcγ-receptor-bearing effector cells *in vitro*. Figure 4 shows that wild-type b12 and the KA variant provide better control of virus in the presence of effector cells than the LALA variant.

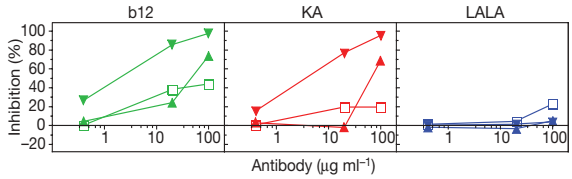

Figure 4 | Antibody-dependent cell-mediated viral inhibition (ADCVI) by b12 and variants. CEM.NKR-CCR5 cells were infected with $SHIV_{SF162P3}$ and incubated for 48 h, washed and combined with effector cells and serially diluted antibody. After a 30-min incubation, cells were re-washed to remove excess antibody and free virus. Viral inhibition was measured after incubation for 7 days. The LALA variant is less effective than wild-type b12 and the KA variant in mediating ADCVI with both human and rhesus effector cells. Squares, no effectors; up-triangles, huPBMCs; down-triangles, rhPBMCs.

A reasonable explanation of the protection experiments described, taking into account the *in vitro* observations made, is as follows. After virus challenge, all infectious virions may be neutralized by antibody and infection of the animal prevented. Alternatively, some cells may become infected and produce large numbers of progeny virus particles. These also may be neutralized and infection prevented. However, as more cells become infected, the risk of infection spinning out of control increases. Lacking Fcγ-receptor-binding function, the LALA variant may be less effective in clearing virions and in suppressing viral release from infected cells. The latter function requires higher antibody concentrations than neutralization[21] (compare Figs 1c and 4) and may contribute to the high concentrations often associated with passive protection against HIV. If activity against infected cells was provided instead by a robust cellular immune response, then considerably lower antibody concentrations may nevertheless provide benefit. In other words, HIV vaccine efficacy may be crucially dependent on a combination of antibody and cell-mediated immunity.

METHODS SUMMARY

Animals used in this study were female Indian rhesus macaques challenged with $SHIV_{SF162P3}$ (refs 25–27). CH variants of b12 were created by site-directed mutagenesis[21]. Recombinant antibodies were expressed in Chinese hamster ovary (CHO-K1) cells, purified using Protein A affinity matrix, and monitored for endotoxin contamination. Plasma viral loads were assessed by quantitative assays for the measurement of SIV RNA using a branched DNA signal amplification assay for SIV[28] (Siemens, formerly Bayer). Antibody concentrations in serum and measurement of binding to $SHIV_{SF162P3}$ gp120 were determined by enzyme-linked immunosorbent assay (ELISA)[19]. Neutralization of monoclonal antibodies and sera were performed either with phytohaemagglutinin-activated peripheral blood mononuclear cells (PBMC) from a single rhesus macaque as target cells[19] or using an HIV-1 envelope pseudotyped luciferase $SHIV_{SF162P3}$ assay by Monogram Biosciences[29]. C1q binding and C3 fixation using b12 or variants and human or macaque serum were measured in ELISA. Human FcγRIa, FcγRIIa (131R), FcγRIIb and FcγRIIIa (158V) and macaque FcγRIa,

FcγRIIa and FcγRIIIa were cloned and expressed as soluble proteins in CHO-K1-SV or HEK293 cells. Binding of antibodies to human and macaque FcγRs was measured in ELISA. Depletion of $CD8^+$ lymphocytes was accomplished using the mouse–human chimaeric anti-CD8 monoclonal antibody, cM-T807[23]. The ADCVI assays were based on methods previously described[24] using target cells, consisting of CEM.NKR-CCR5 cells, infected with $SHIV_{SF162P3}$ and either huPBMCs or rhPBMCs as effectors. Statistical analysis was performed using GraphPad Prism for Windows, version 4.03. Analysis was performed with a repeated measures two-way ANOVA, followed by Bonferroni post hoc test. The alpha level was 0.05.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Macaques. All protocols for female Indian rhesus macaques were reviewed and approved by the Institutional Animal Care and Use Committees. The animals were housed in accordance with the American Association for Accreditation of Laboratory Animal Care Standards. At the start of all experiments except the $CD8^+$ T-cell depletion studies, all animals were experimentally naive and were negative for antibodies against HIV-1, SIV and type D retrovirus. Virus challenges and intravenous antibody transfers are more fully described elsewhere[4].

Challenge virus. The virus used in this study was $SHIV_{SF162P}$ passage 3, which has been described elsewhere[25–27]. $SHIV_{SF162P3}$ retains the R5 phenotype of HIV-1_{SF162}. $SHIV_{SF162P3}$, propagated in phytohaemagglutin (PHA)-activated rhesus macaque peripheral blood mononuclear cells (PBMCs), was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (catalogue no. 6526; contributors: J. Harouse, C. Cheng-Mayer and R. Pal).

b12 and variant antibodies. IgG1 b12 is a human antibody (IgG1, κ) that recognizes an epitope overlapping the CD4-binding site of gp120 (refs 17, 18). Variants of b12 were created by site-directed mutagenesis as previously described[21].

Antibody production. Recombinant IgG1 (wild-type b12, isotype control and b12 variants K322A and L234A,L235A) were expressed in Chinese hamster ovary (CHO-K1) cells in glutamine-free custom formulated Glasgow minimum essential medium (GMEM Selection Media) (MediaTech Cellgro). Two IgG1 isotype control antibodies were used in this study: (1) an anti-dengue NS1 antibody, and (2) an anti-ebola GP antibody. For large-scale tissue culture, media was supplemented with 3.5% Ultra Low Bovine IgG Fetal Bovine Serum (Invitrogen) and grown in ten-layer Cellstacks and Cell Cubes (Corning). Antibodies were purified using Protein A affinity matrix (GE Healthcare), and dialysed against phosphate-buffered saline (PBS). Care was taken to minimize endotoxin contamination, which was monitored using a quantitative chromagenic Limulus Amoebecyte Lysate assay (Cambrex) performed according to the manufacturer's recommendations. Antibody used for the passive transfer experiments contained <3 IU of endotoxin per ml.

Plasma viral loads. Quantitative assays for the measurement of SIV RNA were performed at Siemens Medical Solutions (formerly Bayer Diagnostics) using a branched DNA signal amplification assay for SIV[28].

Serum antibody ELISAs. Antibody concentrations in macaque sera were determined by two different ELISAs: (1) recombinant monomeric HIV-1 $gp120_{JR\text{-}FL}$ (provided by Progenics), and (2) the B2.1 peptide. B2.1 is a homodimer of the peptide HERSYMFSDLENRCI-(biotinylated Orn)-KK (synthesized by AnaSpec). This peptide binds the b12-antigen-binding site with high specificity as described in detail[30]. The ELISAs are fully described elsewhere[19].

SHIV capture ELISA. Measurement of binding to $SHIV_{SF162P3}$ gp120 was determined in Rhesus (no. 355) PBMC-derived viral culture lysate by capture with the anti-gp120 antibody D7324 (International Enzymes) in ELISA. Microtitre plates (Corning) were coated overnight at 4 °C with D734 at a concentration of 5 μg ml^{-1} diluted in phosphate-buffered saline (PBS). All subsequent steps were performed at room temperature. Unless otherwise specified, plates were washed four times after each step with PBS supplemented with 0.05% Tween. After incubation and washing, coated plates were blocked for 1 h with PBS supplemented with 3% bovine serum albumin (BSA). Blocking solution was decanted and viral culture supernatants diluted 1:3 in PBS containing 1% BSA and 0.02% Tween (PBS-B-T) were added and incubated for 2 h. After washing, antibodies serially diluted in PBS-B-T (5 μg ml^{-1}–0.32 ng ml^{-1}) were added and incubated for 2 h. Plates were again washed and alkaline-phosphatase-conjugated goat anti-human IgG $F(ab')_2$ (Pierce) was added (diluted 1:500 in PBS-B-T) and incubated for 1 h. Plates were washed and developed with *p*-nitrophenyl phosphate (Sigma) and absorbances were read at 405 nm at 10 min, 20 min and 30 min. The assay volume for each step was 50 μl.

Neutralization assays. Neutralization of antibodies and sera was assessed by two different methods. Neutralization of the primary isolate $SHIV_{SF162P3}$ was performed using phytohaemagglutinin (PHA)-activated PBMCs from a single rhesus macaque (no. 355) as target cells. Cells from this animal replicate $SHIV_{SF162P}$ efficiently. Neutralization assessment was carried out as described previously[19]. Neutralization titres of animal sera were reported by Monogram Biosciences after preparation of an HIV-1 envelope pseudotyped luciferase $SHIV_{SF162P3}$ capable of single-round replication. The pseudovirus-based neutralization assay was performed as previously described[29].

C1q binding and C3 fixation assays. The ability of b12 and variants to bind C1q or cause fixation of C3 present in human and macaque serum was measured in ELISA. To assess the affinity for C1q of b12 and variants present in human and macaque serum, antibodies were serially diluted (62.5–0.2 μg ml^{-1}) and coated onto a microtitre plate and incubated overnight at 4 °C. Plates were blocked with 0.5× PBST/1% gelatin followed by the addition of human or rhesus serum (3%). After washing, rabbit anti-human C1q (DAKO) was added at a dilution of 1:1,000. IgG-bound C1q was detected with a peroxidase-conjugated swine anti-rabbit IgG (DAKO) and was subsequently developed with ABTS. For the C3 fixation assay, mouse anti-human C3 (DAKO) was added at a dilution of 1:100 followed by detection with peroxidase-conjugated rabbit anti-mouse IgG (Jackson) diluted 1:1,000. Reactions were stopped with 2% oxalic acid after 10 min. PBST washes were carried out between each step. The assay volume for each step was 100 μl. Unless otherwise specified, incubation times were 1 h at room temperature with gentle shaking.

FcγR binding assays. All FcγR proteins contained a histidine tail for purification. Binding of antibodies to human and macaque FcγRs was measured in ELISA by capturing the recombinant FcγRs with an anti-poly-histidine antibody (Research Diagnostics) at 4 μg ml^{-1} diluted in PBS, coated onto a microtitre plate and incubated overnight at 4 °C. FcγRs were added and incubated at a final concentration of 2 μg ml^{-1} in PBST. After washing, serial dilutions of b12, variants and isotype controls were added. HRP-labelled $F(ab')_2$ fragments of goat anti-human $F(ab')_2$ fragments of IgG (Jackson) were used as the detection conjugate (incubation for 90 min). The results were visualized with ABTS and the reaction was stopped with 2% oxalic acid. PBST washes were carried out between each step. The assay volume for each step was 100 μl. Unless otherwise specified, incubation times were 75 min at room temperature.

$CD8^+$ T-cell depletions. Depletion of $CD8^+$ lymphocytes was accomplished using the mouse–human chimaeric anti-CD8 monoclonal antibody, cM-T807 (provided by the NIH Nonhuman Primate Resource, Beth Israel Deaconess Medical Center, Boston, Massachusetts). Administration of the antibody was given on days 0, 3 and 7 at doses of 10 mg kg^{-1} on day 0, followed by doses of 5 mg kg^{-1} on days 3 and 7. Methods for depletion and confirmation of depletion are fully described elsewhere[23].

ADCVI assays. The ADCVI assays were based on methods previously described[24]. Targets, consisting of CEM.NKR-CCR5 cells, were infected with $SHIV_{SF162P3}$ and washed to remove cell-free virus and incubated for 48 h. (CEM.NKR-CCR5 cells were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (catalogue no. 4376; contributor: A. Trkola.) Effector cells, either huPBMCs or rhPBMCs, were combined with serially diluted antibodies and added to target cells for a 30-min incubation. Cells were re-washed and incubated for 7 days at 37 °C in 5% CO_2. Supernatant was collected and assayed for p27 by ELISA (Zeptometrix).

Statistics. The control group consisted of 4 animals ($n = 4$), and each of the treated groups consisted of 9 animals ($n = 9$). For practical reasons, the experiment was divided into two parts with exactly the same set up. In experiment 1, there were 2 animals in the control group and 4 animals in each of the treated groups. In experiment 2, there were 2 animals in the control group and 5 in each of the treated groups. Statistical analysis was performed using GraphPad Prism for Windows, version 4.03 (GraphPad Software, 2005). Group differences in viral load in time and group differences in neutralization titres were determined after normalization of data by $\log_{10}$ transformation.

Serum antibody levels (μg/ml) and half-lives (days)

mAb	b12								
Animal	**N475**	**T738**	**AG71**	**R471**	**R315**	**BH34**	**H283**	**T119**	**T541**
Day									
0	602	395	780	551	649	385	435	668	600
4	265	113	301	396	378	295	419	414	585
7	94	74	298	269	377	288	364	335	512
10	n/a	n/a	n/a	n/a	n/a	213	208	300	354
14	57	42	146	246	132	122	110	139	212
21	25	47	147	123	91	81	45	93	102
28	10	30	65	77	43	n/a	n/a	n/a	n/a
Half-life (days)	**5.0**	**9.2**	**9.2**	**10.3**	**7.2**	**8.9**	**5.9**	**7.2**	**7.6**

Average half-life for b12-treated group is 7.8 ± 1.7 days.

mAb	KA								
Animal	**N483**	**BB01**	**CC10**	**L981**	**AC55**	**CA89**	**BT89**	**BA57**	**T365**
Day									
0	639	355	571	1,023	502	520	525	1029	381
4	356	272	362	845	392	505	545	801	330
7	340	161	305	439	149	431	645	502	348
10	n/a	n/a	n/a	n/a	n/a	242	284	284	299
14	164	114	153	187	146	253	142	164	110
21	58	62	39	66	125	78	98	56	82
28	12	42	32	54	88	n/a	n/a	n/a	n/a
Half-life (days)	**5.8**	**10.3**	**7.6**	**6.0**	**9.2**	**7.6**	**7.2**	**4.8**	**8.5**

Average half-life for KA-treated group is 7.4 ± 1.7 days.

mAb	LALA								
Animal	**CD75**	**V276**	**BD75**	**M900**	**R111**	**DG81**	**AG91**	**CB86**	**CA20**
Day									
0	400	910	477	310	436	595	557	523	600
4	428	478	311	216	269	212	308	187	261
7	338	306	268	161	214	224	256	65	263
10	n/a	134	n/a	n/a	n/a	n/a	105	10	126
14	277	126	219	144	147	161	83	0	55
21	134	83	63	125	87	80	68	0	38
28	91	n/a	10	92	55	3	n/a	n/a	n/a
Half-life (days)	**9.8**	**6.0**	**5.3**	**12.6**	**8.7**	**4.3**	**6.5**	**2.0**	**5.1**

Average half-life for LALA-treated group is 6.7 ± 3.2 days

Supplementary Information, Table 1 *In vivo* kinetics of transferred antibodies and calculated half-lives in serum. Numbers represent transferred antibody concentration (μg/ml) at the indicated day post challenge. Half-lives were calculated based on the slope of the linear regression fitted into the semi-logarithmic representation of the antibody concentrations over time. Group averages are reported with the standard deviation. n/a indicates a timepoint where no serum sample is available.

Neutralizing antibody titers in sera (IC_{50})

mAb	b12								
Animal	N475	T738	AG71	R471	R315	BH34	H283	T119	T541
Day									
0	3,929	3,116	3,604	3,170	2,814	2,828	2,909	4,308	4,601
4	1,991	1,745	1,661	1,982	1,415	1,740	2,032	2,612	2,261
7	2,008	1,667	1,436	2,025	1,029	1,414	2,031	1,752	1,852
14	1,357	658	998	1,468	745	1,037	1,067	1,089	1,298
Protection	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

mAb	KA								
Animal	N483	BB01	CC10	L981	AC55	CA89	BT89	BA57	T365
Day									
0	4,881	2,506	3,891	4,324	2,586	2,924	3,796	4,363	3,221
4	3,047	1,458	2,139	2,242	1,900	1,805	2,761	3,612	2,299
7	2,261	1,292	1,433	1,985	2,071	2,174	3,166	2,200	2,132
14	1,641	722	904	1,471	1,109	1,237	2,378	1,545	1,429
Protection	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No

mAb	LALA								
Animal	CD75	V276	BD75	M900	R111	DG81	AG91	CB86	CA20
Day									
0	3,980	3,136	3,022	1,762	2,755	3,593	2,009	1,592	2,705
4	2,031	1,639	1,898	1,016	1,191	1,245	1,604	2,484	1,578
7	1,553	1,332	1,904	910	824	1,209	1,501	498	1,083
14	1,163	892	1,070	948	948	692	1,005	213	848
Protection	Yes	Yes	Yes	Yes	Yes	No	No	No	No

Supplementary Information, Table 2. Neutralizing titers in the serum of all experimental animals on the day of challenge and during the two following weeks. Numbers represent reciprocal dilution of serum producing 50% neutralization.

Supplementary Information, Table 3. Supplementary statistical methods.

1. Plasma viral loads. Due to ethical reasons, no more than 4 animals were included in the control group. In each of the treated groups, 9 animals were included. After plasma viral loads/ml were measured, data were normalized by $\log_{10}$ transformation. Mean, SEM and N is given in Table 1 for each treatment group, on each day. The alpha level was 0.05. All data were analyzed by use of Graph Pad Prism for Windows, version 4.03 (Graph Pad software Inc., San Diego, CA).

Table 1. Mean, SEM and N of $\log_{10}$ data for viral loads of all experimental animals on each day.

IgG1 control			**b12**			**LALA**			**KA**			
Days	**mean**	**SEM**	**N**	**mean**	**SEM**	**N**	**mean**	**SEM**	**N**	**mean**	**SEM**	**N**
0	2.100	0.000	4	2.100	0.000	9	2.140	0.040	9	2.100	0.000	9
4	2.100	0.000	4	2.100	0.000	9	2.100	0.000	9	2.100	0.000	9
7	2.600	0.289	4	2.211	0.111	9	2.100	0.000	9	2.100	0.000	9
10	4.448	0.814	4	2.100	0.000	9	2.156	0.056	9	2.100	0.000	9
14	6.578	0.333	4	2.100	0.000	9	2.431	0.266	9	2.100	0.000	9
18	7.118	0.416	4	2.100	0.000	9	2.931	0.553	9	2.100	0.000	9
21	6.518	0.312	4	2.100	0.000	9	3.487	0.694	9	2.100	0.000	9
25	6.083	0.432	4	2.244	0.144	9	3.848	0.730	9	2.100	0.000	9
28	5.848	0.550	4	2.400	0.300	9	3.861	0.695	9	2.100	0.000	9
31	5.450	0.732	4	2.500	0.400	9	3.763	0.693	9	2.133	0.033	9
35	4.968	0.849	4	2.533	0.433	9	3.579	0.661	9	2.134	0.034	9
38	4.780	0.962	4	2.456	0.356	9	3.530	0.634	9	2.356	0.256	9
42	4.883	1.025	4	2.411	0.311	9	3.450	0.617	9	2.520	0.420	9
49	4.808	1.011	4	2.367	0.267	9	3.686	0.643	9	2.431	0.331	9
56	4.868	1.015	4	2.156	0.056	9	3.370	0.633	9	2.384	0.284	9
70	5.838	0.402	4	2.100	0.000	9	3.326	0.661	9	2.318	0.183	9
84	4.955	1.007	4	2.113	0.013	9	3.176	0.667	9	2.271	0.169	9

A repeated measures 2-way ANOVA followed by Bonferroni post test was used to test significance of data from the plasma viral loads. An extremely significant effect of treatment ($p<0.0001$), of time ($p<0.0001$), and of interaction ($p<0.0001$) was found. The results of the Bonferroni post test are depicted in Table 2.

Table 2. Results of Bonferroni post test.

Day	IgG vs b12	IgG vs LALA	IgG vs KA	b12 vs LALA	b12 vs KA	LALA vs KA
0	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
7	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
10	P<0.01**	P < 0.05*	P<0.01**	P > 0.05	P > 0.05	P > 0.05
14	P<0.001***	P<0.001***	P<0.001***	P > 0.05	P > 0.05	P > 0.05
18	P<0.001***	P<0.001***	P<0.001***	P > 0.05	P > 0.05	P > 0.05
21	P<0.001***	P<0.001***	P<0.001***	P > 0.05	P > 0.05	P > 0.05
25	P<0.001***	P < 0.05*	P<0.001***	P < 0.05*	P > 0.05	P < 0.05*
28	P<0.001***	P > 0.05	P<0.001***	P > 0.05	P > 0.05	P < 0.05*
31	P<0.001***	P > 0.05	P<0.001***	P > 0.05	P > 0.05	P < 0.05*
35	P<0.01**	P > 0.05	P<0.001***	P > 0.05	P > 0.05	P > 0.05
38	P< 0.05*	P > 0.05	P<0.01**	P > 0.05	P > 0.05	P > 0.05
42	P<0.01**	P > 0.05	P<0.01**	P > 0.05	P > 0.05	P > 0.05
49	P<0.01**	P > 0.05	P<0.01**	P > 0.05	P > 0.05	P > 0.05
56	P<0.01**	P > 0.05	P<0.01**	P > 0.05	P > 0.05	P > 0.05
70	P<0.001***	P<0.01**	P<0.001***	P > 0.05	P > 0.05	P > 0.05
84	P<0.001***	P > 0.05	P<0.01**	P > 0.05	P > 0.05	P > 0.05

*: significant at p<0.05; **: significant at p<0.01; *** significant at p<0.001.

2. Neutralizing titers. Neutralizing titers were measured at several time intervals after viral challenge. Summary of data is given in Table 3, which shows mean, SEM and N of this experiment. After neutralizing titers were measured, data were normalized by ^{10}log transformation. The alpha level was 0.05.

Table 3. Mean, SEM, and N of ^{10}log data of neutralizing titers for all experimental animals in three treated groups.

	b12			b12-KA			b12-LALA		
Days	**mean**	**SEM**	**N**	**mean**	**SEM**	**N**	**mean**	**SEM**	**N**
0	3.532	0.027	9	3.548	0.035	9	3.418	0.046	9
4	3.281	0.026	9	3.358	0.041	9	3.200	0.041	9
7	3.219	0.033	9	3.306	0.038	9	3.051	0.058	9
14	3.021	0.038	9	3.119	0.051	9	2.901	0.075	9

A repeated measures 2-way ANOVA followed by Bonferroni post test was used to test significance of data from the neutralizing titers. A very significant effect of treatment ($p=0.0021$), and an extremely significant effect of time ($p<0.0001$), but not of interaction was found. The results of the Bonferroni post test are depicted in Table 4. Differences in neutralizing titers are also shown in Figure 1.

Days	b12 vs LALA	b12 vs KA	LALA vs KA
0	P > 0.05	P > 0.05	P > 0.05
4	P > 0.05	P > 0.05	P > 0.05
7	P > 0.05	P < 0.05*	P<0.001***
14	P > 0.05	P > 0.05	P<0.01**

*: significant at $p<0.05$; **: significant at $p<0.01$; *** significant at $p<0.001$.

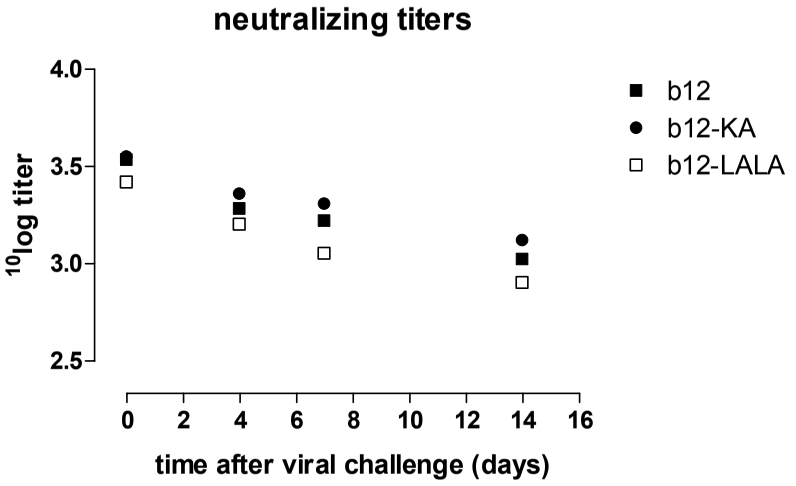
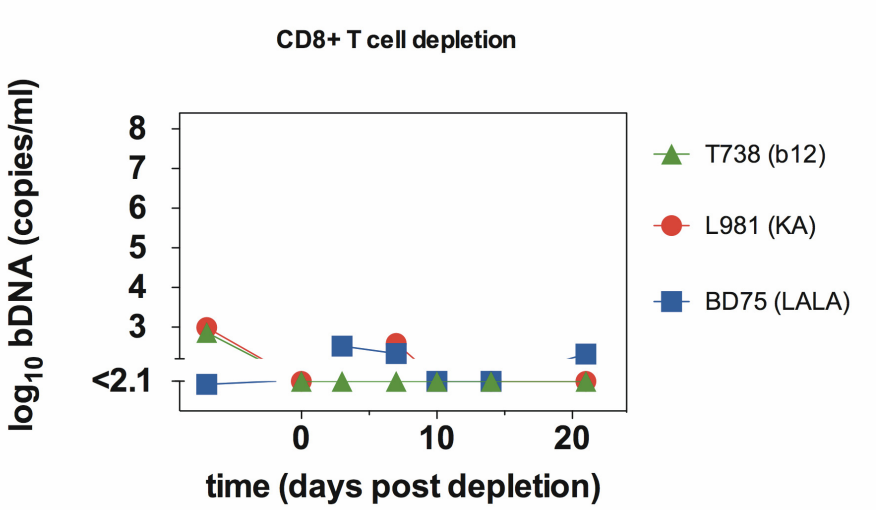
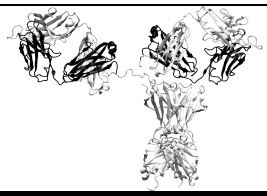


Figure 1. Results of neutralizing titers in three experimental groups.



Supplementary Information, Figure 1. Analysis of viral load following CD8+ T-cell depletion of protected macaques. One protected animal from each of the b12 and variant groups was depleted of CD8+ T-cells using the mouse-human chimeric anti-CD8 antibody cM-T807. Doses of 10 mg/kg were administered on days 0, 3, and 7, followed by doses of 5 mg/kg on days 3 and 7. None of the animals showed consistently detectable virus indicating likely achievement of sterilizing immunity provided by transferred antibodies. The occasional measurement of viral loads just above the detection limit quoted by the manufacturer of the bDNA assay kit is not considered significant.

Chapter 6



Effective, Low Titer, Antibody Protection Against Low-Dose Repeated Mucosal SHIV Challenge in Macaques

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Neutralizing antibodies are thought crucial to HIV vaccine protection but a major hurdle is the high antibody concentrations likely required as suggested by studies in animal models[1]. However, these studies typically apply a large virus inoculum to ensure infection in control animals in single challenge experiments. In contrast, most human infection via sexual encounter probably involves repeated exposures to much lower doses of virus[2-4]. Therefore, animal studies may have overestimated protective antibody levels in humans. To investigate the impact of virus challenge dose on antibody protection, we repeatedly exposed macaques intravaginally to low doses of a CCR5 coreceptor-using SHIV (an HIV/SIV chimera) in the presence of antibody at plasma concentrations leading to relatively modest neutralization titers of the order of 1:5 IC_{90} values in a PBMC assay. An effector function deficient variant of the neutralizing antibody was also included. The results show that a significantly greater number of challenges are required to infect animals treated with neutralizing antibody than control antibody-treated animals, and the notion that effector function may contribute to antibody protection is supported. Overall, the results imply that lower levels of antibody than considered hereto may provide benefit in the context of typical human exposure to HIV-1.

Much of what we know about antibody protection against HIV comes from studies using passively administered broadly neutralizing human monoclonal antibodies or monospecific neutralizing polyclonal antibodies in animal challenge models[5-11], including intravenous (*i.v.*), vaginal and rectal challenge in macaques. The hallmark of most of these studies is that protection, in the form of sterilizing immunity, is achieved at relatively high serum neutralization titers corresponding to high antibody concentrations. The most quantitative of these studies suggest that sterilizing immunity requires serum antibody concentrations at least two orders of magnitude greater than *in vitro* neutralizing concentrations[10,11]. However, this estimate is quite approximate and dependent upon, among other parameters, the neutralization assay used. Even so, the data have convinced many researchers that achieving sterilizing immunity via antibodies alone is extremely challenging and a more realistic goal for vaccine-induced antibodies has been viewed as blunting infection and relying on vaccine-induced cellular immunity to clear, or, failing that, control infection. However, as noted above, a limitation of macaque protection studies is the use of high viral challenge doses to ensure all control animals become infected with a single challenge. Yet, it is well established that the average probabilities for heterosexual transmission in human exposures are low and dependent upon the viral burden in the donor and susceptibility factors associated with the donor and recipient such as the presence of sexually transmitted diseases (STDs). Transmission frequencies on the order of 1:1,000 per coital act have been reported in chronic infection of the donor[2-4,12], increasing by about an order of magnitude in acute infection[2,3,12,13]. The amount of virus, albeit estimated by quantitative PCR rather than infectivity, contained in a typical macaque challenge is much higher than would be found, for example, in the semen ejaculate of an acutely infected man[12-15]. Indeed, viral inoculums typically average 5×10^5 copies per ejaculate, with a reported maximum of about 2×10^7 copies[12], whereas we found that a high-dose 300 $TCID_{50}$ (50% tissue culture infectious doses) inoculum of $SHIV_{SF162P3}$ contains about 10^8 viral copies.

In order to investigate antibody protection against viral challenge doses that may better represent those encountered in human heterosexual exposure, we utilized a low-dose repeated mucosal challenge model[14,16] in which a reduced virus dose requires several challenges to infect untreated animals, but yet eventually infects all animals with a reasonable number of challenges. In this model, we could expect to observe benefit provided by antibody if the number of challenges required for infection in treated animals was greater than the number of challenges required for infection in controls.

The human monoclonal antibody b12 neutralizes a broad range of HIV isolates from a variety of clades[17,18] through recognition of a conserved epitope overlapping the CD4-binding site of gp120[19]. A high serum concentration of b12, corresponding to about 75-fold the IC_{90} in a PBMC assay and 3,000-fold the IC_{50} in a pseudotyped virus assay provided 90% protection against a high-dose vaginal challenge with $SHIV_{SF162P3}$[20]. In addition in that study, the importance for protection of the interaction of b12 with Fc receptors was established by comparison of b12 and engineered b12 variants[20].

Here, we explored the question of whether a relatively low b12 neutralizing antibody titer could provide benefit to macaques in the low-dose repeated challenge model and simultaneously compared protection by the effector function-deficient b12 variant LALA. Based on earlier studies [14,16,21], we began the experiment with repeated 3 $TCID_{50}$ $SHIV_{162P3}$ vaginal challenge. With only a single animal infected after 11 challenges, we increased the viral dose to 10 $TCID_{50.}$ This dose corresponds to approximately 2.65 x 10^6 viral RNA (vRNA) copies, an amount somewhat higher than typically found in human semen during acute infection[12,13] but substantially lower than traditional high-dose challenges with $SHIV_{SF162P3}$[14].

The study involved a total of 14 animals, consisting of 4 isotype control-treated animals, 5 animals receiving wild-type b12, and 5 animals receiving the LALA variant, which has similar neutralizing activity as b12 but does not mediate Fc effector functions [20]. Animals were *i.v.*-treated weekly (Thursday) with 1 mg/kg of antibody to maintain serum levels, based on previously reported half-lives[20]. This dose of b12 antibody is far less than the 25 mg/kg dose that provides 90% protection against high-dose challenge with $SHIV_{SF162P3}$ [20] and provides negligible protection against high-dose challenge with $SHIV_{SF162P4}$[10]. Intravaginal challenges were administered twice weekly (Friday and Monday) and blood drawn regularly to monitor viral infection, passively transferred antibody levels and serum neutralizing activity. **Supplementary Figure 1** details the entire treatment course for each animal and **Supplementary Table 1** summarizes antibody treatments, viral challenges, detection and day-of-peak viremia in plasma.

As shown in **Figure 1** notably more challenges were required to infect b12-treated than control animals and also suggests that somewhat fewer challenges may be required to infect LALA variant-treated than wild-type b12-treated animals. One animal (b12-treated, BF68) remained uninfected after 40 consecutive 10 $TCID_{50}$ challenges.

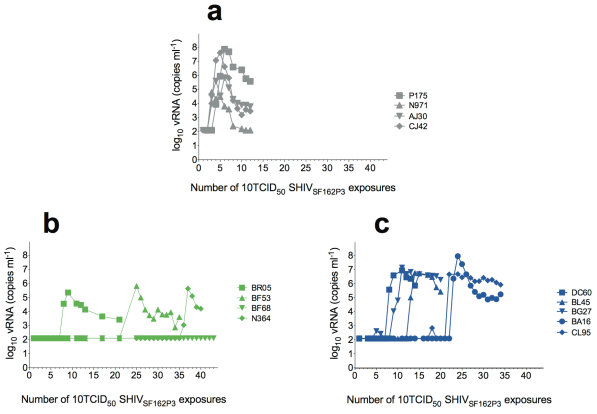

Figure 1. Protection by b12 and variant LALA during vaginal low-dose repeated challenge with $SHIV_{SF162P3}$. Female Indian rhesus macaques were treated weekly with 1 mg kg^{-1} of either b12 or b12 effector function variant LALA or an isotype control antibody (anti-dengue, DEN3) and challenged vaginally twice weekly. The viral challenge dose began at 3 $TCID_{50}$ and was subsequently increased to 10 $TCID_{50}$ $SHIV_{SF162P3}$. **a,** All animals in the isotype control groups became virus positive after a maximum of 4 challenges of 10 $TCID_{50}$. 4 out of 4 animals were infected after a total number of 10 challenges of 10 $TCID_{50}$. **b**, 3 b12-treated animals were virus positive after 6, 23, and 38 viral challenges of 10 $TCID_{50}$, respectively, and 1 animal (BF68) remained virus negative after 40 challenges. 3 out of 4 animals were infected after a total number of 107 challenges of 10 $TCID_{50}$.

BK10 was infected after 6 challenges of 3 TCID $_{50}$. (See **Suppl. Fig. 2**.) **c,** Plasma virus was observed in the LALA-treated animals following 6, 8, 12, 17, and 23 viral challenges, respectively. 5 out of 5 animals were infected after a total of 66 challenges of 10 $TCID_{50}$. Viral challenges and *i.v.* antibody treatments were suspended after positive detection of virus in plasma but the course of infection was monitored for several weeks. The SIV viral RNA (vRNA) assay detection limit is 125 copies ml^{-1} (log 2.1).

We investigated the magnitude of protection using three approaches. First, we used an adapted Kaplan-Meier analysis (**Fig. 2**) in which the percent of animals remaining uninfected is plotted against the number of 10 $TCID_{50}$ viral challenges. To prevent positive bias, we also included the animal BK10 that was infected in the 3 $TCID_{50}$ challenge series as if it was infected by the first 10 $TCID_{50}$ challenge (see above and **Suppl. Fig. 2**). The three survival curves are significantly different (p=0.0377). A comparison of the individual pairs of Kaplan-Meier curves reveals that LALA is significantly different from control (p=0.0027) while a (borderline) non-significant difference for b12 versus control (p=0.056) is seen due to the strong penalty incurred by including BK10 in the analysis. The same analysis excluding BK10 would indicate a significant difference (p=0.0058). The LALA and b12 groups did not differ significantly from each other.

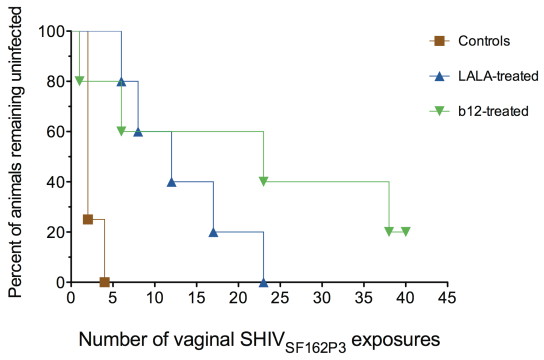

Figure 2. Kaplan-Meier analysis of protection in low-dose (10 $TCID_{50}$) repeated challenge by b12 and LALA treatment. The percent of animals remaining uninfected is plotted against the number of 10 $TCID_{50}$ viral challenges (compare Fig. 1). A single animal (BK10; b12-treated) became infected during the initial repeat 3 $TCID_{50}$ challenge (see Suppl. Fig. 2). To allow inclusion of this animal in the analysis, it is included as if it was infected in the first 10 $TCID_{50}$ challenge. The Kaplan-Meier survival curves are significantly different from each other (p = 0.0377; Log-rank (Mantel-Cox) test).

Second, we calculated hazard ratios for b12 and LALA-treated animals with a Cox-proportional hazard model that estimates the relative risk of infection for each of the treatment groups versus controls. Treatment with b12 was found to reduce the infection risk by about 20-fold. The risk reduction for LALA treatment was approximately 10-fold (**Table 1a).** Third, we calculated the reduction in infection susceptibility as described by Regoes, et al [22] by tallying the total number of 10 $TCID_{50}$ virus challenges required to infect all animals within each group (within the limits of the experiment). As shown in **Table 1b** b12-treated animals (p=0.0016) as well as LALA-treated animals (p=0.0145) only became infected after a significantly larger

number of challenges compared to the control group. It should be noted that this number is underestimated for b12 in this type of analysis, as one b12-treated animal remained uninfected at the end of the experiment. Overall, our analyses suggest that there is a significant difference in the protection afforded by the repeated administration of 1 mg/kg of both b12 antibody and LALA variant as compared to treatment with the isotype control antibody. The approximately two-fold difference in b12 and LALA hazard ratios and the observation that b12-treated animals resisted nearly twice as many challenges as LALA-treated animals (104 versus 61) reflects the trend previously described in a high-dose virus challenge for the effector function-crippled LALA variant to be less effective in protection than the fully effector function-competent wild type b12 antibody[20].

a	Cox-proportional hazard model	
Group	*Hazard ratio*	*95% CI of ratio*
b12 vs control	21.3*	1.7; 260.9
LALA vs control	10.1	1.0; 101.0
b	Infection susceptibility	
Group	*Number of 10 $TCID_{50}$ challenges leading to infection*	*Number of 10 $TCID_{50}$ challenges not leading to infection*
Control	4	6
b12	4*	104
LALA	5	61

Table 1. Statistical analyses comparing relative risk of infection between treatment groups. (**a**) The hazard ratios for b12 and LALA-treated animals are calculated using a Cox-proportional hazard model. It shows that b12 and LALA treatment significantly reduced the risk of infection at each challenge by a factor of 21 and 10 times, respectively. (**b**) The reduction in infection susceptibility [22] is also demonstrated by comparing the total number of challenges resulting in infection to the total number of challenges not leading to infection. *To prevent positive bias, BK10 has been included in this analysis as if it was infected in first challenge.

An analysis of peak viremias suggests a trend towards lower peak viremias in the b12-treated group compared to controls although this difference does not achieve significance (**Suppl. Fig. 3**). However, there is a significant difference ($p = 0.016$), about 2 orders of magnitude, between peak viremias in the b12 and LALA–treated animals, again consistent with an impact of effector function on anti-viral activity.

We determined antibody serum concentrations throughout the course of the experiment by ELISA (**Suppl. Fig. 4**). Considerable variations in individual serum concentration were found, but no significant correlation was found between average concentration and the number of challenges to infection. Likewise, the appearance of

infection did not correlate with the magnitude of the antibody concentration at the estimated time of infection (10 – 17 days prior to detection of virus). Neutralizing antibody titers in sera were assessed in a pseudovirus assay and were as expected based on previous studies[10,20] given the antibody concentrations measured by ELISA (**Suppl. Table 2**). Average b12 concentrations for challenges not resulting in infection was relatively low, about 40 μg/ml, corresponding to an average 1:200 IC_{50} titer in a pseudovirus assay and to an estimated 1:5 IC_{90} titer in a PBMC assay (**Table 2**). MHC genotyping revealed that there was no apparent correlation with the allelic profiles of the animals in this study that would account for any unusual ability to resist infection (**Suppl. Table 3**).

Animal	# of *i.v.* Ab treatments without infection	Average serum Ab [µg/ml][1]	Average IC_{50} pseudovirus assay[2]	Average IC_{90} PBMC assay[3]
LALA-treated				
DC60	9	25	1:125	1:3
BG27	11	26	1:130	1:3
BL45	13	46	1:230	1:6
CL95	15	33	1:165	1:4
BA16	18	37	1:185	1:5
b12-treated				
BK10	5	31	1:155	1:4
BR05	9	25	1:125	1:3
BF53	19	60	1:300	1:8
N364	27	53	1:265	1:7
BF68[4]	28	40	1:200	1:5

Table 2. Average serum antibody concentrations and neutralization titers in macaques repeatedly challenged with a low dose of $SHIV_{162P3}$ in the period before they became infected. [1]Average serum concentration of transferred b12 and LALA for each macaque prior to infection. [2]Average neutralization titer estimated from the average serum concentrations and b12 and LALA IC_{50} values in the pseudovirus assay (=0.2 μg/ml). [3]Average neutralization titer estimated from the average serum antibody concentration and b12 and LALA IC_{90} values in a PBMC-based assay (=8 μg/ml). [4]BF68 did not become infected after 40 challenges at 10 $TCID_{50}$.

In summary, we have shown that neutralizing antibody can provide clear benefit against repeated low-dose SHIV challenge in the macaque model at low serum antibody concentrations corresponding to modest neutralization titers. There is a concern that low-dose challenge models may be "lowering the bar" too much in terms of the requirements for protection. In this context, we note that oral chemoprophylaxis is possibly less, and certainly not more, protective against $SHIV_{SF162P3}$ challenge in the low-dose repeated challenge model, arguing that the model is not intrinsically and universally more susceptible to protective intervention[21]. If translated into protection against HIV infection in humans, the

findings are a promising development for HIV vaccine design. Serum neutralizing antibody titers in the approximate range of 1:200 (IC_{50} values in a pseudovirus assay) corresponding to about 1:5 (IC_{90} values in a PBMC assay) increased the number of low-dose challenges to achieve infection here by at least an order of magnitude. If vaccination in humans led to a similar decrease of transmission rate, then one might expect a significant impact on the pandemic. Neutralizing titers above are near or below those described in the sera of a significant proportion of HIV-infected donors against multiple isolates from different clades[23-27] suggesting that such titers may be achieved with appropriate immunogens. Finally, the data further support the contribution of effector function in antibody resistance to HIV infection, underscoring the notion that the ability of an immunogen to elicit extra-neutralizing antibody activities in addition to neutralization should be assessed in vaccine evaluation.

METHODS SUMMARY

Animals used in this study were female Indian rhesus macaques challenged intravaginally with $SHIV_{SF162P3}$[28,29]. An Fc variant of b12, LALA, was created by site directed mutagenesis[30]. Recombinant antibodies were expressed in Chinese hamster ovary (CHO-K1) cells, purified using a Protein A affinity matrix, and monitored for endotoxin contamination[10]**.** Plasma viral loads were assessed by quantitative assay for the measurement of SIV RNA using QRT-PCR[31,32]. MHC genotyping by sequence-specific PCR was performed by the University of Wisconsin Genotyping Core[33,34]. Antibody concentrations in serum and measurement of binding to $SHIV_{SF162P3}$ gp120 were determined by ELISA[10]. Neutralization of mAbs and sera were performed using an HIV-1 envelope pseudotyped luciferase $SHIV_{SF162P3}$ assay by Monogram Biosciences[35]. Statistical analyses were performed using Graph Pad Prism for Mac Software, Version 5.0a, San Diego, CA, USA.

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AUTHOR CONTRIBUTIONS

Project planning was performed by A.J.H., L.H., P.A.M., D.R.B.; experimental work by A.J.H, L.H., M.H., D.T.; data analysis by A.J.H., L.H., P.P, W.B., P.W.H.I.P, D.R.B.; and A.J.H., P.P., P.W.H.I.P and D.R.B. composed the manuscript.

METHODS

Macaques. All protocols for female Indian rhesus macaques were reviewed and approved by the Institutional Animal Care and Use Committees. The animals were housed in accordance with the American Association for Accreditation of Laboratory Animal Care Standards. At the start of all experiments, all animals were experimentally naïve and were negative for antibodies against HIV-1, SIV, and type D retrovirus. Virus challenges and *i.v.* antibody protocols are more fully described elsewhere[10].

Challenge virus. The virus used in this study was $SHIV_{SF162P}$ passage 3, which has been described elsewhere[28,29]. $SHIV_{SF162P3}$ retains the R5 phenotype of $HIV\text{-}1_{SF162}$. $SHIV_{SF162P3}$·propagated in phytohemagglutin (PHA)-activated rhesus macaque peripheral blood mononuclear cells (PBMC), was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Cat. No. 6526; Contributors: Drs. Janet Harouse, Cecilia Cheng-Mayer, and Ranajit Pal).

b12 and variant antibody LALA. IgG1 b12 is a human antibody (IgG1,) that recognizes an epitope overlapping the CD4 binding site of gp120[17,19]**.** Variants of b12 were created by site-directed mutagenesis as previously described[30].

Antibody production. Recombinant IgG1 (wild type b12, isotype control, and b12 LALA variant (L234A, L235A) were expressed in Chinese hamster ovary (CHO-K1) cells in glutamine-free custom formulated Glasgow minimum essential medium (GMEM Selection Media) (MediaTech Cellgro) [10]. The isotype control antibody DEN3, an anti-Dengue NS1 human IgG1 antibody, was used in this study. For large-scale tissue culture, media was supplemented with 3.5% Ultra Low Bovine IgG Fetal Bovine Serum (Invitrogen) and grown in 10-layer Cellstacks and Cell Cubes (Corning). Antibodies were purified using Protein A affinity matrix (GE Healthcare), and dialyzed against phosphate-buffered saline (PBS). Care was taken to minimize endotoxin contamination, which was monitored using a quantitative chromagenic Limulus Amoebecyte Lysate assay (Cambrex) performed according to the manufacturer's recommendations. Antibody used for the passive transfer experiments contained less than 1 IU of endotoxin mg^{-1}.

Plasma viral loads. The quantity of SIV viral RNA genomic copy equivalents (vRNA copy Eq/ml) in EDTA-anti-coagulated plasma was determined using a quantitative reverse-transcription PCR (QRT-PCR) assay as previously described[32]. Briefly, vRNA was isolated from plasma using a GuSCN-based procedure as described[31]. QRT-PCR was performed using the SuperScript III Platinum® One-Step Quantitative RT-PR System (Invitrogen, Carlsbad, CA). Reaction mixes did not contain bovine serum albumin (BSA). Reactions were run on a Roche LightCycler 2.0 instrument and software. vRNA copy number was determined using LightCycler 4.0 software (Roche Molecular Diagnostics, Indianapolis, IN) to interpolate sample crossing points onto an internal standard curve prepared from 10-fold serial dilutions of a synthetic RNA transcript representing a conserved region of SIV *gag*.

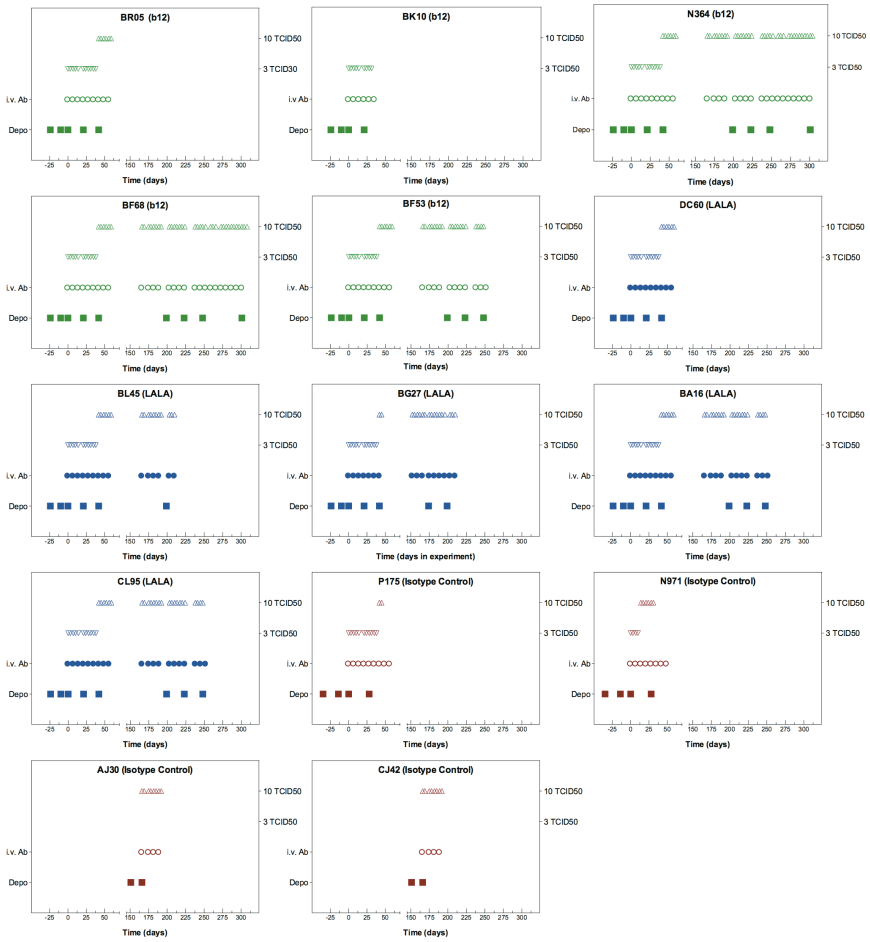
ELISA. b12 and variant antibody concentrations in macaque sera were determined by ELISA against recombinant monomeric HIV-1 $gp120_{JR\text{-}FL}$ (kindly provided by Progenics) and is fully described elsewhere[10].

Neutralization assays. Neutralization titers in animal sera were reported by Monogram Biosciences after preparation of an HIV-1 envelope pseudotyped luciferase $SHIV_{SF162P3}$ capable of single-round replication and performed as previously described[35].

MHC genotyping. MHC genotyping by sequence-specific PCR was performed by the University of Wisconsin Genotyping Core with support of NIH grant 5R24RR16038-6 awarded to David I. Watkins and previously described [34].

Statistics. The isotype control groups consisted of a total of 4 animals (n=4), and each of the treated groups consisted of 5 animals (n=5). Statistical analyses were performed using Graph Pad Prism for Mac Software, version 5.0a (Graph Pad Software Inc., San Diego, CA, 2005). A Kaplan-Meier Survival Analysis was performed for Figure 2. The alpha level was 0.05.

Supplementary Information Figures and Tables

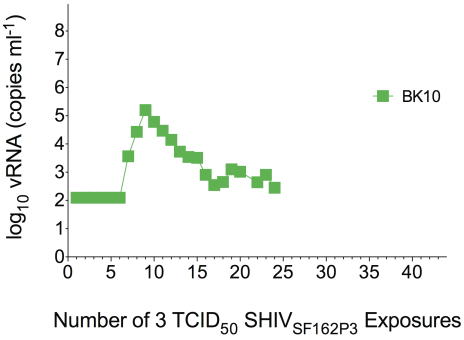


Supplementary Figure 1. Course of treatment for macaques in the low-dose repeated challenge experiment. For all treatment groups, antibody was passively transferred intravenously each week. Viral challenges are separated according to dosage of inoculums (3 $TCID_{50}$ and 10 $TCID_{50}$ doses). Time is representative of actual calendar days from the beginning of the experiment. The main experiment was paused at day 66 and resumed on day 151 and is depicted as a break in the x-axis. *Symbol key*: Depo-Provera treatments, squares; Antibody treatments, open circles; 3 TCID50 viral challenges, down triangles; 10 TCID50 viral challenges, up triangles

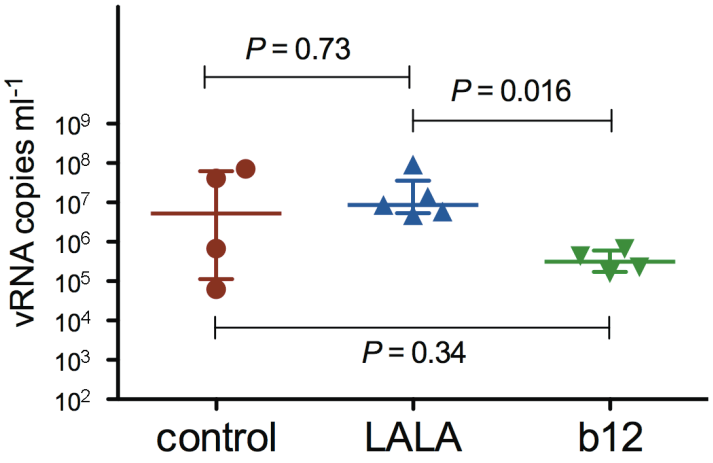
The viral dose that requires several inoculations to infect untreated control animals but yet eventually infects all animals, was estimated to be in the range of 2 to 10 $TCID_{50}$. The experiment was initially designed for repeated vaginal challenges of Depo-Provera-treated

female macaques at a dose of 3 $TCID_{50}$ $SHIV_{SF162P3}$. Depo-Provera is used to synchronize menstrual cycles of the animals. Each Depo-Provera administration is shown as a solid square. Experimental animals received weekly injections of antibody on Thursdays (Day 0) and were virally challenged on Fridays (Day +1) and again on Mondays (Day +4), i.e. weekly antibody treatment and twice weekly viral challenges. The half-life of b12 in macaques is approximately 7 days so this regimen was expected to maintain a relatively stable serum antibody titer. When viremia was detected in only 1 animal (b12-treated BK10) after 11 challenges at 3 $TCID_{50}$, the viral inoculum was increased to 10 $TCID_{50}$. The controls (N971 and P175) at this stage of the experiment were infected after 2 and 4 challenges of 10 $TCID_{50}$, respectively. No additional b12 and LALA-treated animals were infected at this time.

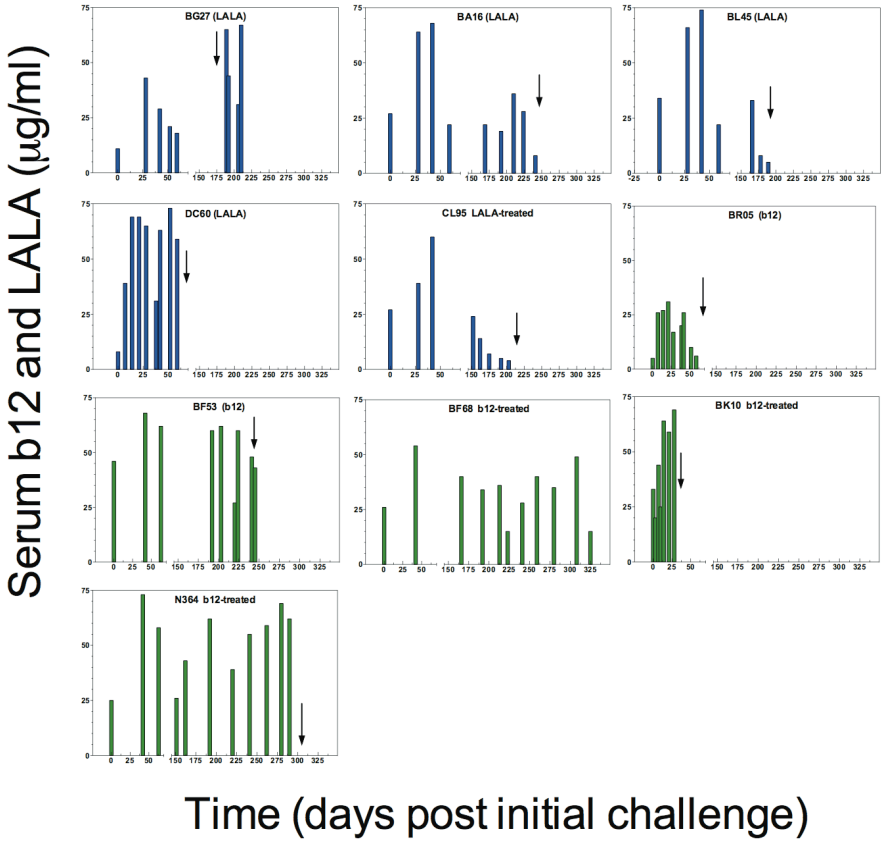
The study design provided for challenges and *i.v.* antibody treatments to cease for those animals when plasma viremia was detected. However, a preliminary monitoring by non-quantitative PCR monitoring led to 4 animals (LALA-treated BG27, CL95 and b12-treated BF53, N364) being erroneously designated as infected and the animals were removed from the treatment and challenge regimen and the experiment was paused at day 66 and resumed on day 151. Subsequent SIV vRNA plasma evaluations revealed that the animals were not viremic and a treatment schedule for these animals was re-established to equalize the number of challenges and antibody transfers among all animals remaining in the experiment. When the full protection experiment resumed, a new group of two control animals was incorporated into the experiment. Depo-Provera treatments were re-established and treatment schedules for antibody administration and viral challenges began in the same manner as described above. Both new control animals (AJ30 and CJ42) became PCR+ for vRNA after 2 challenges. At the conclusion of the experiment, no detection of plasma SIV vRNA for b12-treated BF68 had been observed after receiving 40 challenges at 10 $TCID_{50}$.



Supplementary Figure 2. BK10 was infected during the 3 $TCID_{50}$ challenge period. A single experimental animal, b12-treated BK10, was infected after 6 viral exposures of 3 $TCID_{50}$. Viral challenges and *i.v.* antibody treatments were suspended after positive detection of virus in plasma but the course of infection was monitored for several weeks. SIV viral RNA (vRNA) assay detection limit is 125 copies ml^{-1} (log 2.1).



Supplementary Figure 3. Comparison of peak viremias in control, b12 and LALA-treated macaques. 2 of 4 isotype control animals experienced peak viremia above 5 x 10^7 SIV vRNA copies ml^{-1}. Peak viremias in all LALA-treated animals ranged from 5 x 10^6 to 9 x 10^7 vRNA ml^{-1}. The difference between the b12 and control antibody-treated group does not achieve significance (p=0.34) but a significant difference is observed between the b12 and LALA-treated group (p = 0.016). Analyses performed with One-way ANOVA followed by a Bonferroni posttest, in Graph Pad Prism Software for Mac, Vresion 5.0a.



Supplementary Figure 4. Serum b12 and LALA antibody concentrations during repeated challenge experiment. Serum b12 and LALA antibody concentrations were measured by ELISA at several time points from samples drawn prior to each *i.v.* antibody transfer (day 0) and on days of viral challenge (days = +1 and +4). Animals treated with b12 maintained somewhat higher serum antibody concentrations overall than LALA-treated animals, but no direct correlation is seen between antibody concentration and length of time to infection. Arrows indicate first detection of plasma virus.

Treatment Group	Animal	Antibody Treatments	10 $TCID_{50}$ challenges	Day of Viremia Detection	Day of Viremia Peak
Isotype controls	P175	9	4	56	62
	N971	8	[4]2	49	52
	AJ30	2	2	[3]175	185
	CJ42	2	2	[3]175	185
LALA-treated	DC60	9	6	169	182
	BG27	11	8	175	182
	BL45	13	12	189	192
	CL95	15	17	213	217
	BA16	18	23	241	245
b12-treated	BK10	5	[1]0	28	35
	BR05	9	6	66	73
	BF53	19	23	245	245
	N364	27	38	304	308
	BF68	28	[2]40	n/a	n/a

Supplementary Table 1. Treatment summary.
Total number of *i.v.* antibody treatments and viral challenges before detection of viremia are indicated. The day of viremia detection is the day SIV vRNA was first detected in EDTA-treated plasma following the initial virus challenge. The day of peak viremia is the measurement of highest vRNA ml^{-1} in the plasma on the day indicated.
[1]BK10 was viremic after 6 challenges of 3 $TCID_{50}$.
[2]BF68 received 40 challenges at 10 $TCID_{50}$ and remained uninfected.
[3]The second phase of the experiment began on day 151. AJ30 and CJ42 were included as new isotype-treated controls.
[4]It is possible that N971 became infected during the initial course of 3 $TCID_{50}$ viral challenges. Virus was detected in plasma 10 days following the last challenge at 3 $TCID_{50}$.

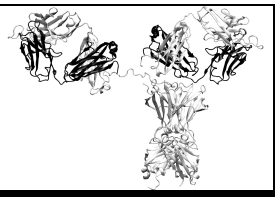
Supplementary Table 2 LALA-treated				
Animal	**Day**	**IC_{50}[1]**	**IC_{90}[1]**	**SerAb [µg/ml][2]**
DC60	0	152	19	8
	49 (-17d)	426	45	63
	52 (-14d)	395	43	73
	56 (-10d)	259	30	59
BG27	0	110	13	11
	159 (-17d)	152	21	21
	162 (-14d)	127	11	18
	166 (-10d)	215	28	ND
BL45	0	132	<10	34
	174 (-17d)	47	<10	33
	177 (-14d)	<10	<10	8
	181 (-9d)	<10	<10	0
BA16	0	171	23	27
	220 (-21d)	76	<10	36
	224 (-17d)	165	15	28
	241 (det)[3]	20	<10	36
CL95	0	149	15	27
	192 (-28d)	19	<10	5
	196 (-17d)	<10	<10	4
	213 (det)[3]	16	<10	0
Supplementary Table 2 b12-treated				
BF53	0	139	13	46
	217 (-28d)	319	ND	62
	220 (-25d)	172	31	27
	241 (-4d)	181	22	48
BR05	0	94	11	5
	49 (-17d)	132	17	ND
	52 (-14d)	62	<10	10
	56 (-10d)	74	<10	6
N364	0	214	18	25
	280 (-24d)	471	58	69
	283 (-21d)	487	57	ND
	290 (-14d)	397	41	55
BK10	0	152	15	33
	11 (-17d)	148	16	25
	14 (-7d)	206	26	64
	28 (det)[3]	391	38	31
BF68[4]	0	143	14	26
	42	277	32	54
	166	254	29	40
	192	189	21	34
	241	214	23	28
	262	130	16	40
	280	215	32	43

Supplementary Table 2. Neutralization titers and antibody concentrations of transferred b12 and LALA in the serum of animals are shown at the day of initial challenge (Day 0) and at the 3 closest time points prior to the day of detection of plasma virus (indicated in parenthesis).
[1]Numbers represent reciprocal dilution of serum producing 50% and 90% neutralization as reported by Monogram Biosciences in a pseudovirus assay. [2]Serum antibody concentrations were determined by ELISA using 3 different formats.
[3](det) refers to day of detection of vRNA in plasma.
[4]BF68 did not become infected after 40 challenges at 10 $TCID_{50}$.
ND means no data.

Animal ID	A01	A02	A08	A11	B01	B03	B04	B08	B17
PI75	-	+	-	-	+	-	-	-	-
N971	+	+	-	-	-	-	-	-	+
AJ30	-	+	-	-	+	-	-	-	-
CJ42	-	-	-	+	-	-	-	-	-
DC60	-	-	-	-	+	-	-	-	-
BG27	-	-	-	+	+	-	-	-	-
BL45	-	-	+	-	-	-	-	-	-
CL95	-	+	-	-	+	-	-	-	-
BA16	-	+	+	-	+	-	-	-	-
BK10	-	-	-	-	-	-	-	-	-
BR05	-	+	-	-	-	-	-	-	-
BF53	-	+	-	-	+	-	-	-	-
N364	+	-	-	-	+	-	-	-	+
BF68	-	-	+	-	-	-	-	-	-

Supplementary Table 3. A panel of 9 class I alleles that have been shown to be important in SIV epitope presentation were included in the evaluation[19,20]. No direct correlation with the allelic profiles of the animals in this study could be made from the results that would account for an unusual ability to resist infection. Further, neither of the *Mamu-B**17 or *Mamu-B**08 alleles known to confer SIV resistance[19,20] were identified to be present in BF68, a b12-treated animal that resisted infection from 40 low dose challenges. Color-coded for treatment groups: Green = b12; Blue = LALA; Brown = Isotype Controls.

Chapter 7



Broadly Neutralizing Human Anti-HIV Antibody 2G12 is Effective in Protection Against Mucosal SHIV Challenge Even at Low Serum Neutralizing Titers

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Abstract

Developing an immunogen that elicits broadly neutralizing antibodies (bNAbs) is an elusive but important goal of HIV vaccine research, especially after the recent failure of the leading T cell based HIV vaccine in human efficacy trials. Even if such an immunogen can be developed, most animal model studies indicate that high serum neutralizing concentrations of bNAbs are required to provide significant benefit in typical protection experiments. One possible exception is provided by the anti-glycan bNAb 2G12, which has been reported to protect macaques against CXCR4-using SHIV challenge at relatively low serum neutralizing titers. Here, we investigated the ability of 2G12 administered intravenously (*i.v.*) to protect against vaginal challenge of rhesus macaques with the CCR5-using $SHIV_{SF162P3}$. The results show that, at 2G12 serum neutralizing titers of the order of 1:1 (IC_{90}), 3/5 antibody-treated animals were protected with sterilizing immunity, i.e. no detectable virus replication following challenge, one animal showed a delayed and lowered primary viremia and the other animal showed a course of infection similar to 4 control animals. This result contrasts strongly with the typically high titers observed for protection by other neutralizing antibodies, including the bNAb b12. We compared b12 and 2G12 for characteristics that might explain the differences in protective ability relative to neutralizing activity. We found no evidence to suggest that 2G12 transudation to the vaginal surface was significantly superior to b12. We also observed that the ability of 2G12 to inhibit virus replication in target cells through antibody-mediated effector cell activity *in vitro* was equivalent or inferior to b12. The results raise the possibility that some epitopes on HIV may be better vaccine targets than others and support targeting the glycan shield of the envelope.

Author Summary

An effective HIV vaccine should elicit broadly neutralizing antibodies, i.e. antibodies that neutralize a wide spectrum of different HIVs *in vitro.* A number of human monoclonal antibodies have been isolated with broad neutralization and shown to protect macaques against vaginal HIV challenge. Protection is generally correlated with neutralization and requires relatively high antibody concentrations that may be difficult to achieve by vaccination. Here, we show that one monoclonal antibody (2G12) is unusually potent in protection relative to its neutralizing ability as hinted at by earlier data. Further studies eliminate an unusual ability of 2G12 to be transported to the vagina (site of infection) as a possible explanation for our observations. Although the precise mechanism is unclear, the studies have important implications for HIV vaccine design in general by suggesting that some vaccine targets on HIV may be better than others and, specifically, by suggesting that the sugar coat of HIV may be a particularly rewarding target if appropriate immunogens can be designed.

Introduction

There is widespread acceptance that eliciting neutralizing antibodies is likely to be an important goal of an effective HIV vaccine [1,2,3]. A good correlation is generally reported between the ability of an antibody to neutralize *in vitro* and to protect *in vivo* against HIV in animal models [4,5,6,7,8,9]. The most quantitative studies have titrated the ability of specific antibodies to protect and found that sterilizing immunity is achieved when the serum concentration of antibody in the challenged animals is many multiples of the *in vitro* neutralization titer [4,8,10]. For instance, Nishimura, *et al*. reported that 99% of macaques were protected against intravenous challenge with an R5 $SHIV_{DH12}$ by a specific polyclonal antibody at a 100% neutralization titer of 1:38 [10]. In another example, we have reported sterilizing immunity against R5 $SHIV_{SF162P4}$ vaginal challenge in 4/4 macaques with a dose of the broadly neutralizing human antibody b12 yielding a serum neutralizing titer of about 1:400 at challenge [8]. The titer corresponded to 90% neutralization in a PBMC assay. Nishimura et al [10] estimated that this titer corresponded to 1:32.5 or greater in their assay system providing good correspondence between the two studies. At an antibody dose giving a serum neutralizing titer of about 1:80 in the Parren, *et al.* study, 2/4 macaques showed sterilizing immunity and the other 2 were infected with a delayed and lower primary viremia as compared to controls. At an antibody dose giving a serum neutralizing titer of about 1:16, no animal was protected but there was a slight delay and some lowering in the magnitude of primary viremia.

Most other studies have not titrated the ability of antibodies to protect but high serum concentrations of antibody relative to neutralizing titer were generally used and shown to provide protection against virus challenge [4,5,6,9,11]. The one notable exception is provided by studies of Mascola and colleagues [7] on protection by the broadly neutralizing human MAb 2G12. In particular 2/4 macaques showed sterilizing immunity when challenged by an X4 SHIV ($SHIV_{89.6P}$) when the serum neutralizing titer, as measured at 90% neutralization in a PBMC assay, was less than 9. In fact, the mean concentration of 2G12 in the sera of the animals at challenge was calculated to provide 90% neutralization only with neat serum (i.e. 1:1 neutralizing titer). The actual concentration of 2G12 in the protected animals at the time of challenge was relatively high, about 200 μg/ml following an *i.v.* administration of 15 mg/kg antibody, but 2G12 is relatively poor at neutralization of $SHIV_{89.6P}$ (IC_{90} ~200μg/ml) hence the low neutralizing titer. The authors also carried out protection experiments with mixtures of antibodies, including 2G12. These experiments when taken together again suggested that 2G12 may provide protection that is unusually effective relative to its neutralizing titer.

Monoclonal human IgG1 2G12 is a very interesting and unique antibody. It is broadly neutralizing, particularly against clade B HIV-1 isolates [12,13,14]. It has a domain-exchanged structure that leads to closely proximal antibody combining sites that are well suited to the recognition of a cluster of oligomannose residues on the glycan shield of HIV [12,15,16,17,18]. 2G12 belongs to a small set of human MAbs that are described as broadly neutralizing and that recognize distinct epitopes on the HIV envelope spike. The MAb b12 recognizes an epitope overlapping the CD4 binding site "on the side" of the spike and the MAbs 2F5, 4E10 and Z13e1 recognize gp41 very close to the viral membrane, whilst 2G12 recognizes an epitope which is more on the "top" of the spike [19,20,21].

Given the suggestion that 2G12 may have unusual prophylactic activities and given the potential importance of this for HIV vaccine design, we decided to carry out a macaque protection study using a virus different from that of Mascola and colleagues and to pursue potential properties of 2G12 that might correlate with protection. Ideally, we would have had available a SHIV that was relatively neutralization sensitive to 2G12 to permit study of a maximum dynamic range of 2G12 concentrations with neutralizing activity. However, currently available SHIVs are relatively resistant to 2G12 and the R5 virus $SHIV_{SF162P3}$ was chosen as the most sensitive to 2G12 neutralization. An R5 virus was thought to be more appropriate for modeling human infection than an X4 virus. The challenge virus was used intravaginally following pre-administration of 2G12 intravenously. The results indicate that 2G12 can provide protection against an R5 virus challenge at a surprisingly low neutralization titer. Unusually efficient transport to the vaginal mucosal surface does not appear to explain the activity of 2G12. The results support targeting the glycan shield through vaccine design.

Results

The ability of 2G12 to neutralize a panel of SHIVs in PBMC and pseudovirus assays was first assessed. A comparison with b12 was included in the study. As shown in **Table 1**, 2G12 was not particularly effective against any of the SHIVs tested. The activity of 2G12 against the R5 $SHIV_{SF162P3}$ was comparable to that against the X4 $SHIV_{89.6P}$ used in previous studies described above and was chosen for macaque studies.

Virus	MAb	Rhesus PBMC-based Neutralization Assay		Pseudovirus-based Neutralization Assay	
		(μg/ml)			
		IC_{50}	IC_{90}	IC_{50}	IC_{90}
SHIV 89.6P	2G12	20	>900	2.6	>50
	b12	300	>900	11.5	>50
SHIV SF162P3	2G12	20	900	7.6	>50
	b12	2	8	0.29	2
SHIV BaL	2G12	>300	>300	1	>50
	b12	<1.2	<1.2	0.02	0.09

Table 1. Comparison of SHIV neutralization by b12 and 2G12 in rhesus PBMC-based and pseudovirus luciferase reporter gene assays. The selection of $SHIV_{SF162P3}$ for the protection study was based on the results of 2G12 neutralization of rhesus PBMCs and pseudovirus assays against the panel shown.

Figure 1 depicts the outcome of the protection study that was carried out with five 2G12-treated animals, two antibody isotype (anti-Dengue NS1 IgG1, DEN3) treated control animals and two antibody-untreated control animals. The Indian rhesus macaques were first treated with Depo-Provera to thin the vaginal epithelium and to synchronize menstrual cycles [8,22]. One day before vaginal challenge with 500 $TCID_{50}$ (50% tissue culture infectious doses) of $SHIV_{SF162P3}$, each animal was given an intravenous dose of 40 mg/kg of either 2G12 or the isotype control antibody. Prior to the protection experiment, two additional control animals were challenged with 500 $TCID_{50}$ $SHIV_{SF162P3}$ without administration of antibody to verify the infectivity of the viral stock. Blood was drawn from the animals at regular intervals following challenge to monitor viral infection, serum levels of passively administered antibody and serum neutralizing activity. The 4 control animals became infected with peak viremias of approximately 10^7 virus copies per ml between days 14 and 21 as is generally noted in this system [9]. Two of the five 2G12-treated animals also became infected. One was infected with viral kinetics closely similar to that of the control animals. The second had a notably delayed and lower peak viremia at day 35. Three of the five 2G12-treated animals were protected and showed no detectable viremia at day 55. In order to determine whether breakthrough infection may be associated with selection of antibody escape mutants, we attempted to sequence the env gene from plasma virus of the unprotected animals. Env from animal 95113 could not be amplified but interestingly sequence analysis of animal 90154 env revealed a T388A mutation disrupting the position 386 N-glycosylation and consistent with 2G12 escape. The characterization of a 2G12 escape mutant in one of the unprotected animals raises the possibility that such variants already exist in the inoculum. Alternatively, in the presence of Ab, in particular at the suboptimal concentration achieved in the study, a certain level of viral replication may take place following challenge, allowing in some cases for the generation and selection of an escape mutant. Such scenario would suggest that the mechanism of antibody protection is not only to prevent cell infection but also to abort an already ongoing infection, presumably though effector functions as discussed below. These two possible scenarios for escape are currently under investigation.

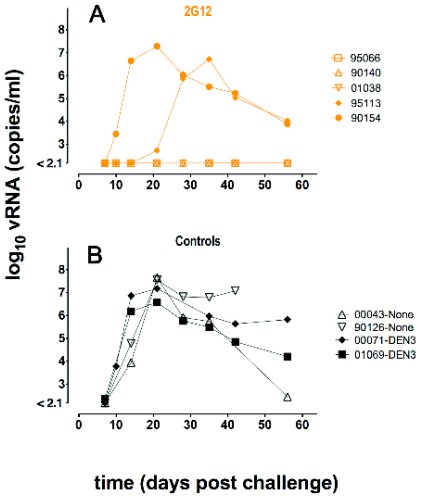

Figure 1. Plasma viral loads following $SHIV_{SF162P3}$ vaginal challenge of 2G12-treated and control macaques. A total of nine female Indian rhesus macaques were divided into treatment groups of five animals for *i.v.* administration of 2G12, two animals to receive the isotype control (Dengue anti-NS1, DEN3), and two additional controls were challenged prior to the beginning of the protection study to confirm viral fitness, but were not treated with antibody. In (**A**) two 2G12-treated (40 mg/kg) animals became infected: 90154 reached peak viremia of 2 x 10^7 on day 21 similar to controls; 95113 showed a one-week delay of infection onset and peak viremia was lower at 5 x 10^6. The remaining three 2G12-treated animals were protected against infection and showed no measurable viremia. In (**B**) all 4 control animals experienced peak viremia between 1 X 10^7 and 4 x 10^7 on day 21. The quantity of SIV viral RNA genomic copy equivalents (vRNA copy Eq/ml) in EDTA-anticoagulated plasma was determined using quantitative RT PCR [52]. The assay minimum detection is 150 copies of vRNA Eq/ml (2.1 log) with a 99% confidence level.

Table 2. 2G12 serum antibody concentrations following passive administration.

Protected				Not Protected			
95066	**gp120**	**Man 4D**	**anti-Id**	**95113**	**gp120**	**Man 4D**	**anti-Id**
Day	**Serum Ab µg/ml**			**Day**	**Serum Ab µg/ml**		
-1	0	0	0	**-1**	0	0	0
0	1215	1241	911	**0**	822	1146	998
3	696	994	733	**3**	571	494	567
7	793	816	599	**7**	494	510	355
10	698	529	500	**10**	463	456	465
15	767	328	446	**15**	402	134	177
22	651	252	335	**22**	230	49	155
90140	**gp120**	**Man 4D**	**anti-Id**	**90154**	**gp120**	**Man 4D**	**anti-Id**
Day	**Serum Ab µg/ml**			**Day**	**Serum Ab µg/ml**		
-1	0	0	0	**-1**	0	0	0
0	954	901	1250	**0**	1252	1174	922
3	640	704	1005	**3**	750	575	545
7	694	545	838	**7**	597	371	448
10	640	398	705	**10**	554	221	348
15	453	452	502	**15**	453	174	328
22	173	394	505	**22**	231	101	342

1038	**gp120**	**Man 4D**	**anti-Id**
Day	**Serum Ab µg/ml**		
-1	0	0	0
0	1203	921	891
3	959	527	717
7	927	413	714
10	898	365	690
15	605	220	496
22	296	103	147

For each animal, the results from the different ELISA formats are shown in separate columns. The concentrations of 2G12 in the macaque sera were determined from the measurement of binding to monomeric JR-FL (gp120), to an immobilized synthetic oligomannose dendron conjugated to BSA [21] (Man4D), and to a highly specific anti-idiotype-2G12 antibody (MIgG1 L13) [23] (anti-id). In all formats, a dilution series of serum was compared to a 2G12 standard curve and the concentration determined using a nonlinear regression curve fit analysis performed in GraphPad Prism Software for Mac, Version 5.0a.

The 2G12 antibody concentrations in the sera of the macaques at different time points were measured using three different ELISA formats. With a few exceptions, the determined serum concentrations derived from the three formats were generally in good agreement (**Table 2,** opposite page). The first format used was an ELISA based on the ability of 2G12 to specifically recognize a relatively conserved cluster of oligomannose glycans. Serum was titrated against an immobilized synthesized oligomannose dendron conjugated to BSA[21]. The second ELISA format was based on a highly specific anti-idiotype-2G12 antibody (MIgG1 L13) that does not block the binding of gp120 or inhibit the neutralizing ability of 2G12 [23]. For comparison, a third ELISA format using monomeric gp120 $_{JR\text{-}FL}$ was used to measure the transferred 2G12 contained in the macaque serum. In all formats, a dilution series of serum was compared to a 2G12 standard curve and the concentration determined using a non-linear regression curve fit analysis.

The *i.v.* transfer of 2G12 at 40 mg/kg resulted in a high 2G12 serum concentration at the time of challenge that varied between 0.9 and 1.2 mg/ml. **Table 3** summarizes the half-life of serum 2G12 that varied between 7.2 and 15.6 days in the 5 macaques. The average half-life of all animals as measured in the three ELISA formats is about 11 days (Table 3). The half-life of 2G12 in rhesus macaques has previously been noted as about 13 days [5].

Table 3.	Half-life (days) of 2G12 in macaque serum					
	Protected			Not Protected		Average
Animal	95066	90140	01038	95113	90154	(days)
gp120 ELISA	12.2	9.2	7.7	9.2	9.9	9.6
Man4D ELISA	10.7	15.6	7.2	10.5	8.4	10.5
L13 anti-idiotype ELISA	12.9	13.4	9.8	10.4	15.1	12.3

Table 3. Half-life of transferred 2G12 in macaque serum. The data represents the half-life ($t_{1/2}$) of serum 2G12 determined from data in three different ELISA formats over a period of three weeks following *i.v.* transfer of 40 mg/kg of 2G12. The half-life of transferred 2G12 ranged between 7.2 and 15.6 days in the 5 macaques with a somewhat shorter half-life observed in animal 01038. The average half-life of all animals as measured in the three ELISA formats is about 11 days. The half-life of 2G12 in rhesus macaques has previously been noted as about 13 days [5]

While for b12, 90% neutralization titers (IC_{90}) of approximately 1:80 in a PBMC assay were associated with protection in 50% of $SHIV_{SF162P4}$ [8] and 90% of $SHIV_{SF162P3}$ [9] challenged animals, a titer of only 1:1 was sufficient to protect 60% of all animals with 2G12 in the experiment described here. It should be noted the IC_{90} for 2G12 neutralization of PBMC is approximately 900 μg/ml and the serum concentration of 2G12 at challenge was approximately 900 - 1,200 μg/ml making it impractical to directly measure neutralization in the PBMC assay. Using the generally more sensitive pseudovirus assay, 90% neutralization was not reached at a 1:50 serum dilution for any of the 2G12-treated animals. Therefore, it does appear that MAb 2G12 can offer substantial protection at relatively low serum neutralizing titers.

We next compared properties of 2G12 and b12 that might help explain the observed differences in protective activity relative to serum neutralization. One possibility is a gross difference in transudation efficiency for the two MAbs. For b12, it has been noted previously that the concentration of antibody at the vaginal surface following passive administration is only a small fraction of that in the serum [8]. If 2G12 was transudated to the vaginal surface much more efficiently than b12 then it is possible that it could achieve comparable neutralizing titers in vaginal fluids. This might lead to improved protection although it should be noted that no correlation between mucosal antibody levels and protection has been established. Earlier data suggests that 2G12 is not transudated unusually effectively although vaginal concentrations can vary widely [7]. We carried out a direct comparison of vaginal concentrations of b12 and 2G12 for a time period of 7 days after *i.v.* administration of 5 mg/kg MAb to 3 control macaques. As shown in **Figure 2**, the concentrations of the two MAbs transudated to the vaginal mucosal surface are similar and thus transudation is unlikely to contribute to protection differences between the MAbs.

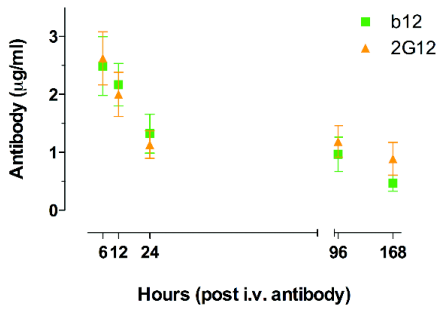

Figure 2. Comparison of b12 and 2G12 transudated to the vagina following intravenous administration. Each antibody treatment group consisted of three female Indian Rhesus macaques which were *i.v.*-administered 5 mg/kg of either b12 or 2G12 following Depo-provera treatment. Vaginal secretions from each animal were absorbed to cellulose wicks. A set of 3 samples per animal was taken at 6 hours, 12 hours, 24 hours, 4 days, and 7 days post *i.v.* antibody administration. The concentration of antibody in mucosal secretions was determined by ELISA from the clarified supernatant extracted from the wicks. Resulting data were compared to the corresponding antibody standard curve using nonlinear regression. Arithmetic means and standard deviations were calculated for each set of triplicate samples per animal. Data points were calculated from all animals at each timepoint and error bars represent the standard error of means. The typical time for viral challenge in protection experiments is indicated. The differences in the mean concentrations of b12 and 2G12 at each timepoint were evaluated in a student's t test and determined to be non-significant. Analyses performed in GraphPad Prism Software for Mac, Version 5.0a.

Evidence has been obtained to suggest that interaction of b12 with Fc receptors can contribute to protection against $SHIV_{SF162P3}$ challenge in macaques [9]. In particular, it was noted that wild-type b12 mediated antibody-dependent cell-mediated virus inhibition (ADCVI) and was effective at protection, whereas a b12 variant lacking FcR binding did not mediate ADCVI and was less effective than the wild-type antibody. If 2G12 were effective at ADCVI then this might contribute to enhanced protective activity. **Figure 3** (opposite page) suggests that 2G12 is somewhat less effective than b12 at ADCVI on the basis of a strict concentration comparison. The significance of this result is discussed further below.

Since two MHC class I alleles (Mamu-B*08 and –B*17) have been associated with elite control of SIV replication, we evaluated all experimental animals by MHC genotyping. We used PCR-SSP to test for a panel of 9 class I alleles previously shown to be important in SIV epitope presentation [24,25,26,27,28] (**Table 4**, opposite page). Protected animal 90140 was positive for *Mamu-A*01*, an allele that appears with high frequency in many colonies and

has been associated with moderate reduction of SIVmac239 replication [27,28,29,30]. Protected animal 95066 expresses the *Mamu-B*01* allele. This allele remains on the panel based on early reports of SIV-derived epitopes [31,32], but studies have shown that Mamu-B*01 does not bind SIV-derived epitopes and has no effect on SIV disease progression [26]. However, even with the presence of the *Mamu-A*01* allele (which is not associated with elite control of SIV replication) in animal 90140, there is no apparent correlation with the allelic profiles of the animals in this study that would account for any unusual ability to resist infection.

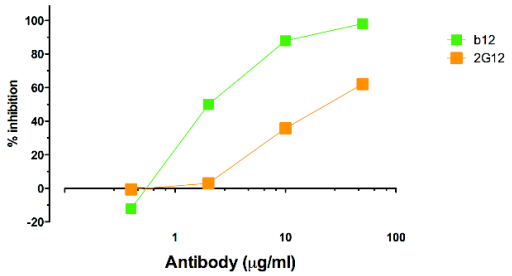

Figure 3. Comparison of antibody-dependent cell-mediated viral inhibition (ADCVI) by 2G12 and b12. Target cells (CEM.NKR-CCR5) were infected with $SHIV_{SF162P3}$ and incubated for 48 hours, washed to remove cell-free virus and combined with Rhesus PBMC effector cells and serially diluted antibody. Viral inhibition was measured after incubation for 7 days. 2G12 is somewhat less effective than b12 in mediating ADCVI for a strict concentration comparison. An unpaired Two-tailed t test (P=0.3285) of b12 and 2G12 ADCVI with an F test comparison of variance reveals no significant difference (P=0.4154). Analysis performed in GraphPad Prism Software for Mac, Version 5.0a.

Table 4. MHC genotyping

Animal	A01	A02	A08	A11	B01	B03	B04	B08	B17
95066^{P}	-	-	-	-	+	-	-	-	-
90140^{P}	+	-	-	-	-	-	-	-	-
01038^{P}	-	-	-	-	-	-	-	-	-
95113^{N}	-	-	-	-	-	-	-	-	-
90154^{N}	-	-	-	-	-	-	-	-	-
00071^{I}	-	-	-	-	-	-	-	-	-
01069^{I}	-	-	-	-	+	-	-	-	-
90126^{C}	-	-	-	-	+	-	-	-	-
00043^{C}	+	-	-	-	-	-	-	-	-

Table 4. MHC genotyping of macaques against MHC Class I alleles. Macaque samples were tested against a panel of nine MHC class I alleles that have previously been shown to be important in SIV epitope presentation or increased resistance to SIV infection [24,25,26]. The alleles are: *Mamu-A*01, Mamu-A*02, Mamu-A*08, Mamu-A*11, Mamu-B*01, Mamu-B*03, Mamu-B*04, Mamu-B*08, and Mamu-B*17.* Animal 90140 is positive for *Mamu-A*01* and animal 95066 was determined to carry the *Mamu-B*01* allele. *Mamu-A*01* has been associated with moderate control of SIVmac239 replication [29,30]. *Mamu-B*01* remains on the panel based on early reports of SIV-derived epitopes [31,32], but subsequent studies show that *Mamu-B*01* does not bind SIV-derived epitopes and has no effect on SIV disease progression [26].
$^{P, N, I, C}$ denotes protected, non-protected, isotype control, and non-antibody treated control animals, respectively.

Discussion

The results presented here lend strong support to the notion that passively administered bNAb 2G12 is able to offer substantial protection against mucosal SHIV challenge at low serum neutralizing titers. In particular, 3 of 5 macaques showed sterilizing immunity on vaginal challenge with a high dose of $SHIV_{SF162P3}$ when serum-neutralizing titers of 2G12 were of the order of 1:1. This result contrasts strongly with protection observed with bNAb b12 when sterilizing immunity for the majority of animals is associated with neutralizing titers of very approximately 1:100 and greater [8,9,33]. The result also contrasts with the quantitative studies of Martin and colleagues [10], which show sterilizing immunity against challenge with the X4 $SHIV_{DH12}$ only at high specific anti-DH12 antibody neutralizing titers. The result is however consistent with studies of Mascola and colleagues who showed that low serum neutralizing titers of 2G12 provided sterilizing immunity for 2 of 4 macaques vaginally challenged with the X4 virus $SHIV_{89.6P}$ [7].

We investigated factors that might help explain the protective efficacy of 2G12, especially in relation to b12. This efficacy might be explained if 2G12 was transported very effectively to the site of infection; the results presented suggest this is not the case. We next noted that, although the neutralizing titers of 2G12 in our experiments are low, the actual serum concentrations of 2G12 are high since the antibody neutralizes the challenge virus $SHIV_{SF162P3}$, and indeed other available SHIVs rather poorly. Therefore, it is possible that the protective efficacy of 2G12 derives from another anti-viral function of antibody that becomes important at high antibody concentration. One such function could be antibody-mediated host cell activity against SHIV-infected cells, which can be measured in the ADCVI assay. The results showed that 2G12 is somewhat less effective than b12 in the ADCVI assay. However, at the serum concentrations achieved in the passive transfer experiments, 2G12 should be able to promote infected cell killing *in vivo*. Therefore, one possible explanation for the differing relationship between neutralization and protection for b12 and 2G12 against $SHIV_{SF162P}$ challenge is that protection is determined not by neutralizing ability but solely by activity against infected cells. An argument against this explanation is provided by the observation that b12 can still provide substantial protection in the complete absence of Fc receptor function and ADCVI [9]. Furthermore, HIVIG, which tends to mediate effective ADCVI (GL and DNF, unpublished observations) is rather ineffective at protection against SHIV challenge [5,7]. An explanation more concordant with the totality of data is that both neutralizing and extra-neutralizing activities are important for protection. Fc-mediated extra-neutralizing activities include, not only host cell activities against infected cells, but also those against free virions such as phagocytosis. It may be that 2G12 is able to compensate for its weak neutralization of $SHIV_{SF162P3}$ by effective extra-neutralizing activities such as ADCVI. Protection studies using a SHIV that is sensitive to neutralization at low 2G12 concentrations but only sensitive to extra-neutralizing activities at high concentrations may help to better separate the contributions of different mechanisms to protection here. Alternatively, a 2G12 mutant lacking effector activity analogous to that generated for b12 [9] may help towards this aim.

One intriguing difference between b12 and 2G12 that might relate to differences in protection has been described in terms of neutralization kinetics (PP, unpublished observations). Thus, it appears that following antibody-virus preincubation *in vitro*, neutralization by 2G12 occurs almost immediately while b12-mediated neutralization slowly progresses with time, reaching 2G12 neutralization levels only after hours. Only at high

concentrations, corresponding to high neutralization titers, do b12 neutralization kinetics match those of 2G12. *In vivo* such kinetic differences may give 2G12 a noticeable advantage over b12: immediate efficiency at preventing target cell infection may be of particular importance *in vivo* as delay in neutralization may lead to cell infection and viral spread spinning out of antibody control. Further studies looking at correlations between antibody neutralization kinetics and protection efficiency should help reveal whether kinetics are important for protection.

A further consideration for 2G12 is that the antibody recognizes high mannose glycans on the envelope gp120 surface. These glycans are also recognized by a number of lectins including DC-SIGN, which has been proposed to have a critical role in transmission by facilitating the transport of virus by dendritic cells to lymphoid tissues [34,35]. Indeed, it has been shown that the addition of an N-glycan site to the V2 loop of SF162P leads to a gain of DC-SIGN binding and that this correlates with enhanced mucosal transmission of $SHIV_{SF162P3}$ [36]. The gp120-DC-SIGN interaction can be perturbed by 2G12 but not b12 as shown in a number of assays including inhibition of whole virus binding to DC-SIGN-expressing cell lines [37,38]. If the HIV-DC-SIGN interaction is critically important for the establishment of infection, then it is possible that 2G12 protection is mediated by inhibiting this interaction. Intriguingly, although 2G12 requires relatively high concentrations to neutralize $SHIV_{SF162P3}$, presumably because of relatively low affinity for the envelope trimer [39,40,41], it binds with high nM affinity to monomeric gp120 from $SHIV_{SF162P3}$. HIV is suggested to express both functional and other forms of envelope including monomeric envelope [42,43]. If DC-SIGN was exploiting nonfunctional as well as or instead of functional envelope on virions then 2G12 might be an efficient competitor for binding to nonfunctional envelope at the *in vivo* concentrations achieved in our passive experiments. Future studies on the potential role of inhibiting the HIV-DC-SIGN interaction in blocking transmission could make use of anti-DC-SIGN antibodies.

In summary, the data presented here, together with earlier data, show conclusively that monoclonal antibody 2G12 can offer protection against mucosal SHIV challenge at low neutralization titers. An explanation based on unusual 2G12 transudation properties to the mucosal surface is ruled out. Viable explanations include: (1) rapid 2G12 neutralization kinetics, (2) a critical role for extra-neutralizing, e.g. Fc-mediated, 2G12 activities under the conditions of the experiment, and (3) a critical role for 2G12 inhibition of virus interaction with lectin, e.g. DC-SIGN, bearing cells. Further *in vivo* protection studies will be required to distinguish these possibilities. Nevertheless, the results are provocative in suggesting the glycan shield as a potentially favorable HIV vaccine target.

MATERIALS AND METHODS

Macaques. All protocols for female Indian rhesus macaques were reviewed and approved by the Institutional Animal Care and Use Committees. The animals were housed in accordance with the American Association for Accreditation of Laboratory Animal Care Standards. At the start of the experiments, all animals were experimentally naïve and were negative for antibodies against HIV-1, SIV, and type D retrovirus. Virus challenge and *i.v.* antibody protocols are more fully described elsewhere [8,44].

Challenge virus. The virus used in this study was $SHIV_{SF162P}$ passage 3, which has been described elsewhere [45,46,47]. $SHIV_{SF162P3}$ retains the R5 phenotype of $HIV\text{-}1_{SF162}$. $SHIV_{SF162P3}$, propagated in phytohemagglutin (PHA)-activated rhesus macaque peripheral blood mononuclear cells (PBMC), was

obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Cat. No. 6526; Contributors: Drs. Janet Harouse, Cecilia Cheng-Mayer, and Ranajit Pal).

Antibodies. Recombinant 2G12 was obtained from Polymun Scientific, Vienna, Austria. The isotype control antibody DEN3, an anti-Dengue NS1 human IgG1 antibody was expressed in Chinese hamster ovary (CHO-K1) cells in glutamine-free custom formulated Glasgow minimum essential medium (GMEM Selection Media) (MediaTech Cellgro). For large-scale tissue culture, media was supplemented with 3.5% Ultra Low Bovine IgG Fetal Bovine Serum (Invitrogen) and grown in 10-layer Cellstacks and Cell Cubes (Corning). The antibody was purified using Protein A affinity matrix (GE Healthcare), and dialyzed against phosphate-buffered saline (PBS). Care was taken to minimize endotoxin contamination, which was monitored using a quantitative chromagenic Limulus Amoebecyte Lysate assay (Lonza) performed according to the manufacturer's recommendations. Antibody used for the passive transfer experiments contained <1 IU of endotoxin/mg.

Plasma viral loads. The quantity of SIV viral RNA genomic copy equivalents (vRNA copy Eq/ml) in EDTA-anticoagulated plasma was determined using a quantitative reverse-transcription PCR (QRT-PCR) assay. Briefly, vRNA was isolated from plasma using a GuSCN-based procedure as previously described [48]. QRT-PCR was performed using the SuperScript III Platinum® One-Step Quantitative RT-PR System (Invitrogen, Carlsbad, CA). Reaction mixes did not contain bovine serum albumin (BSA). Reactions were run on a Roche Lightcycler 2.0 instrument and software. vRNA copy number was determined using LightCycler 4.0 software (Roche Molecular Diagnostics, Indianapolis, IN) to interpolate sample crossing points onto an internal standard curve prepared from 10-fold serial dilutions of a synthetic RNA transcript representing a conserved region of SIV *gag*.

Serum antibody ELISAs. 2G12 antibody concentrations in macaque sera were determined in ELISA by three different methods: (1) by binding to an immobilized synthetic oligomannose dendron [21] conjugated to BSA; (2) by binding to the anti-Idiotype 2G12 mouse IgG1 L13 kindly provided by Polymun Scientific, Vienna, Austria [49]; and (3) by binding to monomeric gp120 JR-FL kindly provided by Progenics [8].

Antibody concentrations in vaginal secretions. The determination of antibody concentration in mucosal secretions was performed as described by Kozlowski, et al. [8,50]. Briefly, vaginal secretions from each animal were absorbed to cellulose wicks (Solan Weck-Cel surgical spears; Xomed Surgical Products, Jacksonville, FL). A set of 3 samples per animal was taken at 6 hours, 12 hours, 24 hours, 4 days, and 7 days post *i.v.* antibody administration. Wicks were weighed before and after secretion absorption. Clarified supernatants extracted from the wicks were used to determine the concentration of antibody in mucosal secretions by ELISA. Resulting data was compared to the corresponding antibody standard curve using nonlinear regression. Arithmetic means and standard deviations were calculated for each set of triplicate samples per animal. The differences in the mean concentrations of b12 and 2G12 at each timepoint were evaluated in a student's t test. Analyses performed in GraphPad Prism Software for Mac, Version 5.0a.

Neutralization assays. Neutralization of antibodies and sera was assessed by 2 different methods. Neutralization of the primary isolate $SHIV_{SF162P3}$, was performed using phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) from a single rhesus macaque (no.355) as target cells. Cells from this animal replicate $SHIV_{SF162P}$ efficiently. Neutralization assessment was carried out as described previously [8]. Neutralization titers of animal sera were reported by Monogram Biosciences, South San Francisco, CA after preparation of an HIV-1 envelope pseudotyped luciferase $SHIV_{SF162P3}$ capable of single-round replication. The pseudovirus-based neutralization assay was performed as previously described [51].

MHC genotyping. MHC genotyping by sequence-specific PCR was performed by the University of Wisconsin Genotyping Core with support of NIH grant 5R24RR16038-6 awarded to David I. Watkins and previously described [25].

Viral sequence amplification: Viral RNA was extracted from 140 μl of monkey serum using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. 8μl of this viral RNA was then used for cDNA synthesis using Superscript III (Invitrogen) primed by primer sf162rtn (5'-TTATAGCAAAATCCTTTCC-3'). 3 ul of the cDNA reaction was then used to amplify the gp160 open reading frame using primers sf162mf (5'–CACCATGAGAGTGAAGGGGATCAGGAAG-3') and sf162rn (5'-TTATAGCAAAATCCTTTCCAAGCCCTGTC-3') in combination with PfuUltra® Hotstart DNA Polymerase from Stratagene. After an initial denaturation step at 95°C for 4 minutes, 35 cycles were performed with 95°C for 30 seconds, 52°C for 30 seconds and 72°C for 3 minutes, before a final elongation at 72°C for 10 minutes concluded the amplification. Sequences were determined after subcloning the PCR products into TOPO vectors.

Statistics. The experiment consisted of a total of 9 animals (n=9) divided into treatment groups as follows: 2 animals (n=2) in the isotope control group, 2 animals (n=2) in the non-antibody-treated controls, and 5 animals (n=5) in the 2G12-treated group. Statistical analyses were performed using Graph Pad Prism for Windows, version 5 (Graph Pad Software Inc., San Diego, CA, 2005).

Protein Sequences: GenBank accession locus for 2G12 is 10M3_H (heavy chain, Fab 2G12 unliganded) and 10M3_L (light chain, Fab 2G12 unliganded). GenBank accession locus for IgG1 b12 is AAB26306.

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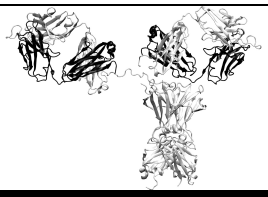
Chapter 8



Broadly Neutralizing Monoclonal Antibodies 2F5 and 4E10, Directed Against the Human Immunodeficiency Virus Type 1 (HIV-1) gp41 Membrane Proximal External Region (MPER), Protect Against SHIV $_{Ba\text{-}L}$ Mucosal Challenge

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The membrane-proximal external region (MPER) of HIV-1, located at the C-terminus of the gp41 ectodomain, is conserved and crucial for viral fusion. Three broadly neutralizing monoclonal antibodies (bnMAbs), 2F5, 4E10 and Z13e1, are directed against linear epitopes mapped to the MPER, making this conserved region an important potential vaccine target. However, no MPER antibodies have been definitively shown to provide protection against HIV challenge. Here, we show that both MAb 2F5 and 4E10 can provide complete protection against mucosal SHIV challenge in macaques. MAb 2F5 or 4E10 was administered intravenously at 50 mg/kg to groups of 6 male Indian rhesus macaques one day prior to and again one day following intrarectal challenge with $SHIV_{Ba\text{-}L}$. All animals in each antibody-treated group were fully protected. The study confirms the protective potential of 2F5 and 4E10 and supports emphasis on HIV immunogen design based on the MPER region of gp41.

Eliciting broadly neutralizing antibodies is an important goal of HIV vaccine design efforts and the study of broadly neutralizing monoclonal antibodies (bnMAbs) can assist in that goal. Human bnMAbs have been described against both gp120 and gp41 of the HIV-1 envelope spike. Three bnMAbs to gp41, 2F5, 4E10, and Z13e1, have been identified and shown to recognize neighboring linear epitopes on the membrane proximal external (MPER) region of gp41 (3, 20, 21, 32, 41). In a comprehensive cross-clade neutralization study by Binley, et al., 2F5 neutralized 67% and 4E10 neutralized 100% of a diverse panel of 90 primary isolates (2). Similar broad neutralization was seen against sexually transmitted isolates cloned from acutely infected patients (18). Both MAbs 2F5 and 4E10 were originally obtained as IgG3 antibodies in hybridomas derived from peripheral blood mononuclear blood lymphocytes (PBMCs) of HIV-1 seropositive non-symptomatic patients and were later class-switched to IgG1 to enable large scale manufacturing and to prolong *in vivo* half-life (3, 5, 28). Although less potent, the monoclonal antibody Z13, isolated from an antibody phage display library derived from a bone marrow donor whose sera was broadly neutralizing (41) has cross-clade neutralizing activity. Z13e1 is an affinity-enhanced variant of the earlier characterized MAb Z13 that is directed against an access-restricted epitope between and overlapping the epitopes of 2F5 and 4E10.

Despite the interest in the MPER as a vaccine target, there is limited information on the ability of MPER antibodies to act anti-virally *in vivo* either in established infection or prophylactically. A study using the huPBL-SCID mouse model showed limited impact from 2F5 when the antibody was administered in established infection (27). Passive administration of 2G12, 2F5, and 4E10 to a cohort of acutely and chronically infected HIV-1 patients provided little direct evidence of 2F5 or 4E10 anti-viral activity, whereas the emergence of escape variants indicated unequivocally the ability of 2G12 to act anti-virally (15, 34). Indirect evidence did however suggest that the MPER MAbs may have impacted virus replication as evidenced by viral rebound suppression in a patient known to have a 2G12-resistant virus prior to passive immunization (34). In prophylaxis, an early 2F5 passive transfer study in chimpanzees suggested the antibody could delay or lower the magnitude of primary viremia following HIV-1 challenge (6). Further protection studies in rhesus macaques using $SHIV_{89.6PD}$ challenge did not provide definitive direct evidence for MPER antibody-mediated protection. One of 3 animals was protected against *i.v.* challenge when 2F5 was administered in a cocktail with HIVIG and 2G12 (16) but all 3 animals treated with 2F5 alone at high concentration became infected. In a vaginal challenge study with $SHIV_{89.6PD}$ (17), 4 of 5 animals were protected with a cocktail of HIVIG/2F5/2G12 but a 2F5/2G12 combination protected only 2 of 5 animals. Further protection studies have used MPER MAbs in combination with other MAbs leaving the individual contribution of these antibodies uncertain (1, 7).

In our previous studies, we successfully used the SHIV/macaque model to demonstrate neutralizing antibody protection against mucosal challenge and we have begun to explore how that protection is achieved (10, 26). Here, we conducted a protection study with the two broadly neutralizing MPER-directed antibodies 2F5 and 4E10 that demonstrates the antibodies can prevent viral infection and confirms that the MPER is in this respect a suitable vaccine target.

MATERIALS AND METHODS

Macaques. All protocols for male Indian rhesus macaques were reviewed and approved by the Institutional Animal Care and Use Committee. The animals were housed in accordance with the American Association for Accreditation of Laboratory Animal Care Standards. At the start of all experiments, all animals were experimentally naïve and were negative for antibodies against HIV-1, SIV, and type D retrovirus. For intrarectal inoculations, animals were sedated with ketamine hydrochloride (10 mg/kg *i.m.*) and placed within a biosafety cabinet in ventral recumbency with the hindquarters elevated. The tail was elevated dorsally and 2000 $TCID_{50}$ $SHIV_{Ba\text{-}L}$ contained in 1ml of sterile tissue culture medium was administered atraumatically inserted into the rectum. Following virus administration, the tail was lowered assuring complete delivery, and the hindquarters remained elevated for 15 minutes before reversing the anesthesia.

Challenge virus. The virus used in this study was $SHIV_{Ba\text{-}L}$, as described elsewhere (25). $SHIV_{Ba\text{-}L}$ retains the R5 phenotype of HIV-$1_{Ba\text{-}L}$. $SHIV_{Ba\text{-}L}$ propagated in phytohemagglutin (PHA)-activated rhesus macaque peripheral blood mononuclear cells (PBMC), was obtained through Advanced Bioscience Labs, Inc., Kensington, MD.

Antibodies. Recombinant IgG1 2F5 and 4E10 were obtained from Polymun Scientific, Vienna, Austria (3, 5, 28, 32). The isotype control antibody DEN3, an anti-Dengue NS1 human IgG1 antibody was expressed in Chinese hamster ovary (CHO-K1) cells in glutamine-free custom formulated Glasgow minimum essential medium (GMEM Selection Media; MediaTech Cellgro, MD). For large-scale tissue culture, media was supplemented with 3.5% Ultra Low Bovine IgG Fetal Bovine Serum (Invitrogen, Carlsbad, CA) and grown in 10-layer Cellstacks and Cell Cubes (Corning, Corning, NY). The antibody was purified using Protein A affinity matrix chromatography (GE Healthcare, Chalfont St. Giles, UK), and dialyzed against phosphate-buffered saline (PBS). Care was taken to minimize endotoxin contamination, which was monitored using a quantitative chromagenic Limulus Amoebecyte Lysate assay (Cambrex Corp., East Rutherford, NJ) performed according to the manufacturer's recommendations. Antibody used for the passive transfer experiments contained <1 IU per mg of endotoxin.

Plasma viral loads. The quantity of SIV viral RNA genomic copy equivalents (vRNA copy Eq/ml) in EDTA-anticoagulated plasma was determined using a quantitative reverse-transcription PCR (QRT-PCR) assay as previously described (9). Briefly, vRNA was isolated from plasma using a GuSCN-based procedure as described (39). QRT-PCR was performed using the SuperScript III Platinum® One-Step Quantitative RT-PR System (Invitrogen, Carlsbad, CA). Reaction mixes did not contain bovine serum albumin (BSA). Reactions were run on a Roche Lightcycler 2.0 instrument and software. vRNA copy number was determined using LightCycler 4.0 software (Roche Molecular Diagnostics, Indianapolis, IN) to interpolate sample crossing points onto an internal standard curve prepared from 10-fold serial dilutions of a synthetic RNA transcript representing a conserved region of SIV *gag*.

Serum antibody ELISAs. 2F5 and 4E10 antibody concentrations in macaque sera were determined in ELISA by three different methods: (1) by binding to M41xt, a construct comprised of the short peptide, GELDKWASLC, and a fusion protein of the ectodomain of $gp41_{JR\text{-}FL}$ linked to the C terminus of the maltose binding protein and more fully described elsewhere (40); (2) by binding to epitope-specific peptides synthesized from the HIV-1 MN Env sequence by AnaSpec, San Jose, CA (HIV-1 MN Env-165, Catalog no. 28760, for 2F5 serum ELISAs and HIV-1MN Env-168, Catalog no. 28763, for 4E10 serum ELISAs; and (3) by binding to recombinant gp41 (Vybion, Ithaca, NY). For each ELISA, serially diluted serum was used to determine the concentration by comparison to the corresponding antibody standard curve using nonlinear regression.

Neutralization assays. Neutralization of antibodies was assessed by 2 different methods. Neutralization of the primary isolate $SHIV_{Ba\text{-}L}$, was performed using phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) from a single rhesus macaque (no.355) as target cells. Cells from this animal replicate SHIV efficiently. Neutralization assessment was carried out as

described previously (26). Neutralization titers of animal sera were also conducted and reported by Monogram Biosciences, South San Francisco, CA after preparation of an HIV-1 envelope pseudotyped luciferase $SHIV_{Ba-L}$ capable of single-round replication. The pseudovirus-based neutralization assay was performed as previously described (29).

Antibody-dependent cell-mediated viral inhibition (ADCVI). ADCVI antibody activity was measured using methods similar to those described previously (8, 10). Briefly, target cells (CEM.NKR-CCR5 cells infected with virus for 48 hours) were incubated with mAb and with fresh human PBMC effector cells (effector:target ratio = 10:1). Seven days later, p27 was determined from supernatant by ELISA. Percent virus inhibition at each concentration of test mAb was determined in comparison to negative control mAb (DEN3).

MHC genotyping. MHC genotyping by sequence-specific PCR was performed by the University of Wisconsin Genotyping Core with support of NIH grant 5R24RR16038-6 awarded to David I. Watkins and previously described (12, 36).

RESULTS

Selection of SHIV for protection study. We first evaluated MAbs 2F5 and 4E10 in PBMC and pseudovirus neutralization assay formats against SHIVs that have previously been characterized in macaques. As protection *in vivo* is generally correlated with neutralization *in vitro*, we sought a SHIV that was reasonably neutralization sensitive to both MAbs. A summary of the neutralization data is shown in **Table 1**. The R5 $SHIV_{BaL}$ (23, 25) was chosen over the X4 $SHIV_{89.6P}$ due to its greater sensitivity to neutralization by 2F5 and 4E10 and because an R5 virus is more representative of those involved in human transmission than an X4 virus.

Virus	MAb	Rhesus PBMC-based Neutralization Assay		Pseudovirus-based Neutralization Assay	
		(μg/ml)			
		IC_{50}	IC_{90}	IC_{50}	IC_{90}
SHIV 89.6P	2G12	20	>900	2.6	>50
	b12	300	>900	11.5	>50
SHIV SF162P3	2G12	20	900	7.6	>50
	b12	2	8	0.29	2
SHIV BaL	2G12	>300	>300	1	>50
	b12	<1.2	<1.2	0.02	0.09

Table 1. SHIV neutralization by 2F5, 4E10, and b12 in two assay formats: (1) rhesus PBMC-based assay and (2) pseudovirus luciferase reporter gene assay. The selection of $SHIV_{BaL}$ for the protection study was based on the results of 2F5, 4E10, and b12 in a comparison of neutralization in Rhesus PBMCs and pseudovirus assays against the SHIV panel shown. The R5 $SHIV_{BaL}$ was chosen over the X4 $SHIV_{89.6P}$ to better represent human transmission and due to the lower sensitivity to neutralization

by 2F5 and 4E10. Further consideration was that an IC_{90} of 4E10 was not achieved by 4E10 against the R5 $SHIV_{162P3}$ in the generally more sensitive pseudovirus assay.

Experimental design of protection study. A total of 16 male Indian rhesus macaques were intrarectally challenged with 2000 $TCID_{50}$ (50% tissue culture infectious doses) of $SHIV_{Ba\text{-}L}$. One day prior to challenge, each animal was given an intravenous dose of 50 mg/kg of either 2F5, 4E10, or the isotype control IgG (anti-Dengue NS1, DEN3). Experimental animals were divided into treatment groups of 6 animals for administration of 2F5 and 4E10. The isotype control group consisted of 2 animals, and 2 additional untreated animals that were challenged prior to the beginning of the protection study to confirm viral fitness. We decided to use a relatively high antibody dose to provide the best opportunity of observing protection based on the observation that many multiples of the neutralizing titer of the antibody (IC_{90}) measured in a PBMC assay have been described previously as required for protection (22, 26). Based on earlier pharmacokinetic observations with 2F5 and 4E10, we expected the serum antibody half-life to be relatively short (4 to 5 days)(11, 16), so a second intravenous antibody treatment was given at day +1 following viral challenge in order to ensure serum antibody concentrations were maintained long enough to favor a positive outcome. Blood draws were conducted at regular intervals following challenge to monitor viral infection, serum levels of passively administered antibody and serum neutralizing activity.

2F5 and 4E10 provide complete protection against infection by $SHIV_{Ba\text{-}L}$. The outcome of the protection study is depicted in **Figure 1.** The two isotype control-treated animals became infected with peak viremia of approximately 10^7 virus copies per ml between days 14 and 21. The two control animals (96068, 2025), that were challenged prior to the beginning of the protection study to confirm viral infectivity, but not treated with antibody, were also infected with similar onset and peak viremias as isotype control-treated animals.

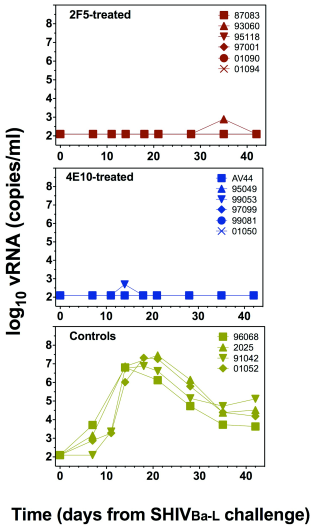


Figure 1. Protection by 2F5 and 4E10 against mucosal $SHIV_{Ba\text{-}L}$ challenge. A total of 16 male Indian Rhesus macaques were divided into 2 antibody treatment groups of 6 for administration of 2F5 and 4E10. The control group was comprised of 2 isotype control-treated animals and two control animals (96068, 2025) that were challenged prior to the beginning of the protection study to confirm viral infectivity, but not treated with antibody. All animals were intrarectally challenged with 2000 $TCID_{50}$ $SHIV_{Ba\text{-}L}$. Animals in the antibody treatment groups received an intravenous dose of 50 mg/kg of either 2F5, 4E10, or isotype control antibody 1 day prior to viral challenge (day -1) and again one day following viral challenge (day +1). All control animals (4/4) experienced peak viremia on the order of 10^7 between days 14 and 21. All 2F5 and 4E10-treated animals were completely protected. A single animal from each group (2F5-treated 93060, and 4E10-treated 99053) experienced very low-level transient viremias, but at the end of 6 weeks there was no detectable virus in any of the animals. The assay minimum detection limit is 150 SIV RNA copies per ml (2.1 $\log_{10}$ vRNA copies/ml) with a 95% confidence level.

In contrast, all 2F5 and 4E10–treated animals were completely protected against the establishment of infection. In each group, in 4 of 5 animals, no viremia could be detected at any time point during the 6 weeks follow-up after challenge. Of note, a single animal from each group, 2F5-treated 93060 and 4E10-treated 99053, experienced one very low-level transient viremia, close to the 150 RNA copies per ml detection threshold of the quantification method, of 2.7 x 10^2 copies per ml on day 14 and 4.0 x 10^2 copies per ml on day 35, respectively.

Serum antibody concentrations. The 2F5 and 4E10 antibody concentrations in the sera of the macaques were measured using three different ELISA formats: (1) by binding to the M41xt construct (40); (2) by binding to epitope-specific peptides: HIV-1 MN Env-165 for 2F5 and HIV-1MN Env-168 for 4E10 (AnaSpec, San Jose, CA); and (3) by binding to recombinant HIV-1 gp41 (Vybion, Ithaca, NY). In all formats, a dilution series of serum was compared to the appropriate antibody standard curve and the concentration determined using a non-linear regression curve fit analysis. The serum concentrations derived from the three formats were generally in good agreement. **Table 2** and **Table 3** summarize the serum antibody concentrations of 2F5 and 4E10-treated animals. The data shown in **Figure 2** represents the mean serum antibody concentrations from all animals in each group and from each ELISA format. Following the first *i.v.* antibody transfer at 50 mg/kg, the mean concentration of 2F5 and 4E10 at the time of challenge was 742 μg/ml and 866 μg/ml, respectively.

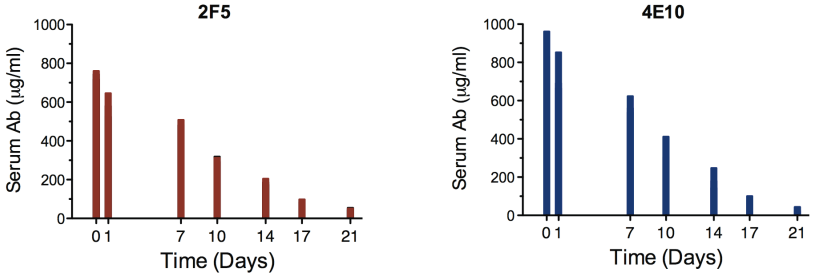


Figure 2. Mean serum concentrations of 2F5 and 4E10 on day of challenge in macaque serum. For each antibody treatment group, the data shown represent mean serum antibody concentrations averaged over all the animals in the group and averaged over 3 ELISA formats beginning at the time of virus challenge and continuing through day 21.

2F5-treated macaques							
87083	**gp41**	**M41xt**	**Peptide**	**97001**	**gp41**	**M41xt**	**Peptide**
Day	Serum Ab μg/ml			Day	Serum Ab μg/ml		
-1	0	0	0	-1	0	0	0
0	760	860	753	0	464	631	597
1	775	771	849	1	366	566	507
7	554	570	690	7	316	478	460
10	455	367	554	10	269	271	188
14	209	224	221	14	175	159	116
17	125	83	134	17	82	187	79
21	55	81	77	21	25	73	69
93060	**gp41**	**M41xt**	**Peptide**	**1090**	**gp41**	**M41xt**	**Peptide**
Day	Serum Ab μg/ml			Day	Serum Ab μg/ml		
-1	0	0	0	-1	0	0	0
0	887	911	907	0	475	444	456
1	609	843	747	1	417	463	368
7	587	669	460	7	421	363	325
10	285	369	288	10	276	289	223
14	201	233	160	14	205	189	191
17	90	107	100	17	138	90	127
21	43	8	15	21	98	79	83
95118	**gp41**	**M41xt**	**Peptide**	**1094**	**gp41**	**M41xt**	**Peptide**
Day	Serum Ab μg/ml			Day	Serum Ab μg/ml		
-1	0	0	0	-1	0	0	0
0	882	881	919	0	843	844	840
1	468	566	563	1	524	673	453
7	408	478	329	7	617	498	624
10	332	271	251	10	305	318	259
14	202	219	135	14	134	213	230
17	51	95	120	17	26	36	35
21	38	52	78	21	8	17	16

Table 2. Serum antibody concentrations in 2F5-treated macaques. Concentrations of transferred antibody were determined in ELISA using three assay formats: (1) by binding to the M41xt construct (40); (2) by binding to the HIV-1 MN Env-165 containing the sequence ELDKWA, AnaSpec, San Jose, CA); and (3) by binding to recombinant gp41, Vybion, Ithaca, NY. The determined serum concentrations derived from the three formats were generally in good agreement. In all formats, a dilution series of serum was compared to the appropriate antibody standard curve and the concentration determined using a non-linear regression curve fit analysis.

4E10-treated macaques							
87083	**gp41**	**M41xt**	**Peptide**	**97001**	**gp41**	**M41xt**	**Peptide**
Day	Serum Ab μg/ml			Day	Serum Ab μg/ml		
-1	0	0	0	-1	0	0	0
0	984	973	1025	0	629	930	755
1	881	938	916	1	501	860	601
7	757	793	770	7	430	569	516
10	338	323	645	10	279	374	334
14	279	216	386	14	126	68	151
17	103	54	77	17	25	53	91
21	5	29	35	21	25	21	36
93060	**gp41**	**M41xt**	**Peptide**	**1090**	**gp41**	**M41xt**	**Peptide**
Day	Serum Ab μg/ml			Day	Serum Ab μg/ml		
-1	0	0	0	-1	0	0	0
0	941	893	947	0	955	994	546
1	679	744	863	1	911	824	493
7	520	292	552	7	701	688	541
10	250	200	326	10	459	482	550
14	159	69	239	14	185	320	522
17	111	10	89	17	136	179	59
21	59	4	33	21	58	54	27
95118	**gp41**	**M41xt**	**Peptide**	**1094**	**gp41**	**M41xt**	**Peptide**
Day	Serum Ab μg/ml			Day	Serum Ab μg/ml		
-1	0	0	0	-1	0	0	0
0	828	990	777	0	644	997	773
1	793	885	651	1	388	873	466
7	526	642	632	7	302	762	362
10	271	469	326	10	238	631	286
14	233	95	70	14	105	99	126
17	155	60	62	17	82	33	98
21	56	9	53	21	70	28	84

Table 3. Serum antibody concentrations in 4E10-treated macaques. Concentrations of transferred antibody were determined in ELISA using three assay formats: (1) by binding to the M41xt construct (40); (2) by binding to HIV-1 MN Env-168 containing the sequence NWFDITN, AnaSpec, San Jose, CA); and (3) by binding to recombinant gp41, Vybion, Ithaca, NY. The determined serum concentrations derived from the three formats were generally in reasonable agreement. In all formats, a dilution series of serum was compared to the appropriate antibody standard curve and the concentration determined using a non-linear regression curve fit analysis.

Serum antibody half-lives. The antibodies were transferred in 2 doses: first on day -1 before viral challenge and again on day +1 following viral challenge. To calculate the antibody half-lives, it was necessary to use a formula to account for the decay of antibody between the doses and the resulting boost in antibody concentration at day 2. **Figure 3** models the decay following the first antibody dosing and the spike resulting from the second bolus using the 2-dose one phase decay formula: (Y= IF(X<X0, Plateau+(Y0-Plateau)*exp(-K*(X)), Plateau+(Y1-Plateau)*exp(-K*(X-X0))), where X and Y=time of experimental intervention; K=rate constant). The half-life of each antibody for 3 weeks following challenge in all animals was then calculated from the overall mean of the concentrations from the three ELISA formats (Analysis in GraphPad Prism). From this analysis, we determined the half-life of 2F5 to be 4.4 days and 4E10 at 4.1 days.

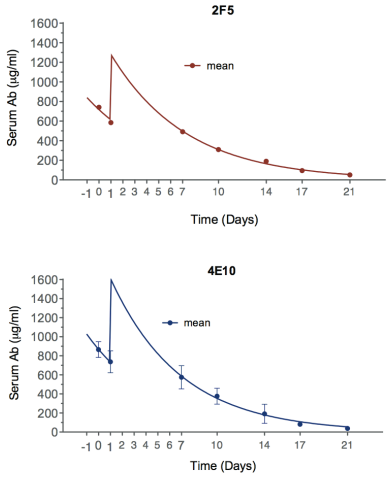


Figure 3. Model of 2F5 and 4E10 half-life ($t_{1/2}$) in macaque serum. For each antibody treatment group, the data shown represents mean serum antibody from each animal averaged over 3 ELISA formats beginning at day 0 following the first *i.v.* transfer of 50 mg/kg of each antibody, 2F5 or 4E10. The half-lives were determined using the 2-dose one phase decay formula: (Y= IF(X<X0,Plateau+(Y0 Plateau)*exp(-K*(X)), Plateau+(Y1-Plateau)*exp(-K*(X-X0))), where X and Y = time of experimental intervention; K = rate constant). The model accounts for the decay following the first antibody dosing and the spike resulting from the second antibody dose. The half-life of each antibody for 3 weeks following challenge was then calculated from the overall mean of the concentrations from the three ELISA formats (Analysis in GraphPad Prism Software). The average half-life of 2F5 was determined to be 4.4 days and the average half-life of transferred 4E10 was determined to be 4.1 days.

Serum antibody neutralization titers. Serum samples were assayed from all animals beginning on the day of the first antibody *i.v.* transfer and continuing for the following 3 weeks in a $SHIV_{Ba-L}$ pseudovirus single round assay established by Monogram Biosciences (29). The 50% (IC_{50}) and 90% (IC_{90}) serum neutralizing titers of all 2F5 and 4E10 treated macaques are shown in **Table 4** and **Table 5**, respectively. In both the 2F5 and 4E10 groups, IC_{50} values were similar and on the order of 1:2,000 on the day of viral challenge and IC_{90} values were on the order of 1:200.

2F5-treated macaques						
Animal	**87083**	**93060**	**95118**	**97001**	**1090**	**1094**
Day	**IC_{50} (1/diln)**					
-1	<50	<50	<50	<50	<50	<50
0	2049	2332	2419	1421	1226	2311
1	2181	1825	1433	1339	1279	1291
7	1831	1723	1280	1906	1086	1667
10	1693	807	1203	760	1768	967
14	596	67	581	715	772	613
17	555	263	417	579	543	49
21	327	49	249	544	308	49
4E10-treated macaques						
Animal	**AV44**	**95049**	**99053**	**97099**	**99081**	**1050**
Day	**IC_{50} (1/diln)**					
-1	<50	<50	<50	<50	<50	<50
0	1813	1833	1148	1741	2091	1492
1	1457	1444	1654	2520	1841	789
7	1119	1317	1201	1162	1549	845
10	974	878	796	669	1286	380
14	529	514	616	539	716	537
17	505	484	476	287	472	261
21	299	401	334	75	404	271

Table 4. Neutralizing titers of transferred antibodies 2F5 and 4E10 in macaque serum. Numbers represent reciprocal dilution of serum producing 50% neutralization as reported by Monogram Biosciences in an HIV-1 pseudotyped Env luciferase assay.

2F5-treated macaques						
Animal	**87083**	**93060**	**95118**	**97001**	**1090**	**1094**
Day	**IC_{90} (1/diln)**					
-1	<50	<50	<50	<50	<50	<50
0	227	273	273	186	174	210
1	204	191	193	159	201	175
7	219	197	185	170	168	292
10	177	125	146	125	184	164
14	104	104	84	92	137	91
17	69	<50	68	74	75	<50
21	<50	<50	<50	73	52	<50
4E10-treated macaques						
Animal	**AV44**	**95049**	**99053**	**97099**	**99081**	**1050**
Day	**IC_{90} (1/diln)**					
-1	<50	<50	<50	<50	<50	<50
0	194	192	189	189	220	182
1	161	139	214	214	238	102
7	152	139	192	192	194	92
10	107	142	98	98	172	<50
14	68	70	55	55	93	<50
17	63	64	<50	<50	69	<50
21	<50	<50	<50	<50	<50	<50

Table 5. Neutralizing titers of transferred antibodies 2F5 and 410 in macaque serum. Numbers represent reciprocal dilution of serum producing 90% neutralization as reported by Monogram Biosciences in an HIV-1 pseudotyped Env luciferase assay.

Antibody-dependent cell-mediated viral inhibition (ADCVI). Studies have indicated that 2F5 and 4E10 bind somewhat weakly to infected cells (30, 41) and therefore, may not efficiently mediate activities such as ADCC. Because FcR-mediated antibody effector functions are important for protection by mAb b12 (10), we wished to investigate whether 2F5 and 4E10 could inhibit virus yield from infected cells in the presence of Fc receptor-bearing effector cells *in vitro.* In the ADCVI assay, an infected cell culture is incubated with antibody and effector cells, supernatant containing excess antibody and free virus is removed, and then viral infection is estimated seven days later (8). In **Figure 4** we show that 4E10 is poor at mediating ADCVI while 2F5 displays significant activity only at high concentrations, being overall notably less effective than b12.

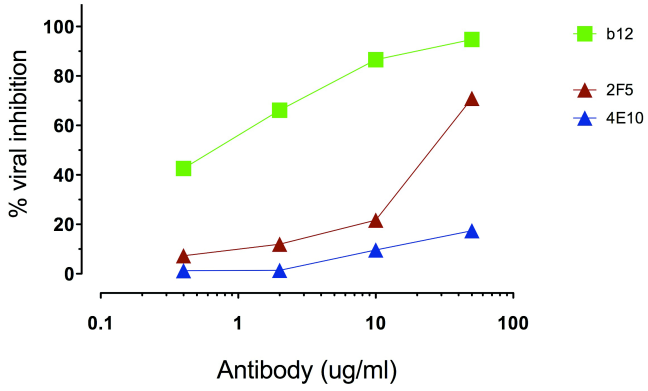


Figure 4. Antibody-dependent cell-mediated viral inhibition (ADCVI) of 2F5, 4E10 in comparison to b12. Target cells (CEM.NKR-CCR5) were infected with $SHIV_{Ba-L}$ and incubated for 48 hours then washed to remove cell-free virus. Serially diluted antibody was added at 50, 2, and 0.4 μg/ml to effector cells (rhPBMCs) and incubated for 30 min. Cells were re-washed and incubated for 7 days at 37°C in 5% CO_2. Supernatant was collected and assayed for p27 by ELISA.

MHC genotyping. Two MHC class I alleles (*Mamu-B*08* and *Mamu-B*17*) have been associated with elite control of SIV replication. We, therefore, evaluated all experimental animals by MHC genotyping using PCR-SSP to test macaque samples against a panel of 9 class I alleles shown to be important in SIV epitope presentation (4, 12, 14, 24, 36). The results shown in **Table 6** reflect that no animals in either antibody treatment group express the *Mamu-B*08* or *Mamu-B*17* alleles. The *Mamu-A*01* allele, a frequently occurring allele in many colonies that has been associated with moderate reduction of SIVmac239 replication (4, 19, 24, 38), appears in 4 macaques in the 2F5 group and 2 macaques in the 4E10 group. *Mamu-A*08*, also expressed at relatively high frequency, is seen in 4 animals treated with 2F5 and 2 animals treated with 4E10. This allele has so far been linked to a single epitope presentation derived from $SHIV_{HXBC2}$ and its role in SIV pathogenesis is unclear (35). The *Mamu-B*01* allele is present in 2 macaques in the 2F5 group and 1 macaque in the 4E10 group. This allele remains on the panel based on early reporting of SIV-derived epitopes (33, 37), but subsequent studies have shown that *Mamu-B*01* does not bind SIV-derived epitopes and has no effect on SIV disease progression (14). Overall, even with the presence of the *Mamu-A*01*, *Mamu-A*08*, and *Mamu-B*01* alleles (which are not associated with elite control of SIV replication) there is no apparent correlation with the allelic profiles of the animals in this study that would account for any unusual ability to resist infection.

2F5	A01	A02	A08	A11	B01	B03	B04	B08	B17
87083	+	-	+	-	-	-	-	-	-
93060	+	-	-	-	-	-	-	-	-
95118	-	-	-	-	-	-	-	-	-
97001	+	-	+	-	+	-	-	-	-
01090	+	-	+	-	-	-	-	-	-
01094	-	-	+	-	+	-	-	-	-
01069	-	-	+	-	+	-	-	-	-
90126	-	-	-	-	+	-	-	-	-
96068[C]	-	-	+	-	-	+	+	-	-
01052[C]	+	-	-	-	-	-	-	-	-
4E10	**A01**	**A02**	**A08**	**A11**	**B01**	**B03**	**B04**	**B08**	**B17**
AV44	-	-	-	-	-	-	-	-	-
95049	-	-	-	-	-	-	-	-	-
99053	+	-	+	-	-	-	-	-	-
97099	-	-	+	-	-	-	-	-	-
99081	+	-	-	-	+	-	-	-	-
01050	-	-	-	-	-	-	-	-	-
91042[C]	-	+	-	-	-	-	-	-	+
2025[C]	+	-	-	-	-	-	-	-	-

Table 6. MHC genotyping of macaques against MHC Class I alleles. Samples from all experimental macaques were tested against a panel of 9 class I alleles shown to be important in SIV epitope presentation (4, 12, 14, 24, 36). Two MHC class I alleles (*Mamu-B*08* and *Mamu-B*17*) have been associated with elite control of SIV replication and no experimental animals in either antibody treatment group express the *Mamu-B*08* or *Mamu-B*17* alleles. Overall, there is no apparent correlation with the allelic profiles of the animals in this study that would account for any unusual ability to resist infection. [C] denotes control animals not antibody-treated.

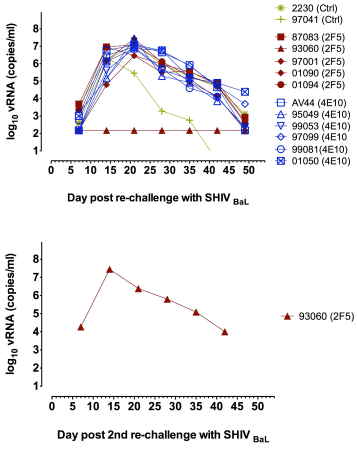
Table 6. (opposite page) MHC genotyping of macaques against MHC Class I alleles. Samples from all experimental macaques were tested against a panel of 9 class I alleles shown to be important in SIV epitope presentation (4, 12, 14, 24, 36). Two MHC class I alleles (*Mamu-B*08* and *Mamu-B*17*) have been associated with elite control of SIV replication and no experimental animals in either antibody treatment group express the *Mamu-B*08* or *Mamu-B*17* alleles. Overall, there is no apparent correlation with the allelic profiles of the animals in this study that would account for any unusual ability to resist infection. [C] denotes control animals not antibody-treated.

Virus re-challenges. To assess the possibility that antibody administration followed by SHIV viral challenge may have initiated a long-term protective immune response and, therefore, to determine if a change in vulnerability to infection existed, re-challenges were conducted with 2000 $TCID_{50}$ $SHIV_{BaL}$ six months after the first SHIV challenge. **Figure 5a** shows that all animals in each antibody treatment group were infected except 2F5-treated animal 93060. Six months later a second re-challenge was conducted with 93060 and the animal became infected with a peak viremia of 2.83×10^7 vRNA copies per ml measured in plasma taken on day 14 following the second viral re-challenge (**Figure 5b**).

Figure 5. $SHIV_{BaL}$ re-challenges of all protected macaques treated with either 2F5 or 4E10.

(a) To assess the vulnerability of the protected animals by SHIV infection, re-challenges were conducted with 2,000 $TCID_{50}$ $SHIV_{BaL}$ 6 months after initial challenge. All animals in each antibody treatment group were infected except 2F5-treated animal 93060.

(b) Subsequently (6 months later), a second re-challenge was conducted with 93060 and the animal became infected. Note: 2F5-treated animal 95118 was not re-challenged. This animal was euthanized for unrelated medical reasons prior to re-challenge.



DISCUSSION

The results of this study show that gp41 MPER antibodies 2F5 and 4E10 can offer full protection against mucosal SHIV challenge.

The serum neutralizing titers at the time of challenge for both MAbs were approximately 1:2,000 and 1:200 measured as IC_{50} and IC_{90} values in a pseudovirus assay. However, based on the PBMC IC_{90} for 2F5 and 4E10 (See **Table 1**), serum concentrations on day of challenge correspond approximately to 1:26 for 2F5 and 1:37 for 4E10 IC_{90} titers in a PBMC based assay. Sterilizing immunity has been associated for other antibodies, viruses and challenge routes with neutralizing titers at very roughly two orders of magnitude higher than the antibody IC_{90} in a PBMC assay (22, 26, 31). Therefore, sterilizing immunity here was observed at a somewhat lower neutralizing titer on the day of challenge. However, it should be noted that in the present study to maximize our chances of observing protection and given concerns about shorter half-lives of MPER antibodies, we gave a second bolus of passive antibody one day after challenge. Pharmacokinetics modeling suggests that peak concentration attained on day 1 were 1282 μg/ml and 1614 μg/ml, for 2F5 and 4E10, respectively, corresponding to PBMC IC_{90} titers of about 1:64 for 2F5 and 1:49 for 4E10. Serum neutralizing titers were maintained at >1:1,000 (pseudovirus assay IC_{50}) through day 7 still corresponding to roughly 1:50 IC_{90} titer in a PBMC assay. A titration of protection against MAb dose will be required to fully understand the minimal antibody neutralizing titers affording protection as has been described for MAb b12 (26) and a monospecific polyclonal antibody preparation (22). We should also note that in a recently concluded study with the anti-gp120 oligomannose-binding bnMAb 2G12, 3 of 5 macaques were completely protected with serum neutralizing titers on the order of only 1:1 based on the PBMC IC_{90} of 2G12 against the challenge virus, $SHIV_{SF162P3}$ (Hessell, et al., 2009, PLoS Pathogens, in press).

Although low levels of transient viremia were detected in a single plasma sample from animals 93060 and 99053, an infection was not established and no additional vRNA was detected in subsequent plasma samples. For 4E10-treated 99053, a 'blip' was seen at day 14 following challenge, a timepoint most often associated with detection of viremia. However, no further traces of virus were detected during 4 additional weeks of vRNA monitoring. The day 14 blip seen in this animal may be an artifact of the viral load measurement procedure or may represent a true episode of viremia that could correspond to a burst of viral replication quickly contained by the remaining neutralizing antibody. Of note, no viral specific anti-Env antibody response or T cell reponses, as measured by Elispot with SIVmac239 gag, nef, vif, and pol peptide pools, were detected (data not shown). For 2F5-treated 93060, the circumstances are somewhat different. The 'blip' was not detected until day 35, after the antibody concentration has waned to undetectable levels. Similar measurements as those done for animal 99053 detected no antibody and T cell responses even at month 6 prior to a viral re-challenge (data not shown). Interestingly, the re-challenge failed to initiate a productive infection. However, a second re-challenge conducted at month 12 led to a regular infection course in the animal and the significance of the absence of infection following the first re-challenge is unclear.

Because antibody was transferred in 2 doses: first on day -1 before viral challenge and again on day +1 following viral challenge, we measured the half-lives using a 2-dose one phase formula that allowed us to account for the decay of antibody between the doses and the boost in antibody at day 2 (Analysis in GraphPad Prism, **Figure 5**). From this analysis, we calculated the half-life of 2F5 to be 4.4 days and 4E10 at 4.1 days, which is in good agreement with previous studies with these antibodies in macaques (16) and in humans (11). The half-lives are at the bottom and of the range described for antibodies in clinical use, i.e., 4.3 to 21.8 days (13).

Another interesting observation here is that the antibodies 2F5 and 4E10, and particularly 4E10, are less effective than b12 at ADCVI *in vitro*. Nevertheless, the concentrations of both MPER antibodies in sera during the protection experiments were high and ADCVI mechanisms may contribute to protection for both antibodies. Further experimentation will be required to better understand the relative contributions of different mechanisms to MPER antibody protection *in vivo*.

The tremendous breadth of HIV-1 neutralization by 2F5 and 4E10 has traditionally made the MPER region of gp41 an interesting target for immunogen design. This study shows that anti-MPER antibodies can provide protection at moderate serum neutralizing titers. There are considerable challenges in eliciting antibodies to recognize a region close to the viral membrane, but if overcome, the relevant immunogen could contribute to HIV vaccine protection.

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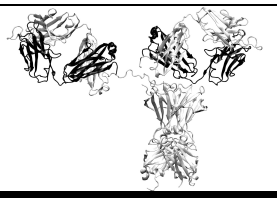
Chapter 9



Inhibition of HIV-1 Infectivity and Epithelial Cell Transfer By Human Monoclonal IgG and IgA Antibodies Carrying the b12 V Region

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Inhibition of HIV-1 Infectivity and Epithelial Cell Transfer by Human Monoclonal IgG and IgA Antibodies Carrying the b12 V Region[1]

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Both IgG and secretory IgA Abs in mucosal secretions have been implicated in blocking the earliest events in HIV-1 transit across epithelial barriers, although the mechanisms by which this occurs remain largely unknown. In this study, we report the production and characterization of a human rIgA$_2$ mAb that carries the V regions of IgG1 b12, a potent and broadly neutralizing anti-gp120 Ab which has been shown to protect macaques against vaginal simian/HIV challenge. Monomeric, dimeric, polymeric, and secretory IgA$_2$ derivatives of b12 reacted with gp120 and neutralized CCR5- and CXCR4-tropic strains of HIV-1 in vitro. With respect to the protective effects of these Abs at mucosal surfaces, we demonstrated that IgG1 b12 and IgA$_2$ b12 inhibited the transfer of cell-free HIV-1 from ME-180 cells, a human cervical epithelial cell line, as well as Caco-2 cells, a human colonic epithelial cell line, to human PBMCs. Inhibition of viral transfer was due to the ability of b12 to block both viral attachment to and uptake by epithelial cells. These data demonstrate that IgG and IgA MAbs directed against a highly conserved epitope on gp120 can interfere with the earliest steps in HIV-1 transmission across mucosal surfaces, and reveal a possible mechanism by which b12 protects the vaginal mucosal against viral challenge in vivo. *The Journal of Immunology,* **2007, 179: 3144–3152.**

Transmission of HIV-1 occurs primarily through sexual contact and breastfeeding in which mucosal surfaces of the genital or gastrointestinal tracts, respectively, are exposed to HIV and/or HIV-infected cells (1, 2). For successful infection to occur, infectious virus must breach the mucosal barrier consisting of stratified squamous or columnar epithelial cells and gain access to target cells permissive for viral uptake and replication (3, 4). Once within the mucosa, HIV-1 establishes both local and systemic viral reservoirs with devastating immunological consequences, including depletion of $CD4^+$ memory T cells and immunodeficiency (5, 6). Moreover, viral reservoirs are resistant to eradication by the cellular and humoral arms of the immune system and by antiviral therapies. The development of strategies to reduce sexual transmission of HIV-1 requires a better understanding of immunological factors that are capable of interrupting attachment, uptake, and transfer of HIV-1 by mucosal epithelial cells.

Evidence from macaque models of simian/HIV (SHIV)[5] infection indicates that Abs directed against envelope spike glycoproteins (i.e., gp120 and gp41) are capable of conferring mucosal defense against HIV-1 (7–10). The most well-characterized anti-envelope Ab in this regard is IgG1 b12, one of only a handful of human mAbs known to be capable of neutralizing a broad range of primary HIV-1 isolates (11–13). IgG1 b12 recognizes a highly conserved epitope on gp120 involved in binding to CD4 (14). Macaques administered IgG1 b12 i.v. or topically (i.e., intravaginally) were protected against SHIV infection by the vaginal route (15, 16). The mechanism(s) by which IgG1 b12 inhibited viral infection of the vaginal mucosa was not determined in these experiments, but it is generally assumed that the Ab interferes with virus-host cell contact.

In humans and macaques, mucosal secretions contain a mixture of IgG and secretory IgA (SIgA) Abs (17). In secretions of the female genital tract, IgG and SIgA are found at roughly equal concentrations, whereas in the gastrointestinal tract, IgG concentrations are 30- to 100-fold lower than SIgA (1, 18–20). SIgA is a polypeptide complex that consists of two (or more) IgA monomers joined at their C termini by a J chain that is covalently associated with secretory component (SC), a 70-kDa glycoprotein derived from the polymeric IgR during transcytosis across epithelial cells (21). Although both IgG and SIgA are capable of neutralizing viruses in mucosal secretions (22), the relative contribution of one Ab type over another in blocking sexual transmission of HIV-1 remains to be determined.

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[5] Abbreviations used in this paper: SHIV, simian/HIV; SIgA, secretory IgA; SC, secretory component; GS, glutamine synthetase; GMEM, glutamine-free MEM; AI, affinity index; $TCID_{50}$, 50% tissue culture-infective dose; MSX, methionine sulfoximine.

To better understand the mechanisms by which Abs against gp120 confer mucosal immunity to HIV, we have produced and characterized a recombinant human IgA_2 mAb that carries the H and L chain variable domains of Ab b12. We demonstrate that monomeric and dimeric forms of recombinant human IgA_2 b12 react with gp120 and neutralize HIV-1 infection of T cells. More importantly, we report that both IgG1 b12 and IgA_2 b12 can prevent HIV-1 attachment, uptake, and transfer by cervical and intestinal epithelial cells. These data demonstrate that both IgG and IgA Abs against gp120 can interfere with the earliest steps in HIV-1 transmission across mucosal surfaces and reveal a possible mechanism by which IgG1 b12 protects the vaginal mucosa against viral challenge in vivo.

Materials and Methods

Construction of mammalian expression vector-encoding IgA_2 b12

The plasmid pSM102 (see Fig. 1) encoding IgA_2 b12 was constructed from pDR12 (12) by replacing the IgG1 constant domains with the IgA_2 constant domains. The genomic DNA encoding human IgA_2m (1) was amplified as a single 1.5-kb *Sac*I-*Sal*I DNA fragment by PCR using plasmid pcDNA3: cα2m (1) as template (23) and custom-designed primers 5′-GTCATCGT GAGCTC**ATCCCCGACCAGCCCCAAGGTG**-3′ and 5′-TTTGACGT CGAC**TTTCCCAAGTGCTGAGACCCTGAGGAT**-3′). The underlined sequences indicate the engineered restriction sites, and the bold sequences indicate the IgA_2m1 coding region. The IgA_2 constant domain was fused in-frame with the b12 V_H coding sequence.

Establishment of stable CHO cell lines secreting monomeric and dimeric/polymeric forms of IgA b12

We used the selectable-amplifiable marker, glutamine synthetase (GS) encoded on plasmid pSM102 (see Fig. 1), to produce stable cell lines secreting high levels of IgA b12. The use of GS as a selectable-amplifiable marker has been described previously (12, 24). CHO-K1 were seeded into 6-well cell culture plates and maintained in glutamine-free MEM (GMEM; Mediatech) containing 5% FCS (HyClone) and methionine sulfoximine (MSX; Sigma-Aldrich) at a range of concentrations (20–120 μM). Supernatants of pSM102-transfected cells were screened by ELISA for the presence of IgA Abs reactive with gp120 (see below). Cells from positive wells were collected by trypsinization and cloned three times by limiting dilution (25). A single clone secreting monomeric IgA_2 b12, herein referred to as 100-02-C9, was chosen for further study based on a number of criteria, including levels of Ab production and stability in culture.

To produce cell lines secreting dimeric IgA_2 b12, clone 100-02-C9 was transfected with plasmid pcDNA3Hygro::J chain encoding human J chain and selected for growth in GMEM-MSX medium containing hygromycin (400 μg/ml) (23). We identified transfectants secreting polymeric forms of IgA_2 b12 by screening cell supernatants using a SC-binding ELISA (26). Two stable transfectants, J2 and J10, were cloned three times by limiting dilution and used for further studies.

Clone 100-02-C9 was maintained in GMEM supplemented with 5% FCS (Ultra Low Bovine IgG FBS; Invitrogen Life Technologies) and 80 μM MSX (Sigma-Aldrich). Clone J1 secreting was maintained in the same medium with the addition of hygromycin B (400 μg/ml). Large scale cell culture was performed using 10-layer CellStacks (Corning).

Immunoprecipitation and Western blot analysis of IgA_2 b12

Supernatants from CHO cell lines (~3 ml) were mixed with polyclonal goat anti-human α-chain antiserum (Cappel/ICN) followed by protein G agarose beads (Pierce), which bind goat IgG but not human IgA (27). Protein G agarose beads (~40 μl) were collected by centrifugation and resuspended in 2× Laemmli sample buffer, boiled for 5 min, and then subjected to SDS-PAGE on 4–18% gradient gel (Bio-Rad). The proteins were transferred to a polyvinylidene difluoride membrane (0.45-μm pore size; Bio-Rad) by electroelution and then probed with biotin-labeled goat anti-human IgA Fab (1/5000) (Tago Immunologicals) and avidin-HRP (Sigma-Aldrich). Membranes were developed using the ECL kit (Amersham Pharmacia) and exposed to Kodak X-OMAT film (Fisher-Scientific). When necessary, polyacrylamide gels were stained with Gel Code Blue (Pierce) to visualize proteins.

Purification of monomeric and dimeric forms of IgA_2 b12

Abs were affinity purified from cell culture supernatants using ImmunoPure Immobilized Protein L (Pierce), which binds human κ L chains, and then were subjected to size exclusion chromatography by a Sephacryl S300 High Resolution column (GE Healthcare) to separate dimeric and monomeric forms of IgA_2 b12. The column was calibrated using the following molecular mass standards: dextran 2000 kDa, ferritan 440 kDa, catalase 220 kDa, and RNase A 10 kDa. Peak fractions of IgAb2 were pooled and purity was checked by SDS-PAGE and concentrations of the IgA molecular forms were determined by ELISA and absorbance spectroscopy (28).

Expression of recombinant human rSC

rSC was produced by transfection of CHO-K1 cells with pCL-2, a plasmid encoding human rSC provided to us by J. Woof (University of Dundee Medical School, Dundee, Scotland; Refs. 29–31). Transfectants were identified by hygromycin B (200 μg/ml; Roche). Stable clones secreting rSC were isolated by limiting dilution and assayed for expression of rSC by ELISA.

gp120 ELISA

HIV-1-specific ELISAs to detect IgA b12 were done by coating Nunc Maxisorp 96-well microtiter plates (Fisher-Scientific) with HIV-1_{IIIB} lysate (Calypte Biomedical) or rHIV-1_{IIIB} gp120 (ImmunoDiagnostics) using 100 ng of protein/well in PBS (pH 7.4). After overnight incubation, plates were blocked with PBS plus BSA (1% w/v) and Tween 20 (0.1%) before the addition of IgA b12 or IgA b12 cell culture supernatants. The plates were washed and then probed with peroxidase-conjugated, affinity-purified goat polyclonal Abs (0.5 μg/ml) specific for human IgA (Southern Biotechnology Associates) or human κ-chain (Sigma-Aldrich). Plates were developed using the one-component tetramethylbenzidine substrate (Kirkegaard & Perry) and read using a spectrophotometer (Molecular Devices). Total IgA levels were determined by sandwich ELISA using plates coated with goat anti-human IgA (Cappel) diluted 1/1000 into PBS and detected using biotin-labeled goat-anti-human, as done previously (18). Human IgA_2 (Calbiochem) was used as a standard.

Ab-binding curves

Ninety-six-well microtiter plates (Corning) were coated with HIV-1_{MN} gp120 (2 μg/ml in PBS (pH 7.4); Immunodiagnostics) overnight at 4°C. The plates were blocked with 4% nonfat dry milk for 30 min at room temperature and then incubated for 2 h at 37°C with serial dilutions of mAbs. The plates were developed by incubation with alkaline phosphatase-labeled goat anti-human κ L chain Abs and the alkaline phosphatase substrate kit (Pierce), then analyzed using a microtiter plate spectrophotometer set at 405 nm. The concentrations of mAbs that gave an OD of 1.0 at 405 nm were used for the avidity determination and competitive inhibition assays (see below).

Ab avidity and affinity for gp120

The avidity (or functional affinity) of each form of b12 mAb was determined by disrupting mAb binding to the solid-phase Ag using increasing amounts of ammonium thiocyanate (NH_4SCN; 0–4.0 M), a mild denaturing agent for 30 min after mAb binding to the solid phase, before adding the labeled conjugate Ab, as described (32, 33). The affinity of each form of b12 mAb was measured by competitive inhibition of Ab binding to the solid phase by preincubation of mAb (concentration yielding OD of 1.0) with serial 3-fold dilutions of soluble HIV-1_{MN} gp120 Ag (0–1 μg/ml) overnight at 4°C, as previously described (34–36). The amounts of unbound mAbs were measured as described above. For both the NH_4SCN and competitive inhibition assays, the ODs in duplicate wells were averaged; the background with no Ag was subtracted. The molar concentrations yielding a 50% inhibition of OD with inhibitor or soluble Ag were determined as the affinity index (AI) and competitive inhibition index, respectively. The latter was used to estimate the dissociation constant (K_D) using the formula $K_D = a_0((A_0/A_0 - A) - 1)$ as described (34).

HIV-1 neutralization assays

HIV-1_{BaL} (R5) and HIV-1_{IIIb} (X4) were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program (NIH ARRRP; nos. 510 and 398, respectively). For conventional neutralization assays, viruses (50 50% tissue culture-infective dose ($TCID_{50}$)/ml) were mixed with IgG1 or IgA_2 b12 (monomeric, dimeric, and polymeric forms) and then added in duplicate to 96-well flat-bottom microtiter plates (Costar) and incubated for 30 min at 37°C. PBMCs from healthy adult donors seronegative for HIV-1 and hepatitis B and C were isolated from

Table I. *Neutralization of HIV-1-pseudotyped virus in luciferase reporter assays*

	Virus					
	JR-FL		JR-CSF		HxB2	
mAb (nM)[a]	IC_{90}[b]	IC_{50}[c]	IC_{90}	IC_{50}	IC_{90}	IC_{50}
IgG1	1.3	0.7	5.3	0.7	0.7	<0.27
mIgA2	4.1	0.6	>58.8	2.4	0.6	<0.24
dIgA2	1.1	0.3	31.3	1.1	0.6	<0.11
pIgA2	1.3	0.3	25.0	0.6	0.3	<0.11
SIgA2	0.9	0.2	16.7	0.2	0.2	<0.1
mAb (μg/ml)	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}
IgG1	0.2	0.1	0.8	0.1	0.1	<0.04
mIgA2	0.7	0.1	>10	0.4	0.1	<0.04
dIgA2	0.4	0.1	10.0	0.4	0.2	<0.04
pIgA2	0.4	0.1	9.1	0.2	0.1	<0.04
SIgA2	0.4	0.1	7.2	0.1	0.1	<0.04

[a] The following molecular masses were used to calculate the molar concentration of each Ab: IgG1 150 kDa; $mIgA_2$ 170 kDa; $dIgA_2$ (with J chain) 360 kDa; $SIgA_2$ b12 (with J chain and SC) 430 kDa. The molecular mass of $pIgA_2$ b12, which is a mixture of monomer, dimer, and higher molecular mass molecules, was assumed to be 320 kDa, the dominant species expressed by clone 1 expressing pIgA2 b12 as determined by gel filtration chromatography.

[b] Concentrations of Ab required to neutralize 90% of the indicated virus, presented in nanomoles or micrograms per milliliter.

[c] Concentrations of Ab required to neutralize 50% of the indicated virus, presented in nanomoles or micrograms per milliliter.

buffy coats by Ficoll-Hypaque gradient centrifugation. Before infection, PBMCs were activated by overnight incubation with PHA (5 μg/ml; Sigma-Aldrich) in RPMI 1640 with 10% heat-inactivated FBS (HyClone), 5% recombinant human IL-2 (Roche), and 50 μg/ml gentamicin (Invitrogen Life Technologies) at 37°C and 5% CO_2. The cells were then washed and cultured for 3 days in RPMI 1640 with 10% FBS and 5% IL-2 before being added to the virus-Ab mixtures. A total of 2×10^5 cells in 100 μl were added to each well and incubated for 2 h at 37°C to permit viral infection. The PBMCs were washed three times to remove unbound virus, and 6 days later the cell supernatants were assayed for p24 levels by ELISA (Beckman Coulter).

Neutralization activity of the various forms of Ab b12 was confirmed using HIV-1 envelope-pseudotyped viruses, which are capable of single-round replication generated by cotransfection of 293T cells with the pNL4-3.luc.RE vector (N. Landau, NIH ARRRP) and the psVIIIex7 *env*-expressing vector, provided by J. Sodroski (Dana-Farber Cancer Institute) (37). The assays were conducted by seeding U87.CD4.CCR5 cells (NIH ARRRP) in totals of 1.5×10^4 cells/well in a 96-well flat-bottom tissue culture-treated plate (Corning). Seeded cells were contained in a 100-μl volume of medium (DMEM containing 10% FBS, 300 μg of G418/ml, glutamine, and penicillin-streptomycin) and incubated for 24 h at 37°C in 5% CO_2. Separately, Ab and virus mixtures were made by adding 60 μl of serially diluted Ab in medium to an equal volume of medium containing 2×10^5 relative light units of pseudotyped virus and incubated for 1 h at 37°C. Following incubation, 100 μl of the Ab and virus mixtures were transferred to the U87 cells and incubated for 3 days at 37°C in 5% CO_2. Cell lysates were generated by adding 60 μl of cell culture lysis reagent (Promega), incubated at room temperature for 5 min, pelleted by centrifugation at $1200 \times g$ for 2 min. A total of 20 μl of lysate was transferred to opaque assay plates (Corning), luciferase reagent was added (Promega), and luciferase activity was measured on a luminometer (Orion; Berthold Detection Systems). The degree of virus neutralization by Ab was determined by measuring luciferase activity. The percent neutralization at a given Ab concentration was express as: ((luciferase activity in the absence of Ab − luciferase activity in the presence of a given Ab concentration)/luciferase activity in the absence of antibody) × 100. The following molecular masses were used to calculate the molar concentration of each Ab (Table I): IgG1 150 kDa; mIgA2 170 kDa; dIgA2 (with J chain) 360 kDa; SIgA2 b12 (with J chain and SC) 430 kDa. The molecular mass of pIgA2 b12, which is a mixture of monomer, dimer, and higher molecular mass molecules, was assumed to be 320 kDa, the dominant species expressed by clone J1 expressing pIgA2 b12 as determined by gel filtration chromatography.

Epithelial cell transfer of HIV-1 to PBMCs

ME180 (HTB-33; American Type Culture Collection (ATCC)) and Caco-2 (HTB-37; ATCC) cells were cultured in Eagle's MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). For the HIV-1 transfer assays, ME180 and Caco-2 epithelial cells were plated in 96-well flat-bottom microtiter plates at 1×10^5 cells/ml (200 μl/well) 2 days before infection. $HIV\text{-}1_{BaL}$ or $HIV\text{-}1_{IIIb}$ (50 $TCID_{50}$/ml) were incubated in the absence or presence of IgG1 or IgA_2 b12, or relevant isotype control Abs for 30 min at 37°C. The virus-Ab mixtures were then applied to confluent epithelial cell monolayers and incubated for 2 h at 37°C. The monolayers were washed three times with medium to remove any unbound virus and overlaid with activated PBMCs (2×10^5/well in 200 μl). Cell-free supernatants were harvested 6 days after coculture and assessed for p24 levels by ELISA. For studies aimed at determining at which step(s) b12 inhibits viral transfer from epithelial cells to PBMCs, mAbs were added 1) concurrently with $HIV\text{-}1_{BaL}$ or $HIV\text{-}1_{IIIb}$ (50 $TCID_{50}$/ml; designated +/−), 2) after epithelial cells had been washed to remove free virus (designated −/+), virus-Ab complexes, or free Ab; 3) or at both steps (designated +/+).

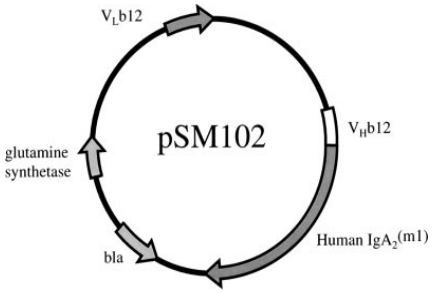

FIGURE 1. Schematic of the IgA_2 b12 expression vector. Plasmid pSM102 encodes the IgA_1 b12 V_H and V_L (κ) chain, GS, as a selectable-amplifiable marker in mammalian cells, and β-lactamase for selection in *Escherichia coli.*

Inhibition of HIV-1_{BaL} attachment and internalization by ME180 cells

We used a protocol adapted from Bobardt et al. (38) to measure HIV-1 attachment and uptake by human cervical ME180 cells. Briefly, ME180 cells grown to confluence in 96-well flat-bottom plates were exposed in duplicate to $HIV\text{-}1_{BaL}$ (20 ng p24/well) in the absence or presence of IgG1 or IgA_2 b12, or relevant isotype control Abs. Cell monolayers were washed twice to remove free virus and virus Ab complexes, and then treated with lysis solution (Beckman Coulter). Levels of p24 Ag were measured in cell lysates by ELISA. The p24 Ag levels were considered representative of the total amount of cell associated virus (i.e., virus on the cell surface, as well as virus that had bee internalized). To determine only the amount of virus internalized by ME180 cells, the monolayers were washed two times to remove free, unbound virus, and then treated with 0.05% trypsin-EDTA (Invitrogen Life Technologies) for 8 min at 37°C to proteolytically remove surface-bound virus (39–41). Preliminary dose-response curves revealed that 0.05% trypsin resulted in the maximal removal of virus from the cell surfaces: higher concentrations of trypsin did not reduce HIV-1 levels further. After treatment with trypsin, the cells were treated with lysis solution and lysates were tested for p24 Ag, as described above.

Results

Production of monomeric and dimeric forms of recombinant human IgA_2 b12

Ab IgG1 b12 was originally engineered from a Fab by cloning the b12 V_H and V_L genes into a mammalian combinatorial expression vector (12). Using this original vector, we switched the isotype of b12 by replacing the IgG1 constant domains with a PCR fragment encoding the constant domains of human IgA_2(m1) (23), resulting in the plasmid pSM102 (Fig. 1). Plasmid pSM102 was then transfected into CHO cells, and several cell lines were established which secreted $mIgA_2$ b12 into cell supernatants. One cell line in particular, 100-02-C9, was chosen for further study because of its stability and robust production of Ab. Clone 100-02-C9 was adapted to protein/serum-free medium and $mIgA_2$ b12 was

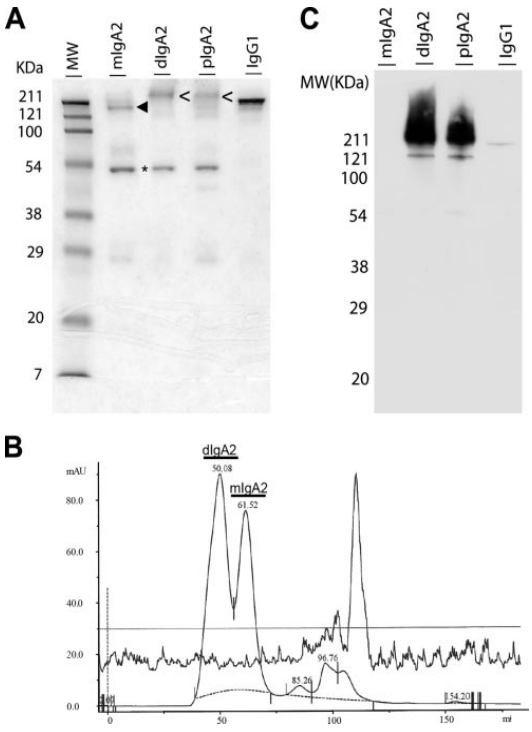

FIGURE 2. Expression and purification of monomeric and dimeric forms of IgA2 b12. *A*, Nonreducing SDS-PAGE analysis of monomeric, dimeric, and polymeric forms of IgA_2 b12, as compared with IgG1 b12. Ab preparations were size-fractionated by nonreducing SDS-PAGE and stained with Coomassie blue. Monomeric IgA_2 b12 migrated with a molecular mass of ~150 kDa (filled arrowhead), whereas higher molecular mass forms (>220 kDa; open arrowheads) were present in the dimeric and polymeric IgA b12 preparations. A protein band of ~50 kDa was present in all IgA b12 preparation (*), and likely corresponds to κ L chain dimers that dissociate from intact Ig (52). IgG1 b12 migrated with a molecular mass of ~160 kDa. *B*, Separation of dimeric and monomeric forms of IgA_2 b12 by FPLC gel filtration. As described in *Materials and Methods*, Abs were eluted from by isocratic elution on HiPrep 16/60 Sephacryl S-300 High Resolution column at a flow rate of 0.5 ml/min. Peaks containing dimeric IgA_2 b12 (50.02 peak) and monomeric IgA_2 b12 (61.52 peak) were determined by ELISA and SDS-PAGE. The following molecular mass standards were used to calibrate the column: dextran 2000 kDa, ferritan 440 kDa, catalase 220 kDa, and RNase A 10 kDa. *C*, Anti-J chain Western blot analysis. Monomeric, dimeric, and polymeric forms of IgA_2 b12, as well as IgG1 b12, were subjected to nonreducing SDS-PAGE, transferred to nitrocellulose, and probed with an anti-human J chain Ab. The minor band observed in the IgG1 b12 lane is probably due to spill over from the adjacent $pIgA_2$ b12 sample.

purified from cell supernatants by protein L affinity chromatography. Analysis of purified $mIgA_2$ b12 by nonreducing SDS-PAGE revealed a protein species of ~150 kDa, which corresponds to the expected molecular mass of $mIgA_2$ b12 (Fig. 2*A*).

To produce dimeric/polymeric forms of IgA_2 b12, clone 100-02-C9 was supertransfected with plasmid pcDNA3Hygro::J chain, encoding human J chain (23). We identified several stable cell lines, notably J2 ("clone 1") and J10 ("clone 2"), which secreted high molecular mass (i.e., dimeric and/or polymeric) forms of IgA_2 b12. Total IgA_2 b12 present in the supernatants of J2 cell cultures, which contain a mixture of monomers, dimers, and higher molecular mass polymers, were purified by protein L affinity column and

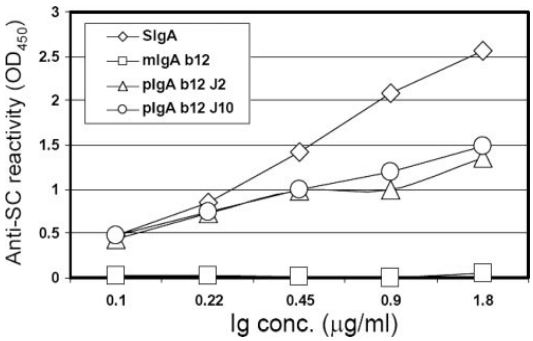

FIGURE 3. SC associates with polymeric IgA_2 b12. Microtiter plates were coated with polyclonal goat anti-human IgA Abs, and then overlaid with human secretory IgA (positive control), monomeric IgA_2 b12 (negative control), or dimeric IgA_2 b12 from CHO cell clones J2 or J10. The plates were then probed with human SC and then developed using anti-human SC Abs. Each data point represents the average of two microtiter wells tested in parallel.

referred to simply as "polymeric" IgA_2 b12 ($pIgA_2$ b12). Dimeric forms of IgA_2 b12 were purified from higher molecular forms IgA_2 b12 by size-exclusion chromatography (Fig. 2*B*). As expected, dimeric IgA_2 migrated with an apparent molecular mass >220 kDa (Fig. 2*A*). Additional minor bands of slightly higher and lower molecular masses (e.g., 200–250 kDa) were also evident by SDS-PAGE, and likely represent $dIgA_2$ b12 with varying degrees of glycosylation (42). Western blot analysis confirmed that J chain was associated with $dIgA_2$ b12, but not $mIgA_2$ b12 or IgG1 (Fig. 2*C*). Additionally, IgA_2 b12 derived from clones J2 and J10 (but not from 100-02-C9) were capable of binding to human SC in a SC solid phase-binding assay (Fig. 3), further demonstrating that J chain was properly incorporated into dimeric/polymeric forms of IgA_2 b12. It should be underscored that the use of the term *polymeric* IgA_2 b12 in this manuscript refers to a mixture of monomers, dimmers, and higher molecular mass polymeric forms of Ab.

Reactivity of IgA b12 with gp120

We used ELISA to confirm that the different forms of IgA_2 b12 retained their ability to bind gp120. The use of anti-κ chain-specific secondary Abs in our ELISAs enabled us to directly compare the binding profiles of different Ab isotypes (i.e., IgA and IgG). As shown in Fig. 4*A*, all three forms of IgA_2 b12 (monomeric, dimeric, and polymeric) reacted with $gp120_{MN}$ in a dose-dependent manner with binding profiles similar to that obtained with IgG1 b12. Identical results were obtained when ELISAs were performed using $gp120_{IIIB}$ and $gp120_{JR\text{-}FL}$ (data not shown).

To assess the relative avidity (i.e., functional affinity) of different forms of IgA_2 b12 for gp120, we determined the amount of ammonium thiocyanate required to elute 50% mAb bound to gp120 (32, 33). From this, we calculated the AI for each mAb preparation, as described in *Materials and Methods*. All three forms of IgA b12 eluted from the plate at roughly equivalent amounts of ammonium thiocyanate (0.2–0.6 M), thereby yielding similar AIs (Fig. 4*B*). Although slightly higher levels of ammonium thiocyanate were required to IgG1 b12, this difference was not significant. The relative affinities of each form of IgA b12 as estimated using a competitive inhibition assay were also similar (Fig. 4*C*). From these experiments, we conclude that monomeric and dimeric forms of IgA_2 b12 recognize gp120 at levels comparable to IgG1 b12.

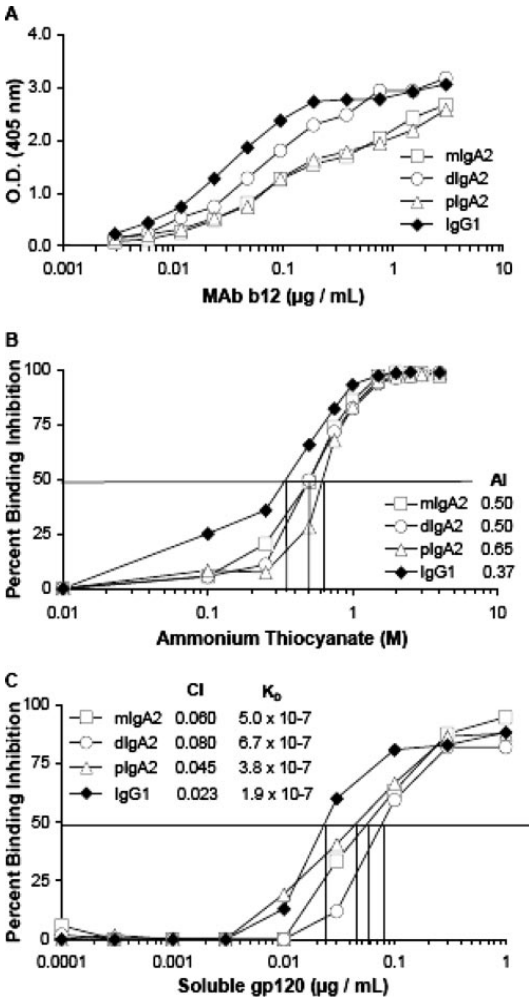

FIGURE 4. Reactivity, avidity, and affinity of IgA_2 b12 for gp120. The relative reactivity, avidity, and affinity of different forms of purified IgA_2 b12 were compared with IgG1 b12. *A*, ELISA analysis examining the reactivity IgA_2 and IgG1 b12 with $gp120_{MN}$. Anti-human κ chain Abs were used as secondary reagents to allow direct comparison of reactivity of different b12 isotypes with gp120. *B*, Functional avidity of IgA_2 and IgG1 b12 for $gp120_{MN}$ as determined by ammonium thiocyanate dissociation (see *Materials and Methods*). The AI (*inset*) is defined as the amount of ammonium thiocyanate (M) required to elute 50% of the Ab from gp120. *C*, The relative affinities of IgA_2 and IgG1 b12 for $gp120_{MN}$ were determined indirectly by competitive inhibition. Abs were mixed with increasing concentrations of soluble $gp120_{MN}$ before being applied to ELISA plates coated with the same Ag. The amount of soluble gp120 required to reduce Ab binding by 50% was defined as the competitive inhibition index (*inset*, *left*). The approximate K_D (*inset*, *right*) was determined as described in *Materials and Methods*.

Neutralization of HIV-1 by IgA_2 b12

We next examined the ability of different forms of IgA_2 b12 to inhibit CCR5-tropic and CXCR4-tropic strains of HIV-1 from infecting PBMCs. Monomeric, dimeric, and polymeric forms of IgA_2 b12 demonstrated a dose-dependent inhibition of HIV_{BaL} (CCR5-tropic) and HIV_{IIIB} (CXCR4-tropic) infection of PBMCs similar to that obtained with IgG1 b12 (Fig. 5). No significant differences in the IC_{50} were observed among the different Abs

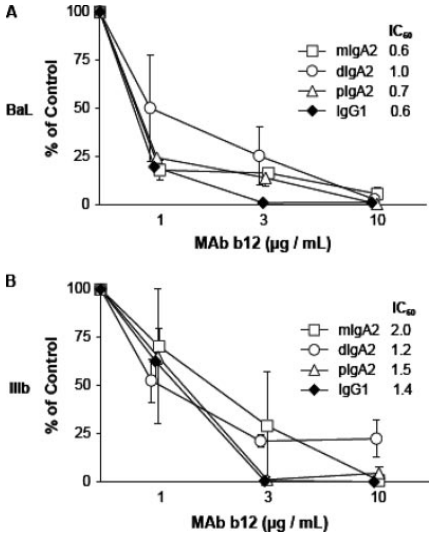

FIGURE 5. Neutralization of HIV-1 infection of PBMCs by IgA_2 b12. Monomeric, dimeric, and polymeric (i.e., a mixture of monomers, dimers, and higher molecular mass polymers) IgA_2 b12, as well as IgG1 b12, at indicated concentrations were incubated with (*A*) $HIV\text{-}1_{BaL}$ or (*B*) $HIV\text{-}1_{IIIb}$ for 30 min before being mixed with activated PBMCs. Levels of p24 in PBMC culture supernatants were determined 6 days later by ELISA, as described in *Materials and Methods*. Viral p24 in the control wells were consistently >14 ng/ml, confirming that the control cells were productively infected with HIV. The mAb concentration (micrograms per milliliter) necessary to inhibit 50% of viral infection, referred to as the IC_{50}, is indicated in the *inset*.

tested in this assay. We also performed pseudovirus infection assays, which are considerably more sensitive than conventional T cell infection assays (37). Three pseudoviruses were tested: $HIV\text{-}1_{JR\text{-}FL}$ and $HIV\text{-}1_{JR\text{-}CSF}$, two CCR5-tropic virus isolates, and $HIV\text{-}1_{HxB2}$, a CXCR4-tropic virus which is virtually the same as HIV_{IIIB}. These studies revealed subtle differences in neutralization activities among the different derivatives of b12 (Table I). These differences were most apparent when mAbs were examined as a function of their molar concentrations used in the neutralization assays, and when we compared IC_{90} values, rather than the less stringent IC_{50} values. For example, monomeric IgA_2 b12 was notably less effective than other forms of b12 at neutralizing $HIV\text{-}1_{JR\text{-}FL}$ and $HIV\text{-}1_{JR\text{-}CSF}$, whereas it was no different at neutralizing HxB2 (Table I). In fact, all forms of IgA_2 b12 were rather poor at neutralizing $HIV\text{-}1_{JR\text{-}CSF}$ for reasons that are not immediately apparent. In contrast, monomeric, dimeric, and polymeric forms of IgA_2 b12 were slightly more effective than IgG1 at neutralizing $HIV\text{-}1_{HxB2}$.

We were also successful in producing a small amount of purified dimeric IgA_2 b12 complexed with human SC (A. Hessell and D. Burton, manuscript in preparation) that enabled us to examine the capacity of $SIgA_2$ b12 to neutralize HIV-1. As shown in Table I, the IC_{50} and IC_{90} for $SIgA_2$ b12 were slightly lower than those obtained with $dIgA_2$ b12, suggesting that the addition of SC may marginally enhance the capacity of IgA b12 to block HIV-1 infection of T cells.

Inhibition of HIV-1 transfer from epithelial cells to PBMCs

Although cervical epithelial cells are not considered permissive for HIV-1 infection per se, they may facilitate virus transfer to other

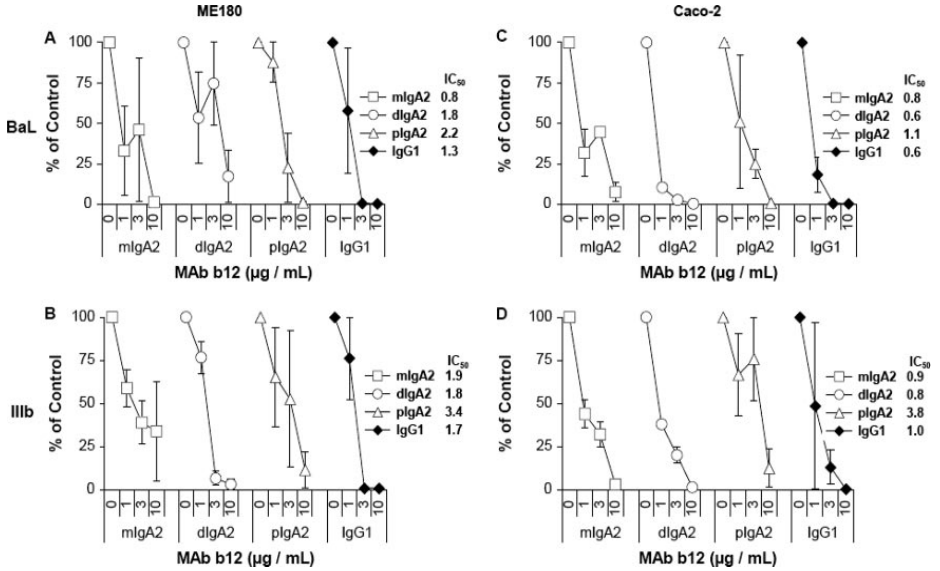

FIGURE 6. Inhibition of HIV-1 transfer from epithelial cells to PBMC target cells by b12 mAbs. Cell-free HIV-1_{BaL} or HIV-1_{IIIb} preparations were incubated with IgG1 or IgA_2 b12, and then applied to confluent monolayers of ME180 cells (*left*, *A* and *B*) or Caco-2 cells (*right*, *C* and *D*). Transfer was assessed by the addition of activated PBMCs and measuring p24 levels in culture supernatants by ELISA. The IC_{50} (micrograms per milliliter) is indicated in the *inset* table. Isotype-matched control Abs of irrelevant specificities showed no inhibition of HIV-1 transfer (data not shown).

cell types within the mucosa (2, 41). To determine whether Ab b12, which is directed against a highly conserved epitope within the CD4-binding site of gp120, can interfere with this step in viral transmission, we incubated HIV-1_{BaL} and HIV-1_{IIIb} with IgG1 b12, or IgA_2 b12, and then applied the mixtures to monolayers of ME180 cells, a human-derived cervical epithelial cell line, or Caco-2 cells, a human-derived intestinal epithelial cell line. HIV-1 "transfer" from epithelial cells to $CD4^+$, chemokine $receptor^+$ cells was assessed by the addition of activated PBMCs to the epithelial cell monolayers. p24 levels were measured in the supernatants of the cocultures 6 days later (see *Materials and Methods*). We found that IgG1 b12, as well as monomeric and dimeric forms of IgA_2 b12, inhibited HIV-1 infection of PBMCs in both the cervical and colonic epithelial cell transfer assay (Fig. 6). To discern whether the mAbs were interfering with viral attachment to and/or uptake by the epithelial cell monolayers ("step 1"), or inhibiting viral transfer from epithelial cells to PBMCs ("step 2"), the transfer assay was modified such that Ab b12 was added concurrently with HIV-1_{BaL} and HIV-1_{IIIb} (as done above), or added after virus had been allowed to attach to, and be internalized by, epithelial cells. As shown in Fig. 7, the addition of IgG1 b12 or dimeric IgA_2 b12 at either step 1 (+/−), step 2 (−/+), or both (+/+) prevented HIV-1 infection of PBMCs, indicating in fact that b12 can block viral attachment of epithelial cells, as well as epithelial-PBMC transfer. Similar concentrations of Ab b12 were required to inhibit step 1 or step 2 of the epithelial transfer assay. It should be noted that at much higher doses of challenge virus (~4000 $TCID_{50}$/ml), Ab applied before viral attachment to epithelial cells was more efficient at blocking infection of PBMCs than was Ab added after epithelial attachment/uptake (data not shown). Thus, b12 can act at either step in the epithelial transfer

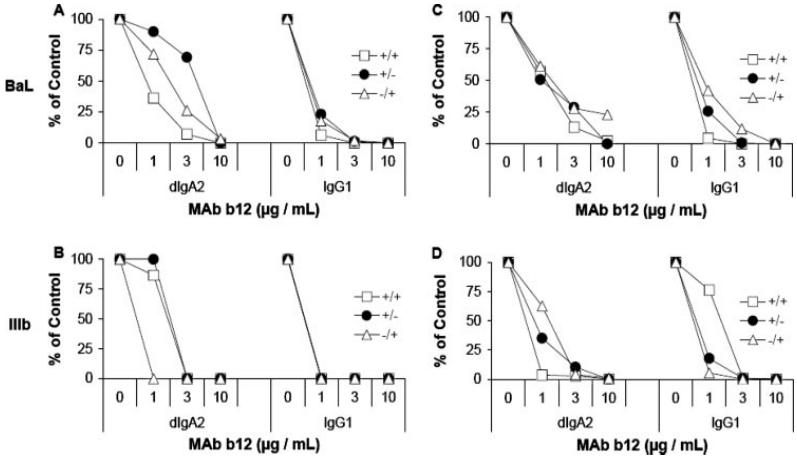

FIGURE 7. b12 mAbs block inhibition of HIV-1 transfer from epithelial cells to PBMCs. Cell-free HIV-1_{BaL} or HIV-1_{IIIb} preparations applied to confluent monolayers of ME180 cells (*left*, *A* and *B*) or Caco-2 cells (*right*, *C* and *D*) were incubated with IgG1 or IgA_2 b12: 1) before epithelial exposure (+/−); 2) after epithelial exposure and washing but before addition of PBMCs (−/+), or both times (+/+), and then transfer was assessed by the addition of activated PBMCs and measuring p24 levels in culture supernatant by ELISA.

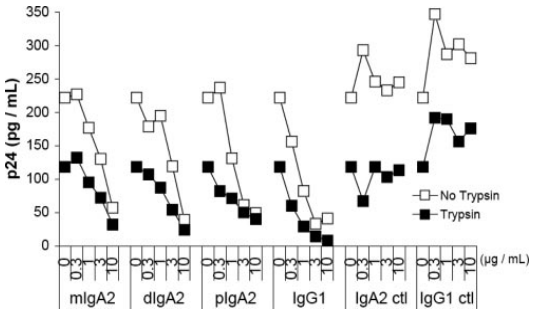

FIGURE 8. Inhibition of HIV-1 BaL internalization in ME180 cells by b12 mAbs. Increasing concentrations of IgG1 or IgA_2 b12 were mixed with HIV-1_{BaL} before application to ME180 cells. After 1 h at 37°C, the cells were washed to remove unbound virus and then lysed. Levels of p24 Ag in cell lysates were considered representative of the total amount of virus bound specifically to cell surfaces, plus the amount of virus that had been internalized (□). Alternatively, ME180 cells were treated with trypsin-EDTA (0.05%) for 8 min before lysis to effectively remove virus bound to the cell surfaces. Levels of p24 Ag in cell lysates from trypsin-treated cells were considered representative of the only amount virus internalized (■).

assay, but appears more efficient when added before viral epithelial exposure.

Inhibition of HIV-1 attachment to and uptake by cervical epithelial cells

In primary intestinal epithelial cells, it was reported that HIV-1 is internalized by endocytosis before being transferred to $CD4^+$, co-$receptor^+$ cells permissive for viral replication (4). From our experiments, it was unclear whether Ab b12 in step 1 was interfering with virus attachment to epithelial cells, and/or blocking virus uptake (i.e., endocytosis) into epithelial cells. To differentiate between attachment and uptake, we modified our transfer assays to include a trypsin digestion step which allowed us to proteolytically inactivate virus bound to cell surfaces, but not affect viruses that had been endocytosed (39–41). Both IgG1 b12 and IgA_2 b12 demonstrated a dose-dependent reduction in the total amount of HIV-1_{BaL} associated with ME180 cells (Fig. 8). In contrast, control IgG1 or IgA_2 Abs had no effect on virus-epithelial interactions. Treatment of HIV-1-exposed ME180 cells with trypsin revealed that ~50% of the total cell-associated virus was resistant to digestion, suggesting that a fraction of HIV-1 was located intracellularly. The amount of HIV-1 recovered from trypsin-treated ME180 cells was reduced when HIV-1_{BaL} was treated with IgG1 b12or IgA_2 b12, inferring that b12 is capable of inhibiting virus uptake, as well as virus attachment to cervical epithelial cells.

Discussion

Understanding the molecular mechanisms by which IgG and IgA Abs against gp120 interfere with HIV-1 transmission across mucosal surfaces has important implications for the design of effective vaccines and topical antivirals. In this study, we have successfully produced and purified recombinant monomeric and dimeric human monoclonal IgA_2 Abs carrying the V regions of gp120-specific Ab b12 and characterized their functional activity in vitro. Human J chain was properly associated with dimeric IgA_2 b12 as evidenced by Western blot analysis and by the ability of dimeric IgA_2 b12 to bind human SC in a solid phase-binding assay. Although dimeric/polymeric forms of IgA can occur in the absence of J chain, these complexes cannot bind SC (43). rIgA_2 b12 recognized gp120 by ELISA and neutralized HIV-1 infection of both activated PBMC and in a T cell line at levels comparable to IgG1 b12, indicating that both monomeric and dimeric forms of IgA_2 b12 are fully functional. Finally, we have demonstrated for the first time that both IgG1 and IgA_2 b12 are capable of preventing HIV-1 attachment to and uptake by colonic and cervical epithelial cells, revealing a possible mechanism by which Ab b12 protects mucosal compartments from HIV-1 infection in vivo.

Although two studies have demonstrated the ability of IgG1 b12 to protect macaques against mucosal SHIV challenge, the mechanism by which Ab b12 neutralized HIV-1 in vivo was not determined (15, 16). Results from our current study indicate that Ab b12 is capable of interfering with the earliest steps in mucosal transmission of HIV-1; attachment to and uptake by epithelial cells. These data are somewhat surprising because b12 recognizes an epitope overlapping the CD4-binding site on gp120, and the interaction of HIV-1 with epithelial cells is independent of CD4 (2, 4). Indeed, neither Caco2 nor ME180 epithelial cells express CD4 as detected by immunofluorescence or flow cytometry (E. N. Janoff and J. Palaia, unpublished data). Therefore, it is likely that Ab b12 interferes with viral attachment by steric hindrance or altering virus-host cell interactions through other means (44). Several groups have proposed that galactosyl ceramide serves as the primary receptor for HIV-1 on intestinal epithelial cells (45, 46). Galactosyl ceramide is a glycosphingolipid that protrudes only a few nanometers from the plasma membrane, so occupancy of IgG or IgA b12 on the surface of gp120 would be expected to interfere with virus-membrane contact. Although the receptor used by HIV-1 on cervical epithelial cells is currently unknown, the virus-epithelial cell interaction most certainly involves gp120.

Two other broadly neutralizing anti-HIV-1 human Abs, 2F5 and 2G12, have been class switched from IgG1 to IgM and IgA_1 (47). 2G12 binds a conserved cluster of oligomannose side chains on gp120, and 2F5 targets a membrane proximal region of gp41. The neutralization activities of 2F5 and 2G12 were unchanged by isotype switching, except for 2G12 IgM, which inhibited HIV-1 infection of PBMCs ~28 times more efficiently than corresponding IgG (47). Whereas we converted Ab b12 to a human subclass IgA_2, Wolbank et al. (47) converted 2G12 and 2F5 to subclass IgA_1. IgA_1 and IgA_2 (of which there are two allotypic variants, m1 and m2) are found at roughly equal concentrations in mucosal secretions of the female genital tract and the large intestine (48). Although slight structural differences exist between IgA_1 and IgA_2 (e.g., IgA_1 has an extended hinge region as compared with IgA_2), there are currently no known functional differences between the two subclasses (17). In another report, Liu et al. (49) isotype switched the human mAb F425B4e8 from an IgG2 to other IgG subclasses (i.e., IgG1,3,4), as well as IgA_1. mAb F425B4e8 recognizes an epitope at the base of the V3 loop of gp120 (50). Overall, the isotype variants of F425B4e8 performed similarly to each other with respect to recognition of gp120 and HIV-1 neutralization activity in vitro.

The feasibility of producing large amounts of IgA_2 b12 in cell culture may have a number of practical applications. Most notable is the possibility of including IgA_2 b12 in a topical microbicide aimed at reducing HIV-1 transmission across the mucosal surfaces of the vagina and ectocervix. In macaque models, IgG1 b12 applied vaginally in PBS or in a hydroxymethyl cellulose gel protected animals against a vaginal SHIV challenge, although protection was short-lived and required relatively high concentrations of Ab (16). We postulate that IgA_2 b12, when complexed with SC, may provide better passive protection than that afforded by IgG1 b12 because SIgA is protease resistant and has a propensity to anchor in the mucus layer overlying epithelial cell surfaces (21). In fact, studies are underway to investigate the potential of SIgA2 b12

as a topical microbicide in the macaque model. Another application of IgA_2 b12 is as a standard in ELISAs designed to detect anti-HIV-1 IgA Abs in human secretions. Accurately measuring anti-viral IgA titers in mucosal secretions is inherently difficult, and has been plagued, in part, by variation in ELISAs from laboratory to laboratory (20). Providing IgA_2 b12 as a standard to public health and research laboratories would better enable the results of ELISAs from different laboratories around the world to be compared.

Determining the role of IgG and SIgA in conferring immunity to HIV-1 on mucosal surfaces has implications for HIV vaccine design and delivery. SIgA Abs are induced almost exclusively following mucosal vaccination, and are not elicited at any appreciable levels by systemic (e.g., i.m.) immunizations (1, 51). Whereas mice are considered to have a "common" mucosal immune system (i.e., immunization at one mucosal site stimulates a SIgA response at both local and both distant local mucosal responses), in humans, the shared mucosal immune system is more restricted. For example, oral immunization stimulates Abs in the proximal small intestine, but not in the distal large intestine or female genital tract (18). However, Abs in the female genital tract can be induced following vaginal or more practically intranasal vaccination. Delivery of mucosal vaccines also poses significant challenges, including the need for better adjuvants and formulations capable of withstanding the harsh environment of the respiratory and gastrointestinal tracts. The availability of fully functional IgG1 and IgA_2 Abs that have identical V regions directed against a conserved neutralizing epitope on gp120 will enable us to define experimentally in animal models the "division of labor" that certainly exists between these two different Ab isotypes in mucosal tissues.

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Disclosures

The authors have no financial conflict of interest.

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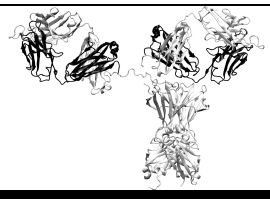
Chapter 10



Summary, Discussion, and Conclusions

The vaccine challenge

Direct evidence of how vaccines work in humans remains elusive, but it has long been believed that the main gatekeeper in most vaccination strategies is neutralizing antibodies. Traditionally, a major component of most vaccines is the capacity to raise robust neutralizing antibody titers that can be correlated to protection (4, 33, 56), however, bnAbs against HIV are rare and their presence in the sera of infected individuals is low (36, 47). This success of HIV-1 to limit the generation of bnAbs due to the arrangement and accessibility of nonfunctional forms of virus surface proteins creates a barrier to design a vaccine based on inactivated virus or virus-like particles. Further, a major obstacle in vaccine design is inducing antibodies that would be able to bind and neutralize the many variations in the virus present in the global pandemic (21).

A study of broadly neutralizing sera from patient cohorts has shown, however, that it is possible for a potent IgG response to be elicited against one of the major vaccine targets, the conserved CD4 co-receptor binding site (25). In contrast, bnAbs have yet to be induced with any vaccine approaches (14). Despite the disappointing results of these attempts, vaccine researchers are now able to employ a wealth of knowledge accumulated from divergent groups within the HIV research community who have provided structural details from x-ray crystallography, and spatial and visual clues from cryo-electron microscopy tomography (5, 18, 20, 23, 50, 52-55, 58). Because of the extreme diversity of HIV-1 isolates in structure and sequence, the favored vaccine targets are the most conserved epitopes of the Env spike that are accessible to antibody. Unequivocally, an eventual successful vaccine therefore will address the conserved epitopes on both gp120 and gp41. Advances in X-ray crystallography have led to decisive insights through structural imagery of both gp41 and gp120 (10, 18, 23, 48, 49). Structures of viral surface proteins bound to neutralizing antibodies or (co-)receptor molecules continue to provide important clues for immunogen design (22, 23, 30, 37, 53).

Strategic and central elements in a designer approach for a vaccine against HIV-1 therefore are the bnMAbs, discussed here. These bnMAbs have been characterized in great detail both structurally, biochemically, and in terms of neutralization (2, 6, 8, 32, 39, 44). Epitope mimics of the conserved Env sequences of the bnMAbs are being engineered to focus antibody responses on their epitopes.

Summary and discussion

The chapters in this thesis trace studies involving four broadly neutralizing antibodies (bnMAbs) against HIV-1 that allow us to examine the potential mechanisms by which antibodies protect against HIV-1 infection. These antibodies thereby provide us with tools to qualitatively and quantitatively investigate the requirements for an antibody component of an HIV-1 vaccine. While each antibody is of the same isotype, the nature and location of their cognate epitope on infectious Env spikes identify unique avenues for virus neutralization and prevention of infection. Our protection studies confirm the potential of these antibodies to blunt viremia upon infection or even provide sterile immunity by prevention or abrogation of any viral replication. When taken together the data from these studies suggest that these antibodies may differ in the mechanisms by which protection is provided and that the serum concentrations of each antibody required for protection may vary.

Further, we know from previous studies, including our own experiments with b12, that passively administered antibody provides protection to naïve animals only when the serum concentrations are nearly the same as the concentration required for 100% neutralization *in vitro*. The benchmark for this measurement in HIV-1 is the standard PBMC neutralization assay providing 90% neutralization. Therefore, a serum neutralizing titer in protected animals can be expected to exceed the antibody IC_{90} about 100-fold (29, 31, 42, 45). However, based on the 2G12 and 2F5/4E10 studies discussed in this thesis, there may be exceptions to this common rule-of-thumb.

It is now reasonable to assume that the requirement for more or less antibody may be linked to the mechanism or mode of protection. For example, we observed that when the correlate described above is applied, somewhat lower serum neutralizing titers were required for the anti-MPER antibodies, 2F5 and 4E10, to provide protection of all challenged animals. More notably is that the bnMAb 2G12 protected at serum neutralizing titers equal to the PBMC IC_{90} of 2G12. Thus a serum neutralizing titer only in the order of 1:1 provided 60% protection which suggests a 100 times greater *in vivo* potency than expected. In comparison, a b12 serum neutralizing titer of 1:80 is required to provide 50% protection and complete protection by b12 required neutralization titers of ~1:400. This outcome, which so far seems unique to antibodies directed against the glycan shield of gp120, provides a justifiable basis to include the sugars covering the Env spike to design immunogens aimed at eliciting protective antibody responses. Similarly, the protection observed by 2F5 and 4E10 with serum neutralizing titers on the order of 1:50 based on the 90% antibody neutralization titer in PBMCs validates the interest in immunogen design targeted at the MPER of gp41.

Mechanisms of b12 protection

The epitope for b12 overlaps the highly conserved CD4 binding site which is only partially accessible by antibody, but critical for virus attachment to a host cell making the site a true vulnerability for HIV-1. Virus neutralization is a Fab-mediated mechanism, which in the case of b12, may be explained by steric hindrance. Antibody

b12 alone can prevent free virus from interacting with CD4 on the surface of a T-cell, thereby inhibiting viral entry. We have seen that b12 may also act against HIV-1 via Fc-mediated effector function. Once HIV-1 infection has established, it can spread both via free virus and also via cell-to-cell spread. We have observed that b12 alone is fairly inefficient at preventing such cell-to-cell transmission of the virus. Effector-mediated functions such as ADCC and CDC may therefore be especially important for interrupting established HIV-1 infection. Once bound to an infected cell or virus *in vivo*, b12 may mobilize, via its Fc fragment, a number of responses that potentially help control virus spread. These responses may include binding and activation of complement and lysis of virions or infected cells by CDC, or binding and activation of IgG Fc receptors (FcγR) to induce ADCC. Immune complexes formed by b12 binding of virus or infected cells can also activate phagocytes via FcγR. The resulting phagocytosis also leads to virus or cell killing. Therefore, b12 can in principle protect by binding to free virus and by directly preventing contact with host cells in addition to full-scale mediation of effector functions to act against virions or infected cells.

The macaque mucosal challenge model was chosen to determine a protective dose of b12 against viral challenge (Chapter 3). The study was designed to examine a titration of b12 concentrations in a high-dose vaginal challenge using an R5 SHIV. The study established, with a high degree of certainty, the amount of b12 required to provide complete protection. At 25 mg/kg complete protection was seen with 4 of 4 macaques; 5 mg/kg protected 2 of 4 macaques, but at 1 mg/kg 4 of 4 macaques became infected and only negligible benefit was seen as manifested by a small delay in onset of viremia. Not surprisingly, and in accordance with the 100x rule discussed above, the serum neutralizing titers were approximately 1:400 for the protected group of animals given 25 mg/kg. The titers dropped to about 1:80 when 2 of 4 were protected and at less than 1:20, no protection was seen (Table 1) with only negligible benefit from delayed onset and lower peak viremias.

A major obstacle in antibody protection against HIV-1 has been the perception that achieving sterile immunity via immunization is not practical because of the high concentration of antibody required as indicated by many studies in animal models, including our own b12 protection study just described. However, this perception is mostly based on studies using a typical single high-dose viral challenge that ensures the immediate infection of all controls. Yet, the dose of virus used for typical high-dose macaque challenge is much higher than the dose involved in human sexual contact even when considering high-risk circumstances such as the presence of STDs or during acute infection. Building on the results of the b12 titration protection study, we therefore decided to investigate the impact of virus challenge dose on antibody protection. Thus we designed an experiment to study whether low b12 neutralizing antibody titers might protect against lower-dose challenge, i.e. closer to that experienced in human sexual exposure events. This study was enabled by an innovative antibody dosing and virus challenge design, in which we maintained the neutralizing antibody titer constant by weekly dosing with 1 mg/kg b12 and challenged repeatedly with low doses of virus. Interestingly, we observed that the dose of 1 mg/kg of b12 that failed to protect in the high-dose challenge model, provided significant protection against low-dose challenge by considerably increasing the number of exposures leading to productive infection. More specifically, the model uses a low viral inoculum of 10 $TCID_{50}$ (containing approximately 2.65 x 10^6 vRNA copies), which corresponds to an amount of virus only somewhat higher than is

found in human semen during acute infection, yet substantially lower than the amount used in the traditional high-dose SHIV challenges. All 4 controls became infected after 10 total challenges, whereas the group of 5 animals treated with a low dose of b12 resisted infection for a total of >100 challenges. Notably, 1 macaque even remained uninfected after 40 consecutive low-dose challenges. The effective serum neutralization titer for the protected low-dose animals was only of the order of 1:5; a titer which provided negligible benefit in the high-dose viral challenge setting (Table 1). We attempted to quantify and compare the protection provided by b12 in the various studies by calculating a Protection Efficacy which estimates the reduction in infection susceptibility of b12-treated versus non-treated animals under the conditions tested. Thus in the high-dose challenge model employed in chapters 3 and 5, we required high b12 neutralization titer of ~1:300 to ~1:400 to achieve protection efficacies of 89% and 100%, respectively. Whereas a ~1:5 neutralization titer of b12 antibody was sufficient to achieve a similar protection efficacy of 91% in the low-dose model (Table 1).

The significance of the repeated low-dose challenge study is that serum neutralizing titers in the range of 1:5 may be achievable by vaccination of humans with an appropriate immunogen. Notably, neutralization titers of similar magnitude have been described against various isolates and clades in seropositive individuals. Therefore, our study provides promise and a long-sought optimism for vaccine development.

We next compared protection by wild type and Fc variant b12 in SHIV vaginal challenge in macaques. When Fc variants of b12 were used in conjunction with wild-type b12, we observed that antibody effector functions play an important role in protection against HIV infection. A higher rate of infection and a primary viremia similar to that of isotype control-treated animals was observed for a b12 variant (LALA) that lacked Fc receptor and complement binding. However, a variant that lacked only complement binding (KA) was equally effective in providing protection as wild-type b12. Therefore, we conclude that Fc receptor binding, but not complement activation, is essential to antibody protection against HIV-1. A number of mechanisms can be invoked that could contribute to these experimental observations, including FcγR-mediated activity against virions (e.g. phagocytosis), FcγR-mediated activity against infected cells (e.g. ADCC and phagocytosis) and FcγR-mediated transport of antibody to a critical anatomical site, e.g. mucosal surface, to prevent propagation of viral infection. We showed that the effector-function deficient b12 variant LALA was unable to mediate ADCVI whereas wild-type b12 could, which is consistent with the notion that ADCVI might be crucial in protection. Further, in the low-dose challenge model, we again observed greater protection with b12 as compared to the LALA variant. Available evidence does not currently support a role for FcγR-mediated transport of antibody to mucosal surfaces in protection.

Finally, we have also evaluated the b12 epitope in context of an isotype-switched IgA antibody. Future studies are planned to determine if this change in isotype will prove to be an advantage at the mucosal surface.

Table 1. Summary of b12 protection studies

bnMAb	b12				
Epitope	gp120 CD4bs epitope				
Study description	Antibody titration study Chapter 3			Effector function study Chapter 5	Low-dose repeated challenge study Chapter 6
Challenge virus[1]	$SHIV_{162P4}$			$SHIV_{162P3}$	
Viral challenge route	*intra vaginally*				
Viral challenge dose	300 $TCID_{50}$				10 $TCID_{50}$
i.v. Ab dose (mg/kg)	25	5	1	25	1
Protection record (# of challenges leading to infection/total # challenges)[4]	0/4	2/4	4/4	1/9	4/108
Serum Ab Concentration (μg/ml)[2]	764	223	16	563	40
Antibody IC_{90} (PBMC) (μg/ml)	2			8	
Serum neut titer (PBMC IC_{90})[2]	400	80	16	nt	nt
Serum Ab Concentration/ PBMC Ab IC_{90} [3]	382	112	8	70	5
Protection Efficacy[5]	100%	50%	0%	88.9%	91%

Table 1. [1]Each of the three protection studies were conducted in the macaque mucosal challenge model. Both R5 $SHIV_{162P4}$ and $SHIV_{162P3}$ are closely related and are very similar in sensitivity to b12 neutralization. [2]Serum concentrations and neutralization titers represent averages of all animals in each treatment group at the time of viral challenge. The standard of protection correlate requires serum neutralization titers to be at least 100 times the concentration of antibody required for 90% neutralization in a standard PBMC assay (i.e. serum neut titer of 1:100). Serum neut titers were directly measured in a PBMC-based assay (for some experiments) as indicated. [3]The neutralization titer or protection correlate was also calculated by dividing the serum antibody concentration by the antibody IC_{90} in a PBMC-based assay. [4]The protection record reflects the number of challenges leading to infection relative to the total number of challenges. [5]From the protection record, we calculated a Protection Efficacy that describes the protection afforded by the neutralizing antibody under the conditions tested. Protection Efficacy is calculated by the formula: 100% – ((infection rate b12-treated/infection rate controls) x100%).

The glycan shield: no longer the silent face of gp120

2G12 recognizes a highly conserved dense cluster of oligomannose glycans on the surface of gp120. The crystal structure of Fab 2G12 revealed an unusual interlocked domain-swapped dimer that facilitates binding to its carbohydrate epitope. An antibody response to the carbohydrates on gp120 is surprising because HIV virions are generated within the host cell. Therefore, the sugar moieties would expectedly be excluded by "self" tolerance mechanisms. However, 2G12 evolved immunological solutions to overcome the usual barriers for human antibodies to recognize and be effective against carbohydrates. The intertwined structure of the Fab arms of 2G12 provides a nearly continuous surface and is energetically favored to bivalently recognize two oligomannose chains with nanomolar avidity (7).

All previous protection studies involving 2G12 have been performed in combination with other MAbs, leaving the assessment of protection by 2G12 alone unknown. However, intriguing results from an earlier study with 2G12 by Mascola, et al (26) in macaques suggested that 2G12 may have unusual protection capabilities. We therefore designed an experiment to examine the potency of 2G12 protection and investigate properties of the antibody that might unveil protection correlates. In our study in **Chapter 7**, we observed that 2G12 was able to provide protection against a high-dose viral challenge at unexpectedly low neutralizing antibody serum titers. As reiterated above, protection has been generally thought to require high serum neutralizing titers on the order of about 100 times the antibody neutralizing concentration. Yet, with a serum neutralizing titer of a meager 1:1, 2G12 achieved a protection efficacy of 60% (i.e. provided sterilizing immunity to 3 of 5 macaques) (Table 2). Notably, 2G12 neutralizes the SHIVs used in our study, as well as the study by Mascola et al mentioned above (26), rather poorly *in vitro*, which suggests that the protection provided by 2G12 may primarily stem from other anti-viral activities.

Anti-gp41 antibodies 2F5 and 4E10: plausable fusion inhibitors

Another conserved region, the MPER of gp41, is a region that would be expected to disfavor eliciting an antibody response because of its proximity to the lipid membrane. However, the bnMAbs 2F5 and 4E10 directed against epitopes on the MPER have been suggested to protect via a unique mechanism. The sequences of these antibodies are enriched with hydrophobic residues that may allow insertion into the viral membrane to reach their respective epitopes. The antibodies directed to this epitope may provide the chance for a viral infection to be aborted after CD4 attachment but before the full conformational changes can be achieved to permit viral and host cell fusion. This unique mechanism may provide a benefit in protection as demonstrated by the high efficacy of protection afforded by 2F5 and 4E10 at relatively low neutralization titers. Thus a protection efficacy of 100% is achieved for both antibodies at neutralizing antibody titers on the order of 1:30 to 1:40. The protection studies conducted with 2F5 and 4E10 are summarized in **Table 2**.

Table 2. Summary of 2G12, 2F5 and 4E10 protection studies: bnMAbs 2G12, 2F5, and 4E10 achieve unexpected high protection efficacy at low neutralization titers

bnMAb	2G12	2F5	4E10
Epitope	Oligomannose cluster (gp120) Chapter 7	gp41 MPER Chapter 8	
Challenge virus[1]	$SHIV_{162P3}$	$SHIV_{BaL}$	
Viral challenge route[1]	*intra vaginally*	*intra rectally*	
Viral challenge dose	500 $TCID_{50}$	2000 $TCID_{50}$	
i.v. Ab dose (mg/kg)	40	50	
Protection record (# of challenges leading to infection/total # challenges)[4]	2/5	0/6	0/6
Serum Ab Concentration (μg/ml)[2]	1053	742	866
Antibody IC_{90} (PBMC) (μg/ml)	900	20	33
Serum Ab Concentration/ PBMC Ab IC_{90} [3]	1.2	37	26
Protection Efficacy[5]	60%	100%	100%

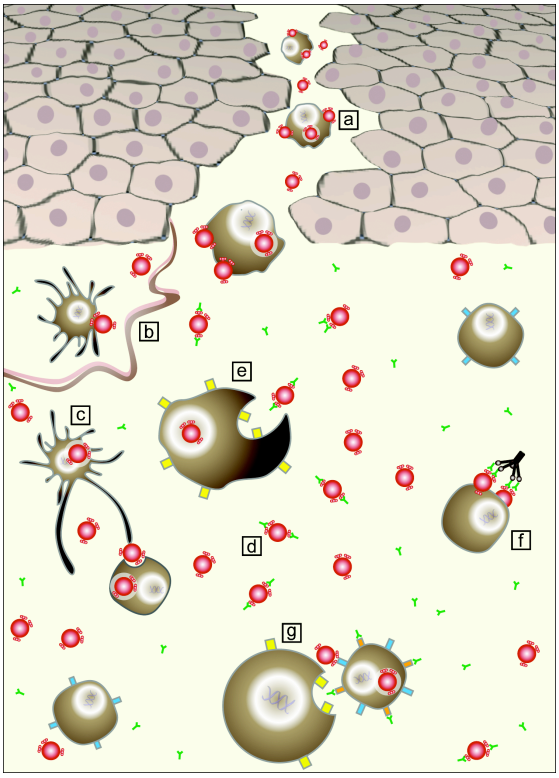
[1]All studies were conducted in the macaque mucosal challenge model by 2 different challenge routes. SHIVs were selected based on *in vitro* Ab neutralization data. [2]Serum concentrations represent averages of all animals in each treatment group at the time of viral challenge. [3]The standard of protection correlate requires serum neutralization titers to be at least 100 times the concentration of antibody required for 90% neutralization in a standard PBMC assay (i.e. serum neut titer of 1:100). [3]The neutralization titer or protection correlate was calculated by dividing the serum antibody concentration by the antibody IC_{90} in a PBMC-based assay. [4]The protection record reflects the number of challenges leading to infection relative to the total number of challenges. [5]From the protection record, we calculated a Protection Efficacy which describes the protection afforded by the neutralizing antibody under the conditions tested. Protection Efficacy is calculated by the formula: 100% – ((infection rate Ab-treated/infection rate controls) x100%).

HIV Infection and anti-viral antibody activities

Antibodies against HIV-1 probably provide protection by more than one mechanism either directly by neutralization or indirectly by initiating effector-mediated functions. Possible mechanisms by antibody protect against mucosal challenge of HIV-1 as studied in this thesis are summarized in Fig. 1. Neutralizing antibodies are most likely to bind effectively to functional Env spikes on free virus, but non-neutralizing antibodies may be present and may bind to non-functional envelope on the virus. Once neutralizing antibody coating is sufficient, neutralization of the virus is achieved and the target cell is protected from infection (Fig 1). Neutralizing antibodies and in some cases non-neutralizing antibodies also recognize viral proteins expressed on the surface of infected cells. The interaction of antibody Fc domains with Fc receptors forms immune complexes that recruit pathways to destroy infected cells or virus particles. These pathways include phagocytosis, complement dependent cytotoxicity (CDC), and antibody dependent cellular cytotoxicity (ADCC) (Fig 1).

bnAbs: deciphering their role in protection and in vaccines

In view of the above, it is important to underscore the difference in impact that neutralizing antibodies may have on established infection versus the initiation of infection (prophylactic setting) (34). A new viral challenge (recreated in the protection studies discussed here) which potentially consists of a limited number of free virions and limited diversity can be thwarted by an appropriate concentration of neutralizing antibody either provided passively or induced by a vaccine. However, after infection is established and viral diversity is increased along with the existence of latent reservoirs of virus, neutralizing antibody appears to have little chance to eradicate infection. In the study by Poignard et al, (34), HIV-infected hu-PBL-SCID mice, received *i.p* injections of b12, 2G12 and 2F5 achieving neutralizing antibody serum titers that would have resulted in (nearly) complete protection in the prophylactic setting, yet, showed very little impact on viral replication in the established setting. Likely explanations for this include the poor activity of the bnMAbs to interrupt viral propagation via cell-to-cell spread, as well as rapid mutation and neutralization escape by the virus. Even the bnMAbs discussed here therefore likely only modulated HIV-1 infection and eventual disease progression only to a limited extent in the patients from whom they were isolated. As suggested by experiments in the HIV-1 infected hu-PBL SCID mice, once the infection has established and festers, HIV-1 quickly mutates to evade neutralization thereby thwarting both Fab-mediated neutralization as well as infection-blunting Fc receptor-mediated activities. Thus where antibody alone can be highly effective in preventing a novel infection, it is likely that it simply becomes an overwhelming task for neutralizing antibodies alone to quell the infection once it has become established.



The figure (above) illustrates the possible mechanisms by which antibodies act against HIV-1. Human transmission of HIV-1 usually occurs at the mucosal surface, either within the vagina or rectum. Abrasions or tearing can occur during sexual intercourse or can exist as a consequence of STDs. It is through these portals that virus exposure most easily occurs. The virus or infected donor cells become trapped in mucus and migrate along the tear eventually contacting various target cells in the mucosal epithelium or underlying stroma (**a**). Host cells residing within the epithelium can become productively infected and can emigrate into the submucosa and the draining lymphatic or venous microvessels and transported to lymph nodes or enter the blood circulation (**b**). Free virus or infected donor cells can also make contact with stromal dendritic cells (**c**) that can pass newly-budded virions onto CD4+ T-cells. Neutralizing antibodies circulating under the mucosal epithelium can prevent or blunt infection by binding free virus and preventing the infection of CD4+ T-cells (**d**). Some free virus particles may become coated by antibodies and destroyed by phagocytic cells (**e**). Activation of complement by opsonized virus or infected cells may induce complement activation and cell killing; although our studies suggest that this is not a major mechanism for antibody protection (**f**). Some CD4+ T-cells may still become infected, but are stopped before they bud new virions by antibodies that bind to viral antigens on their surface. The antibodies signal for the destruction of these infected cells by Fc-receptor-bearing natural killer cells (**g**).
(Graphics by Christina Corbaci)

Therefore, when considering antibody's role in vaccine design, it is important to recognize that a neutralizing antibody response alone is very good at preventing a novel infection, but at least for HIV-1, seems relatively poor at controlling infection once it has established and diversified. Neutralizing antibody might provide sterilizing immunity when present a sufficiently high titers relative to the viral dose as demonstrated in this thesis. A goal to raise and maintain neutralizing antibody titers of sufficient magnitude with a vaccine to provide sterilizing immunity however seems unachievable. Our studies show that significant protection against (relatively low) real-life viral challenge doses may be achieved by the continued presence of relatively low neutralizing antibody titers. This suggests that strong benefits could potentially result from vaccines that could elicit titers of bnAbs similar to b12, 2G12, 2F5 and 4E10. In addition to this, for viruses breaking the protection barrier, such antibodies might possibly limit the initial burst of viral replication to a degree such that the infection could then be contained by the cellular adaptive immune system, which seems more apt at controlling infected cells, thereby limiting disease symptoms (46). Following blunting of the initial virus infection by neutralizing antibody, expansion and deployment of CD8+ cells could delay or prevent disease (4) and possibly decrease the likelihood of a vaccinated person to transmit virus (35). Vaccines that elicit both neutralizing antibodies and cytotoxic T-cell responses exist for other viruses, but there are still numerous challenges to overcome in order to develop such a type of vaccine against HIV. Further investigation of this notion, however, is beyond the scope of this thesis. Nevertheless in order to fully appreciate the role of antibodies in HIV-1 infection, it is crucial to accept the limitations of antibody alone-based vaccine development ideas. In this thesis, the bnMAbs b12, 2G12, 2F5 and 4E10 were described in detail. These important antibodies have defined a number of critical weaknesses and possible routes of attack against HIV-1. The lessons learned may now be applied to design immunogens aimed to elicit such bnAbs *in vivo*.

gp120: a moving target

The gp120 domains of the envelope spike have been extensively studied in the context of key epitopes for neutralization and protection (6, 40, 53). Functional Env is uniquely adept at evading humoral immunity by its diversity and instability. The most conserved regions that would be a target for neutralizing antibodies are deeply buried or do not become accessible until after engagement with co-receptor, yet the highly variable immunodominant loops are well exposed. A further evasion strategy includes the likely expression of mostly non-functional forms of Env that elicit many non-neutralizing antibodies. The two bnMAbs examined in this thesis, b12 and 2G12, address separate and very different epitopes on gp120. While b12 recognizes an epitope overlapping the CD4bs on gp120 located on what might be described as the side of the virus spike, 2G12 recognizes a unique cluster of sugars near the top of the spike. Both antibodies provide substantial protective abilities against mucosal viral challenge and are proving to be valuable tools in the quest to decipher what may be necessary in an eventual vaccine. We have established that b12 protects by providing steric hindrance and preventing infection of the host cell in addition to mediating effector functions. The effector function efficacy of 2G12

protection has yet to be established, but the high serum concentrations achieved in our study which are in great excess over the 2G12 concentration required to effect ADCVI *in vitro*, suggest that 2G12 should be able to enable infected cell killing *in vivo*. In fact, this may help to explain the difference in b12 and 2G12 protection in relation to their neutralization titers, i.e. 2G12 neutralization may not be as important in protection as its infected-cell killing ability or other Fc-mediated actions such as phagocytosis. In this way, 2G12 might overcome its relatively poor neutralization potency. An additional advantage of 2G12 may stem from its recognition of the glycans on gp120 because these glycans are also recognized by DC-SIGN. It is probable that DC-SIGN plays a critical role in HIV transmission by assisting in the transport virus by dendritic cells to lymphoid tissues (15, 43). This concept is more fully explained in the discussion section of Chapter 7, but in essence 2G12 may inhibit the interaction of HIV with DC-SIGN thereby blocking transmission of virus especially if 2G12 is a competitor for DC-SIGN's exploitation of non-functional Env. Both b12 and 2G12 have begun to illustrate their respective importance in understanding the elements required for protection against HIV infection that probably include both neutralizing and extra-neutralizing properties.

MPER: a challenging vaccine design target

Several recent studies have provided a better understanding of the gp41 MPER both structurally and functionally increasing the interest in vaccine design efforts (3, 9, 13, 19, 24, 38, 57). One of the main reasons for the invigorated interest in the gp41 MPER as a target for vaccine design is appreciation of the tremendous breadth of HIV-1 primary isolate neutralization by 2F5 and 4E10. Another characteristic of the MPER that makes it an appealing template for vaccine design is that antibodies can access the region prior to Env interaction with CD4 and before prefusion conformational changes occur (12, 13, 27, 57). Of further note is that 2F5 and 4E10 can remain bound through late stages of fusion and thereby act similarly to fusion inhibitors (13, 16). Even though the advantages are clear, there are also potential obstacles to overcome in designing an immunogen based on the MPER of gp41. First, the epitopes are very close to the viral membrane and antibody may be required to interact with the membrane. Second, the MPER shows a high degree of hydrophobicity and the antibody binding sites of 2F5, and particularly 4E10, are relatively hydrophobic. This may lead to some weak cross-reactivity with a range of antigens that may hinder the elicitation of the corresponding antibodies (1, 17, 28, 38, 41). Indeed, 2F5 and 4E10–like antibodies are rare in the sera of HIV-1-infected individuals indicating that the MPER is probably poorly immunogenic in natural infection (11, 51).

Conclusions

Although the focus of the individual studies described in this thesis was to determine the protective abilities of the bnMAbs, each study emphasizes the enormous challenge to develop an effective HIV-1 vaccine. The structural study included here on b12 (**Chapter 2**) represents a key step in understanding neutralization of the virus on a molecular level. This level of understanding is critical in order to address vaccine development with a knowledge-based approach. The

protection studies (**Chapters 3, 5, 6, 7 and 8**) carried out in the macaque model have provided the basis for understanding the possible ways in which an antibody response raised by immunization will provide protection to humans when exposed to HIV via mucosal routes. Finally, we have begun to fine-tune our understanding of protection and neutralization with Fc-mutated (**Chapters 4, 5 and 6**) and isotype (**Chapter 9**) variants of b12.

Protection by antibodies combines the functions of virus neutralization and effector-mediated destruction of virions and infected cells. The multiple dimensions of antiviral activity of antibodies help prevent infection and lower viral burden and clinical symptoms. These studies provide key insights on the ways in which antibody elicited through immunization can act against HIV-1 successfully to prevent infection and have broadened our thinking about the mechanisms of antibody protection.

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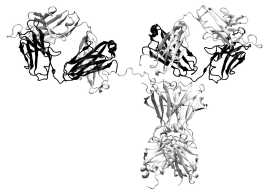


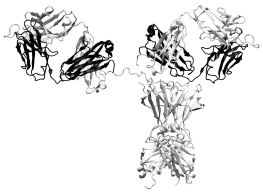
Summary

Antibodies play an important role in protection against infection with pathogens such as bacteria and viruses. The ability of a vaccine to generate inactivating (or neutralizing) antibodies is an important characteristic of a vaccine. Although much progress has been made in understanding AIDS, the disease processes that HIV-1 infection causes, major obstacles have been discovered that make the development of a vaccine against HIV very difficult. These obstacles include the development of reservoirs in which "resting" virus may hide and the ability of HIV to rapidly change shape through mutation and recombination of genetic information. The result is a high degree of diversity in the virus and it is very difficult for the immune system to eliminate the virus. A quarter of a century of intense research has still not succeeded to produce an effective vaccine against HIV-1 and AIDS. For the global epidemic to stop, in which more than 60 million people have been infected and half of whom have died, effective vaccination strategies are essential.

An important branch of research focuses on neutralizing antibodies against HIV-1. Studies have convincingly shown that antibodies can provide effective protection against HIV infection. Worldwide, there are only a handful of known antibodies that can neutralize HIV viruses from different patients. In the chapters of this thesis, the properties of the four major HIV neutralizing antibodies, i.e., b12, 2G12, 2F5 and 4E10, are examined. The investigation in **Chapter 2** examines the structure of the b12 antibody bound to the HIV glycoprotein gp120 and explains in great detail how this neutralizing antibody binds to the major HIV surface protein. In **Chapter 3** it is shown that the antibody b12 can protect against infection in a vaginal infection model in rhesus macaques and that protection correlates with neutralizing antibody titers. **Chapter 4** describes experiments in which a group of variants of b12 were developed containing mutations causing the Fc-dependent effector functions to be disabled. Two variants (KA and LALA) were selected for studies in the rhesus macaque infection model in **Chapter 5**. Treatment of macaques with the original b12 antibody or KA variant protected 8 of the 9 monkeys. When treated with the LALA variant (a variant of b12 that contains mutations causing communication with cells to be switched off) 4 of the 9 monkeys were not protected against infection. This shows that for maximum protection, in addition to virus inactivation qualities, it is necessary for antibodies to activate blood cells to destroy virus-infected cells. Traditionally, in animal studies, the effectiveness of antibodies or vaccines is examined when infection is performed using a high dose of virus. This is necessary in order to achieve a high rate of infection and so that these tests can be implemented with small groups of animals. The dose of virus is much higher than the amount of HIV-1 that occurs in people to cause infection. In **Chapter 6**, a revolutionary new experimental design was used in which antibody protection against lower, more physiological, amounts of virus was studied. The antibody titers that were needed to protect against repeated exposure to a low dose of HIV, representing the amount by which people become infected, were surprisingly low and were in the order of magnitude that a vaccine could be achieved. This study provides the foundation for a new optimism for the

development of an HIV vaccine. The 2G12 antibody addresses an epitope of sugar groups on the HIV surface protein and is capable of neutralizing a large group of HIV viruses. Earlier studies have suggested that low titers of the 2G12 antibody can offer protection against HIV infection. In **Chapter 7**, the protection provided by 2G12 against HIV infection was studied. The 2G12 antibody was found to have unusually protective properties. This study underscores the importance of the sugar shield of the HIV surface protein gp120 as an important goal for vaccine development. In **Chapter 8**, protection by the antibodies 2F5 and 4E10 was investigated. These antibodies recognize a highly conserved region of the gp41 protein that acts as the stalk for the HIV surface protein gp120. Both antibodies fully protected rhesus macaques against HIV infection. This region of gp41, called the MPER, is an excellent third target for vaccine development against HIV. Finally, in **Chapter 9** the effectiveness of the IgA2 isotype variant of the b12 antibody was studied. IgA antibodies have an important role in protecting virus transmission on mucous membranes. The study shows that both IgA2 b12 as does the original IgG1 b12, interferes at the earliest stages of HIV-1 transmission.

The antibodies in this thesis identified a number of unique areas of HIV vulnerability that are major targets for vaccine development. Neutralizing antibodies appear to exploit different mechanisms by which infection with HIV-1 can be interrupted and show how such antibodies produced by a vaccine could offer protection. These studies offer new optimism in the fight against HIV-1 and AIDS.



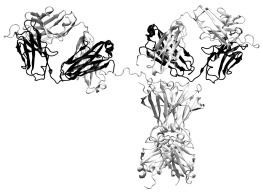
Samenvatting

Antistoffen spelen een belangrijke rol in de bescherming tegen infecties met ziekteverwekkers zoals bacteriën en virussen. Het vermogen van een vaccin om inactiverende (of neutraliserende) antistoffen op te wekken is dan ook een belangrijk kwaliteitskenmerk van een vaccin. Ondanks dat er veel vooruitgang is geboekt in het begrijpen van de ziekteprocessen waarmee HIV-1 infectie AIDS veroorzaakt, zijn er grote obstakels ontdekt die de ontwikkeling van een vaccin tegen HIV erg moeilijk maken. Deze obstakels omvatten o.a. de ontwikkeling van reservoirs waarin "rustend" virus zich kan verstoppen en het vermogen van HIV om razend snel van vorm te veranderen door middel van mutatie en recombinatie van genetische informatie. Het resultaat is een hoge mate van diversiteit in het virus waar het immuunsysteem moeilijk vat op krijgt. In meer dan een kwart eeuw van intens onderzoek, is het daardoor tot op heden niet gelukt om een effectief vaccin tegen HIV-1 en AIDS te produceren. Om de wereldwijde epidemie, waarin al meer dan 60 miljoen mensen geïnfecteerd zijn geraakt en waarvan de helft overleden is, te stoppen zijn effectieve vaccinatie strategieën essentieel.

Een belangrijke tak van onderzoek focusseert op neutraliserende antistoffen tegen HIV-1. Wereldwijd zijn er slechts een handvol antistoffen bekend die HIV virussen afkomstig van verschillende patiënten kunnen neutraliseren. In de hoofdstukken van dit proefschrift zijn de eigenschappen van de vier belangrijkste HIV neutraliserende antistoffen, te weten b12, 2G12, 2F5 and 4E10, onderzocht. De studies laten overtuigend zien dat antistoffen effectieve bescherming kunnen bieden tegen HIV infectie. Het onderzoek in ***hoofdstuk 2*** bestudeert de structuur van de b12 antistof gebonden aan het HIV glycoproteïne gp120 en verklaart in groot detail hoe deze neutraliserende antistof aan het belangrijkste HIV oppervlakte eiwit bindt. In ***Hoofdstuk 3*** wordt aangetoond dat de b12 antistof kan beschermen tegen infectie in een vaginaal besmettingsmodel in rhesus makaken en dat bescherming correleert met neutraliserende antistof titers. ***Hoofdstuk 4*** beschrijft experimenten waarin een groep van varianten van b12 ontwikkeld werden waarin de zogenaamde Fc-afhankelijke effector functies zijn uitgeschakeld. Twee varianten (KA en LALA) werden geselecteerd voor studies in het rhesus makaak besmettingsmodel in ***hoofdstuk 5***. Behandeling van makaken met de oorspronkelijke b12 antistof of de KA variant beschermde 8 van de 9 apen. Bij behandeling met de LALA variant (een variant van b12 die mutaties bevat waardoor communicatie met bloedcellen is uitgeschakeld) waren 4 van de 9 apen niet beschermd tegen infectie. Hieruit blijkt dat voor maximale bescherming antistoffen nodig zijn die, naast virus inactiverende kwaliteiten, ook bloedcellen activeren die virus-geïnfecteerde cellen kunnen uitschakelen. In dierexperimenteel onderzoek waarin de effectiviteit van antistoffen of vaccins wordt onderzocht, wordt besmetting traditioneel uitgevoerd met een hoge dosis virus. Dit is nodig om een hoge graad van infectie te bereiken zodat deze proeven met hele kleine groepen dieren kunnen worden uitgevoerd. De gebruikte dosis is echter veel hoger dan de hoeveelheid virus waarmee HIV-1 infectie in mensen plaatsvindt. In ***hoofdstuk 6*** werd een revolutionaire nieuwe experimentele opzet onderzocht waarin antistof bescherming tegen lagere, meer fysiologische, hoeveelheden virus onderzocht kon worden. De antistof titers die nodig waren om te beschermen tegen

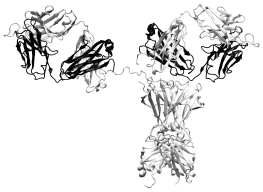
herhaaldelijke blootstelling met een lage dosis HIV, overeenkomend met de hoeveelheid waarmee mensen geïnfecteerd raken, bleken verrassend laag te zijn en lagen in de orde van grootte die met een vaccin bereikt zouden kunnen worden. Deze studie geeft daarmee de basis voor een nieuw optimisme voor de ontwikkeling van een HIV vaccin. De antistof 2G12 ziet een epitoop van suikergroepen op het HIV oppervlakte eiwit en is in staat een grote groep van HIV virussen te neutraliseren. Eerdere studies met 2G12 suggereerden dat een lage titer van deze antistof bescherming tegen HIV infectie kan bieden. In ***hoofdstuk 7*** wordt de bescherming die 2G12 tegen HIV infectie biedt onderzocht. De 2G12 antistof bleek inderdaad over ongebruikelijk beschermende eigenschappen te beschikken. Deze studie onderstreept het belang van het suikerschild van het HIV oppervlakte eiwit gp120 als een belangrijk doel voor vaccin ontwikkeling. In ***hoofdstuk 8*** wordt bescherming door de antistoffen 2F5 en 4E10 onderzocht. Deze antistoffen herkennen een sterk geconserveerde regio in het gp41 eiwit dat de stam vormt voor het HIV oppervlakte eiwit gp120. Beide antistoffen beschermden rhesus makaken volledig tegen HIV infectie. Deze regio in gp41, MPER genaamd, is daarmee een uitstekend derde target voor vaccinontwikkeling tegen HIV. Tenslotte, werd in ***hoofdstuk 9*** de effectiviteit van een IgA2 isotype variant van de b12 antistof onderzocht. IgA antistoffen hebben een belangrijke rol bij de bescherming virus transmissie over slijmvliezen. De studie laat zien dat zowel IgA2 b12 als het originele IgG1 b12 de vroegste stappen van HIV-1 transmissie kan remmen.

De antistoffen in dit proefschrift identificeren een aantal unieke kwetsbare gebieden op HIV die belangrijke doelen vormen voor vaccin ontwikkeling. Neutraliserende antistoffen blijken verschillende mechanismes te kunnen exploiteren waarmee infectie met HIV-1 voorkomen of onderbroken kan worden en laten zien hoe zulke antistoffen opgewekt door een vaccin bescherming zouden kunnen bieden. Deze studies leveren daarmee een nieuw optimisme voor te strijd tegen HIV-1 en AIDS.



Curriculum Vitae

The author of this thesis was born on July 23, 1954 in Bogalusa, Louisiana, USA. After completing high school in 1972, she began studies at the University of Southern Mississippi in the program of Secondary Science Education. During the years of 1973 – 1987, she was primarily employed in legal, business, and politcal organizations. From 1988-1989, she was employed with the Southwest Biomedical Research Institute in Phoenix, Arizona, USA and resumed university studies in science and mathematics. Based on academic achievement, she was awarded a scholarship to begin a program in Biochemistry and Molecular Biology in 1993 at the University of California, San Diego. Completion of the program in June 1996 earned her the degree of Bachelors of Science in Molecular Biology. Immediately following graduation, she joined the laboratory of Prof. dr. Dennis R. Burton to conduct research under the direction of dr. Paul W.H.I. Parren in the Department of Immunology at The Scripps Research Institute in La Jolla, California.



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Ann J. Hessell, Pascal Poignard, Meredith Hunter, Lars Hangartner, David M. Tehrani, Wim K. Bleeker, Paul W.H.I. Parren, Preston A. Marx, and Dennis R. Burton
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Don't walk behind me; I may not lead. Don't walk in front of me; I may not follow. Just walk beside me and be my friend.

Author unknown