Neutralizing antibodies to the HIV-1 envelope glycoproteins
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Chapter 1

Antibodies, Phage Display and Membrane proteins
"Yet it was with those who had recovered from the disease that the sick and the dying found compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice—never at least fatally." (88)

Thucydides, 431 B.C. (Probably the earliest reference to immunity, found in an account of the plague (most likely Pasteurella pestis) that afflicted Athens in 431 B.C.).

Introduction

Although vaccination, i.e. the induction of immunity, was already a proven principle from studies by Edward Jenner (33) and Louis Pasteur (63), it wasn’t until 1890 before the first insights into the mechanism of acquired immunity were obtained from the experimental work by Emil von Behring and Shibasaburo Kitasato (93). They demonstrated that the protective immune state of rabbits vaccinated with diphtheria toxin could be transferred to unvaccinated animals via the serum. The protective agents responsible, then called antitoxins, later turned out to be immunoglobulin (Ig) molecules, i.e. antibodies, and were thus probably the first proteins to be recognized as potential therapeutic agents.

Antibodies

Antibodies are produced by B-cells in response to challenge with foreign substances, i.e. antigens, and consequently antigen-specific antibodies can be found in the serum of the challenged animals. The antibodies act by binding to the antigen (and associated pathogens) and mediate their elimination via complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC) or phagocytosis (38). Additionally bound antibodies may sterically interfere with the biological function of the antigen and thus neutralizing it (57).

During early B-cell development in the bone marrow, a number of mechanisms have evolved to maximize the antibody diversity required to counteract the broad range of potential antigens and pathogens. These include genomic rearrangement of variable-(V), diversity-(D) and joining gene segments (J), somatic mutation of rearranged V(D)J genes, P/- N-nucleotide addition and combinatorial association of heavy- and light chains (Fig. 1.1). After a successful rearrangement of each heavy- and light chain, a process called allelic exclusion ensures that each individual mature B-cell released into circulation expresses membrane-bound antibody molecules with only a single specificity. Upon encountering antigen, the specific B-cells internalize the antigen, where it is degraded into peptides for MHC II presentation on the membrane. In the lymph node or spleen, peptides are recognized by specific CD4* T-cells, i.e. T cells, which in turn trigger the activation of B-cell proliferation. Activation of B-cells leads to additional somatic hypermutations in the V(D)J region followed by positive selection of B-cells with higher affinity for the antigen. This process, called affinity maturation, can result in an increase of 2-4 logs in affinity. Furthermore, during proliferation activated B-cells can switch Ig isotypes under influence of various cytokines secreted by activated T cells. B-cells proliferate into either memory B-cells, capable of mounting an immediate high affinity response upon renewed contact with the antigen, or plasmacells, specialized antibody secreting cells that home to the bone-marrow.

Monoclonal antibodies

As mentioned before, the passive transfer of antibodies in immune sera may confer protection to recipients. However, serum-based treatment in a foreign species, i.e. differing from that of the serum donor, limits repeated administration of the serum as the foreign origin leads to the development of circulating immune complexes, causing a combination of symptoms, generally referred to as serum sickness. Moreover, as individual antibody responses are variable, the exact composition of the polyclonal immune sera is non-reproducible.

A significant advancement in antibody research came with the introduction of the hybridoma technology by Georges Köhler and Cesar Milstein in 1975 (36). With this technique, splenic B-cell from immunized animals are fused with a myeloma cell-line, creating a hybrid cell called a hybridoma, that possesses the immortalized properties of the myeloma cell and secretes antibodies of a predefined single specificity. These monoclonal antibodies (mAbs) can thus be produced in large quantities making them available for use in diagnostic and therapeutic medicine. However, as these mAbs were often of murine origin, their potential as therapeutic agents in human medicine was limited by the emergence of human-anti-mouse antibody responses making long-term immunotherapy difficult. Human mAbs would thus be preferable, but the production of human hybridomas has been difficult, mainly due to the instability of the human hybridomas (7) and the ethical considerations involved with immunizing humans. An alternative for making human mAbs, Epstein-Barr virus (EBV) mediated immortalization of human B-cells, showed similar limitations (18).

One strategy to increase the therapeutic application of mAbs was to genetically engineer the murine mAbs in order to decrease their antigenicity, a process called humanization. Indeed, chimeric antibodies, with murine variable (V)-domains (determining the antigen specificity) and human constant (C)-domains (Fig. 1.1), were less immunogenic. However, the murine V-domains may still induce a considerable immune response. In another approach to humanize mAbs, the CDRs (the actual structures that primarily make up the antigen-binding site; Fig. 1.1B and C) from the murine mAb are grafted onto the human framework regions. This reduces
A) Immunoglobulin heavy chain variable domains (V\textsubscript{H}) are generated by recombination of separate variable (V), diversity (D) and joining (J) gene segments. In humans there are approximately 51 functional VH gene segments (20), 27 D gene segments and 6 JH gene segments (38). Addition and deletion of P- or N-nucleotides at the junctions increases the diversity (17; (38)). The rearranged VDJ segments are next linked to one of five constant domain coding gene segments by DNA rearrangement in an event termed immunoglobulin class switching (86). The figure shows the recombination events of a human heavy chain. For recombination of the human light chain variable region, rearrangements of V\textsubscript{L} (29 V\textsubscript{L} (32) and 40 VK (79)) and J gene segments occur. After light chains rearrangement the VJ segment is linked to one of two constant domains of either \lambda or \kappa subtype. B) At the protein level the V\textsubscript{H} domain is built up of regions that are crucial for the correct antibody structure (FR, framework regions) and regions that interact with the antigen (CDR, complementarity determining region). During affinity maturation, most somatic mutations cluster in the CDR regions (9, 97). The heavy chain CDR3 is thought to be most important for antigen binding. C) The crystal structure of a human IgG1 antibody molecule is shown (75). The right view is rotated 90° along the Y-axis compared to the left view. The antibody molecule is composed of two heavy chains (dark grey) associated with two light chains (light grey). The heavy chain CDR regions (HCDR) are indicated (black). Different antibody fragments are indicated: Fab (fragment antigen binding), Fc (fragment crystalline), Fv (variable regions of both heavy and light chains).

The immunogenicity even further, however, for optimal results a detailed knowledge of the three dimensional structure of the antibody is needed, and the lack of this knowledge often leads to a reduction of the binding affinity of the CDR-grafted antibodies.

More recently transgenic mice have been constructed that express (part of) the human antibody repertoire (27, 40, 89). Upon immunization, these animals elicit a human antibody response and human mAbs can be isolated using conventional hybridoma technology. As a source for diagnostic and therapeutic reagents, this has the added advantage that antibodies to antigens that are not immunogenic in humans (e.g. self-antigens) can be elicited.

Phage display of antibodies

Advances in several areas of biology have resulted in the development of an alternative to hybridoma technology, designated phage display technology. The defining moment in molecular biology that led to the development of phage display libraries was the expression of foreign polypeptides on the surface of filamentous fd bacteriophages (84). Filamentous bacteriophages (genus *Inoviridae*) are a family of single-stranded (ss)DNA viruses that infect Gram-negative bacteria. The F\textsubscript{I} class of filamentous bacteriophages is comprised of the F, fd and M13 phages, which share 98% homology in DNA sequence, and infects *Escherichia coli* (*E. coli*) through its F\textsubscript{I} pili (6, 47, 49, 95). The phage particle has a long cylindrical shape and is composed of five coat proteins (Fig. 1.2). Smith demonstrated that the minor coat protein III, also known as the gene III protein (pili), could tolerate insertions of foreign polypeptides. This, together with the demonstration that functional antibody fragments could be expressed in the periplasmic space of *E. coli* bacteria (10, 83) and the design of human antibody gene-specific primers (45, 68, 78), suggested that this technique was suitable for the expression of entire antibody repertoires (naive or immune) on the surface of filamentous phages (Fig. 1.3). Two human antibody formats, Fab and single chain (sc)Fv (Fig. 1.2), have been displayed on phages (5, 6, 51). Although several phage coat-proteins, e.g. gene
of specific mAbs often depend on the availability with desired specificity (Fig. 1.3). This process is termed (12).

Membrane antigens (12).

Affinity selection (Panning)

Antibody display libraries made from immunized donors may easily contain, $10^9$ to $10^{10}$ members, limited only by the efficiency of the E. coli transformation (13). Given this diversity and the large number of phages that can be obtained in culture (typically $10^{12}$ phages/ml, thus $10^7$ to $10^8$ copies of each member), isolating a desired specificity from the repertoire of cloned antibodies presents the primary challenge. However, the physical linkage between phenotype (expressed protein) and genotype (DNA encoding the expressed protein) in the phage particle, allows for an affinity based selection and amplification of specific clones (56). For selection of antibodies, consecutive rounds of binding, elution and reamplification of the antibody display library can greatly enrich for clones with desired specificity (Fig. 1.3). This process is termed panning, and usually 3-4 rounds of panning are required to isolate a panel of antigen specific high-affinity antibody fragments. Specific phages that were present in the initial library at frequencies in the order of 1 in $10^7$ can thus be revealed (12).

Membrane antigens

Traditional selection strategies for the isolation of specific mAbs often depend on the availability of purified antigen. Some antigens, however, may not be available in purified form, either because their identity is unknown or because the purification procedure influences their physiologically relevant conformation, e.g. integral membrane proteins, which in turn may adversely affect the selected antibody repertoire. Due to the abundance of irrelevant protein, lipid and carbohydrate structures on the cell membrane, selection of phages specific for the target membrane protein is technically challenging and alternative selection strategies for integral membrane proteins have been developed (Table 1.1).

The first strategy involves the partial purification of the target-protein by lysing target-protein expressing cells and capturing the target protein in ELISA plates (74, 77). Although the majority of irrelevant structures can thus be excluded, integral membrane proteins are often dependent on their native environment for their physiological conformation. A strategy involving the partial purification under mild conditions of the target protein, and reconstitution into a membrane environment would allow for more experimental control of the physiological conformation and the ratio of irrelevant structures to target protein. Indeed, a selection with CCR5 (a seven transmembrane, G protein-coupled receptor) captured on paramagnetic beads and reconstituted into proteoliposomes, allowed for the relatively easy selection of specific antibody fragments (48). A prerequisite for these strategies, however, is the preexistence of a specific antibody for capturing of the target-protein.

Other, more widely used strategies are based on whole cells, either fixed or life, as a source of antigen. The efficacies of these strategies largely depend on the expression level of the target antigen (antigen density) (30, 37, 50) and the accessibility of the antigen (30). For antigens that are expressed at high levels and whose antigenic structures are readily accessible, e.g. blood group antigens (44, 81), specific phages can be successfully selected by several rounds of positive selection on antigen-positive cells. Additionally, successful selection strategies based on whole, irradiated or lysed enveloped viruses have been described (46, 59, 96). For...
To construct an antibody display library, RNA is isolated from tissue containing antibody-producing cells and reverse transcribed into cDNA. The DNA fragments encoding the light chain and the Fd parts of the heavy chain (for Fabs) or the variable domains of both light- and heavy chains (for scFvs) are then amplified by means of PCR and cloned into a phagemid vector. This vector is designed to produce the heavy chain fragment as part of a fusion protein with the pilE protein. Upon expression of the phagemid vector in *E. coli* cells both heavy- and light chain fragments or the scFv fragments are targeted to the periplasm of the cell, where the conditions are favorable for antibody assembly and folding. The phagemid DNA is then rescued by super infection with helper-phage. In the resulting antibody display library each phage particle expresses a Fab or scFv molecule on the surface, and harbors the corresponding genes inside. To select specific antibodies the library is incubated with immobilized antigen, after which non-specific phages are washed away. Specific phages are then eluted from the antigen (e.g. by low pH or an excess soluble antigen), reamplified by infection of *E. coli*, and the process is repeated. One round of selection is referred to as a round of panning. By increasing the stringency of the washing procedure in the sequential rounds, high affinity binders are selected. Typically after 3-4 rounds of panning, soluble Fabs or scFvs are expressed by removing the pilE-encoding fragment. Individual clones are grown and screened by means of ELISA or FACS. Clones that are considered positive are then purified and characterized.

Most antigens, however, intricate subtraction and depletion strategies are often incorporated to direct the selection to the target antigens. Depletion of irrelevant phages can be achieved with rounds of negative selection on cells that are target antigen-negative or have lower expression levels. However, due to the propensity of phages to non-specific adherence, negative rounds of selection can result in the loss of specific phages that are present in the library at low starting frequencies. Alternatively, removal of non-relevant clones can be achieved by alternating selection on different antigen sources, thus homing in on target antigen specific clones.

Strategies allowing for simultaneous positive and negative selection have also been developed. These strategies involve labeling of cells, either with an antibody to another target cell-specific membrane protein or via direct labeling of the cell surface (e.g. cell-surface biotinylation), to distinguish between target antigen-positive and -negative cells. For selection, the phage libraries are incubated with mixtures of target antigen-positive and negative cells, prior to isolation of the labeled cells by cell sorting or magnetic separation. These methods allow for the use of complex cell mixtures as a source of panning antigen. Additionally, the potential loss of specific phages due to non-specific adherence in rounds of negative selection is minimized. These strategies are good candidates for the selection of specific phages against antigens with unknown identities.

Two selection strategies based on whole cells are of special note. The first one, the Proximol pathfinder selection or proximity selection strategy, involves the use of a horseradish peroxidase (HRP)-conjugated guiding molecule, either an antibody or ligand, that binds specifically to the target antigen (or associated protein). In the presence of biotin tyramine, the HRP-conjugated molecules catalyze biotinylation of phages binding in close proximity to the target antigen, allowing for recovery of ‘biotin-tagged’ phages from the total population using streptavidin. Using this strategy, phages against carcinoembryonic antigen (CEA), E- and P-selectins and CCR5 could efficiently be isolated within two rounds of selection. Like the strategies involving the capture of target antigens from cell-lysates (see above), however, the preexistence of a specific antibody or ligand is a prerequisite for Proximol.
Table 1.1: Panning strategies for integral membrane proteins

<table>
<thead>
<tr>
<th>Ag Source</th>
<th>Panning Strategy</th>
<th>Ag</th>
<th>Library*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>mAb capture, ELISA plate</td>
<td>HSV gD &amp; gB</td>
<td>I, Fab</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ErbB2</td>
<td>S, Fab</td>
<td>(77)</td>
</tr>
<tr>
<td></td>
<td>mAb capture, Magnetic beads</td>
<td>CCR5 (CD195)</td>
<td>S, scFv</td>
<td>(48)</td>
</tr>
<tr>
<td>Virions</td>
<td>Whole virus, mAb capture, ELISA plate</td>
<td>HIV-1 gp120</td>
<td>I, Fab</td>
<td>(59)</td>
</tr>
<tr>
<td></td>
<td>Irradiated virus, ELISA plate</td>
<td>Ebola GP</td>
<td>I, Fab</td>
<td>(46)</td>
</tr>
<tr>
<td></td>
<td>Viral lysate, ELISA plate</td>
<td>CMV, VZV, HSV-1/2, Rubella</td>
<td>I, Fab</td>
<td>(96)</td>
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<tr>
<td>Fixed cells</td>
<td>Depletion/ Subtraction</td>
<td>m-a-Tac mAb</td>
<td>M(2), Fab</td>
<td>(94)</td>
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<tr>
<td></td>
<td></td>
<td>(? (Epithelium))</td>
<td>S, scFv</td>
<td>(92)</td>
</tr>
<tr>
<td>Life cells</td>
<td>Cells</td>
<td>Kpb</td>
<td>N, scFv</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RhD &amp; RhE</td>
<td>N, scFv</td>
<td>(44)</td>
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<td></td>
<td></td>
<td>ABO B Ag</td>
<td>N, scFv</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RhD</td>
<td>M(2), Fab</td>
<td>(81)</td>
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<tr>
<td></td>
<td></td>
<td>HIV-1 gp120</td>
<td>M(4), Fab</td>
<td>(59)</td>
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<tr>
<td></td>
<td>Depletion/ Subtraction</td>
<td>(? (Melanoma))</td>
<td>I, scFv/ Fab</td>
<td>(17, 67)</td>
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<td></td>
<td></td>
<td>peptide/MHC-1 complex</td>
<td>I, Fab</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGFR</td>
<td>M(2), Fab</td>
<td>(66)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMW-MAA</td>
<td>S, scFv</td>
<td>(52)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FAT (CD36)</td>
<td>N, scFv</td>
<td>(30, 65)</td>
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<td></td>
<td></td>
<td>SSTR</td>
<td>N, scFv</td>
<td>(30)</td>
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<td>DAF (CD55)</td>
<td>N, scFv</td>
<td>(71)</td>
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<td></td>
<td></td>
<td>EGP-2</td>
<td>M(2), scFv</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E-selectin (CD62E)</td>
<td>M(2), scFv</td>
<td>(50)</td>
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<td></td>
<td>HLA-Cw*0602</td>
<td>S, scFv</td>
<td>(43)</td>
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<td></td>
<td></td>
<td>Aminopeptidase N (CD13)</td>
<td>I, scFv</td>
<td>(64)</td>
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<td></td>
<td>FACS</td>
<td>(? (B-cells))</td>
<td>S, scFv</td>
<td>(21)</td>
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<td></td>
<td></td>
<td>(? (Thymic cells))</td>
<td>S, scFv</td>
<td>(55)</td>
</tr>
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<td></td>
<td></td>
<td>(? (DCs))</td>
<td>S, scFv</td>
<td>(39)</td>
</tr>
<tr>
<td></td>
<td>MACS</td>
<td>RhD</td>
<td>I, Fab</td>
<td>(80)</td>
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<td></td>
<td></td>
<td>ABO B Ag</td>
<td>I, Fab</td>
<td>(19)</td>
</tr>
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<td></td>
<td></td>
<td>(? (Platelets))</td>
<td>I, Fab</td>
<td>(72)</td>
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<tr>
<td></td>
<td>Proximol™</td>
<td>CEA (CD66e)</td>
<td>N, scFv</td>
<td>(53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E-selectin (CD62E)</td>
<td>N, scFv</td>
<td>(53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P-selectin (CD62P)</td>
<td>N, scFv</td>
<td>(53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCR5 (CD185)</td>
<td>N, scFv</td>
<td>(54)</td>
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<td></td>
<td>Internalizing phages</td>
<td>ErbB2</td>
<td>M(2)/N, scFv</td>
<td>(8, 69)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TFRC (CD71)</td>
<td>N, scFv</td>
<td>(69)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGRF</td>
<td>N, scFv</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>Tissue section</td>
<td>EPCAM</td>
<td>M(2), scFv</td>
<td>(90)</td>
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<td></td>
<td></td>
<td>EGP-2</td>
<td>M(2), scFv</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Selection <em>in vivo</em></td>
<td>EGP-2</td>
<td>(50)</td>
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<tr>
<td></td>
<td></td>
<td>(? (Endothelium))</td>
<td>S, scFv</td>
<td>(35)</td>
</tr>
</tbody>
</table>

*a* I, immune library; S, (semi-)synthetic library; N, naive library; M(x), model system (x number of distinct phage clones used)

As a consequence this strategy is not suitable for antigens with unknown identity. Furthermore, to our knowledge, no follow-up studies have been published since 1998, making the applicability of this method questionable.

The second selection strategy of note makes use of the property of some cell-surface receptors to undergo endocytosis (6, 28, 69). In this selection strategy specific phages are internalized by the target antigen-positive cells and recovered from the cytosol. Although
this facilitates the efficient removal of irrelevant phages, e.g. by extensively washing the cells, this strategy is limited to antigens that undergo endocytosis under physiological conditions. As internalization is often more efficient when the target antigens are cross-linked, phage display formats that facilitate multivalent display of antibody fragments are preferable for this type of selection strategy. Antibodies identified with this strategy are ideal candidates to be used as targeting molecules for drug delivery. In addition, internalizing antibodies themselves might have a direct therapeutic effect. For instance, it might be possible to use internalization to identify desirable biological effects of the antibodies, such as apoptosis or growth inhibition. Indeed, a growth inhibitory effect of an anti-transferrin scFv identified in the selection on cancer cells was observed (59).

Selections on tissue sections, as an alternative for the physiologically relevant presentation of cell surface antigens, have been described for model libraries (containing 1 specific and 1 non-specific phage clone; (50, 90), but have yet to be tested for full-size libraries. The presence of extracellular matrix components, other (antigen-negative) cell types and even exposed intracellular antigens in such tissue sections, are likely to negatively influence the efficacy of such selection procedures, making subtractive selection strategies necessary.

Finally a strategy based on selection of antibodies in vivo has been developed (35, 50). In this selection strategy, first described for the selection of peptide phages (2, 62, 70), a phage mixture is directly injected into the circulation of an animal followed by the rescue of specific phages by removal of the target tissue (e.g. tumor). The efficacy of this strategy, however, seems to be limited to endothelial cell-specific markers, due to the inability of the phages to cross the endothelial lining of the blood vessels (35, 50, 62). Optimization of the variables influencing the selection (e.g. circulation time, perfusion in vivo, washing in vitro) or modification of the selection strategy (e.g. direct injection into target tissue, local activation of the endothelial cells to allow extravasation) could increase the applicability of this strategy (50).

HIV-1 envelope glycoproteins

In this thesis we used the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) complex (see Chapter 2 for details) as a model for integral membrane proteins. Previously, Env-specific human Fab fragments have been isolated from antibody display libraries using purified Env subunits, gp120 and gp41 (4, 11, 14, 22). One such human Fab obtained from a library prepared from the bone marrow of a HIV-1 seropositive individual, termed b12, is directed against the gp120 CD4-binding site (CD4bs) (3, 14), and has been shown to be exceptionally potent in the neutralization of HIV-1, both in vitro (15, 91) and in vivo (25, 58, 60). This potent neutralizing ability was not correlated with affinity to monomeric gp120 as other Fab fragments bind to overlapping sites on monomeric gp120 with similar affinities, but neutralize weakly. However, b12 was found to bind more efficiently, compared to the other less neutralizing Fab fragments, to cell surface expressed oligomeric gp120 on HIV-1 infected cells (73). Moreover, concentrated virions expressing the oligomeric configuration enriched for b12 (59). This suggests that either the availability of the b12 epitope on monomeric gp120 is limited or that monomeric gp120 has not preserved the conformational structures of the b12 epitope present on oligomeric gp120. Indeed, studies have shown that the oligomeric nature of the HIV-1 envelope protein may strongly influence its antigenic structure (23, 61, 76). Thus the successful rescue of human Fab fragments with HIV-1 neutralizing activity depends on the presentation of envelope proteins in a physiological relevant conformation.

Scope of this thesis

As the interaction between antibody and antigen is closely linked, the scope of this thesis was two-fold: 1) improvement of the antigen presentation, to better mimic the physiologically relevant oligomeric conformation in order to isolate new neutralizing antibodies, and 2) studying the interplay between the (new) neutralizing antibodies and antigens to obtain a better understanding of the immunogenic and structural requirements of physiologically relevant antigen presentations (and thus act as potential HIV-1 vaccine candidates).

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