Abstract

The main purpose of this study was to investigate and optimize techniques for eukaryotic expression of antibody molecules (IgG, Fab and scFv-κ formats), derived from an immune phage displayed Fab library and reactive against HIV-1 glycoproteins gp120 and gp41.

Light and heavy-chain (Fd) DNA from a number of Fab clones were transferred from a prokaryotic phagemid vectors to mammalian expression vectors. COS-7 cells were used for transient expression of Fab-fragments. In addition, by cloning cDNA encoding the Fc fragment downstream of Fd, full length, human IgG1 plasmids were created and transfected into Chinese Hamster Ovary (CHO) cells. Fab fragments of certain clones showed increased virus neutralizing activity when expressed in CHO cells, compared to when expressed in Escherichia coli. Neutralizing capacity of Fab and IgG expressed in mammalian cells were almost the same, indicating that bivalency did not confer improved neutralization efficacy.

Next, conditional expression systems for IgG in mammalian cells were constructed based on the tetracycline operon. HeLa and CHO cell lines that produced prtTA/prtTA-M2s (reverse tet-responsive transcriptional activator) were established. The cDNA for Fab fragments to tetanus toxoid, HIV-1 gp41 and gp120 were transformed into a single chain format, scFv-κ (VH-linker-VL+CL), cloned downstream of the tetracycline responsive element, and transfected into the HeLa-tet on and CHO-tet on cell lines. The gene expression in presence of doxycycline was increased 10 –100 fold; a very low background level was seen when the tetracycline substrate was withdrawn.

To improve yields of secreted proteins, a series of vectors adapted for inducible expression of IgG1 in Drosophila S2 cells were constructed. High expression levels were obtained in stable cell lines: from 0.5-1 mg/l in transient expression, but up to 35mg/l from stable cell lines when accumulated during 10 days. Binding characteristics for IgG1 expressed in S2 cells were indistinguishable of the same clone expressed in mammalian cells, as assessed for anti-HIV and anti-HCV antibodies.

Finally, human Fab antibodies against a putative neutralizing epitope in gp41 of HIV-1 were isolated using overlapping synthetic peptides representing the epitope. The heavy chains were clonally unrelated, despite that almost all clones showed strong specificity to one of the three peptides. The results from E.coli produced Fab indicated that certain clones could neutralize up to 4 of 5 primary HIV-1 isolates. Improved neutralization capacities were found when Fab and/or IgG were expressed in COS, CHO and Drosophila S2 cells. Immunoprecipitation by S2- produced IgG of gp41 and gp160 containing cell lysates, confirmed the binding to the cognate antigen.

In conclusion, human antibodies isolated as Fab fragments could be expressed in eukaryotic cells as Fab or IgG. Several of our clones were improved in their antiviral activities by the expression in mammalian or insect cells, compared to when the same clone was produced in E. coli. The anti-HIV1 antibodies had promising in vitro HIV-1 neutralizing effects and may be further explored as molecular tools for elucidating detailed mechanisms of virus neutralization via the epitopes involved, which may also guide vaccine design. Potent human antiviral antibodies may become useful as prophylaxis or after accidental exposure to the virus.
List of publications

This thesis is based on the following papers, which are referred to by their roman number (I-IV).

Paper I

Paper II
Yari, F. and Persson, M.A.A. Tetracycline regulated scFv-κ expression in eukaryotic cells, (manuscript).

Paper III

Paper IV
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## Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired ImmunoDeficiency Syndrome</td>
</tr>
<tr>
<td>C\textsubscript{H}/C\textsubscript{L}</td>
<td>Constant domains of Heavy/Light chain of antibody</td>
</tr>
<tr>
<td>CHO cell</td>
<td>Chinese Hamster Ovary cell line</td>
</tr>
<tr>
<td>CMV</td>
<td>Cyto Megalo Virus</td>
</tr>
<tr>
<td>COS cell</td>
<td>Simian virus 40 transformed monkey kidney cell line</td>
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<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope</td>
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<tr>
<td>Fab</td>
<td>Fragment antigen binding [of antibodies (\textit{V}<em>{\text{L}} C\textsubscript{L}-\textit{V}</em>{\text{H}} C\textsubscript{H1})]</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystalizable, the constant region on an antibody molecule</td>
</tr>
<tr>
<td>Fd</td>
<td>Fragment diversity, the heavy chain portion of Fab (\textit{V}_{\text{H}} C\textsubscript{H1})</td>
</tr>
<tr>
<td>Fv</td>
<td>Fragment variable, antigen binding part of the antibody (\textit{V}<em>{\text{L}} \textit{V}</em>{\text{H}})</td>
</tr>
<tr>
<td>gp</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical epithelial cancer cell</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal Antibody</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>scFv</td>
<td>single chain Fragment variable</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline Response Element</td>
</tr>
<tr>
<td>tTA/rtTA</td>
<td>tetracycline TransActivator/ reverses tetracycline TransActivator</td>
</tr>
<tr>
<td>\textit{V}<em>{\text{H}}/ \textit{V}</em>{\text{L}}</td>
<td>Variable domain of Heavy/Light chain of antibody</td>
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Aims of the study

The general aim of these studies was to generate strategies for eukaryotic expression of antibodies against envelope glycoprotein of HIV-1. The antibodies were initially isolated from Fab fragment libraries displayed on filamentous phage, but needed to be expressed as whole IgG molecules as well as in the Fab and scFv-κ formats. This implied addressing several objectives:

- To construct and evaluate suitable vectors and cellular hosts for eukaryotic expression of antibodies and/or antibody fragments.

- To express antibodies using the mentioned vectors in different expression system, and assess their properties and HIV-1 neutralization capacity compared to each other as well as to antibodies of the same clones expressed in E. coli.

- To generate an inducible, mammalian expression system, and apply that to future studies of maintenance and kinetics of viral glycoproteins inside the cell.
Introduction

The immune system

We live in a world inhabited by many different varieties of microorganisms like viruses, bacteria, fungi, protozoa and multi-cellular parasites. Many of these microorganisms normally do not cause disease in us as long as the immune system works well but these same organisms can cause infection when the later fails. Thus the immune system protects us from infection by microorganisms.

Physical and chemical barriers on our epithelial body surface like skin and mucous membranes that line the respiratory tract, gastro-intestinal tract, and genital tract are part of our defence system. However these barriers can be breached and allow the microorganisms to enter the tissues and cause harms. Also most of these barriers are only weakly effective against some pathogens. Thus, a second line of defence is necessary and this relies on several types of cells, collectively called leucocytes. Leucocytes as all other cells originate from common ancestor cells, called multipotent stem cells in the bone marrow of the humans as well as other mammals. Multipotent stem cells differentiate to give several lineages of cells. Leucocytes are derived from whether myeloid or lymphoid lineage and provide either innate or specific adaptive immunity.

Innate immunity

Characteristics for this type of immunity which also called natural immunity is that it present from birth and is not specific, which means it acts on many organisms. It also does not show specificity to a certain pathogen and does not become more efficient on subsequent exposure to same pathogen.

Innate immunity includes cells that prevent entry of microorganisms into tissues such as cells that covering the skin and mucous membranes. It also includes antibacterial substances in secretions, lysozome that act immediately when a pathogen's has appeared in the body and eliminate invader prior to the occurrence of disease. Some cells of innate immunity are of special importance for regulating our immune response. They migrate to affected areas and engulf pathogens. This migration of phagocytic cells cause the redness and inflammation associated with infection. These cells can move through out our body, phagocyte all foreign organism, but have special receptors that allow them to distinguish harmless and pathogenic organisms. However, these cells carry fragments of pathogen to lymph nodes where they either prevent or stimulate an adaptive immune response. Other cells involved in innate immunity that should be mentioned are granulocytes (1).
Adaptive immunity

This type of immunity, which also called acquired immunity, or specific immunity occurs in response to infection to a newcomer pathogen, which hasn’t infected the body before. Key cells in adaptive immunity are T and B lymphocytes. T lymphocytes mature in thymus and then entered into the blood circulation, from which they migrate to the peripheral lymphoid organs. There are at least two classes of T cells called T\(_\text{H}\) (T helper cells) and CTL (Cytotoxic T Lymphocyte) each of which has special receptors on their surface, through which and together with signal substances called cytokines, they communicate with other cells of the immune system. In addition each mature T cell displays one of two accessory molecules, CD4 or CD8. Once an antigen is engulfed and processed by a phagocyte, small pieces of the antigen appear on surface of the phagocyte in association with molecules called major histocompatibility complex (MHC) class I or II. These phagocytes are known collectively as antigen presenting cells or APC. Different classes of T cells recognize different antigen/MHC complexes on APC. In general T\(_\text{H}\) cells recognize and bind the antigens associated with MHCII, and CTLs recognize and bind the antigens associated with MHCI. The second subset of T cells, CTLs are usually acting when the invader is a virus. These cells recognise peptides derived from viral antigens on the surface of the infected cell, and secrete cytotoxic cytokines, which kill the infected cells (1).

B cells mature in bone marrow in mammals and like T cells circulate in the bloodstream and enter the peripheral lymphoid organs. B cells express membrane bound molecules called antibodies, which acts as antigen receptors. B cells in contact with antigen can internalise and break it down into small fragments, which are re-expressed at the cell surface in association with MHCII molecules. T\(_\text{H}\) cells can recognize the fragments of antigen on B cells and stimulate these cells to proliferate and differentiate into plasma cells and memory cells. Plasma cells synthesize and secrete large amounts of antibodies that bind the antigen specifically, immobilize it and prevent it from causing infection. Antibodies also facilitate phagocytosis of antigen by a special mechanism called opsonization and participate in so called ADCC (antibody-dependent cytotoxicity). Each B cell expresses antibodies which are specific for only one of the different structures called epitopes on surface of an antigen. During an infection, polyclonal antibodies from a collection of B-cell clones and specific to different epitopes on bacterial surface are produced.

The important concept of memory cells is to insure that a more rapid and stronger immune response will be made upon re-exposure to the same antigen. This form of protection is called immunity and an individual is said to be immune against that particular antigen. Another characteristic for this type of immunity is discrimination between self and none self, which means that the immune system is developed to respond to exogenous antigens, but not to cells and molecules from its own organism (1).

Adaptive immunity traditionally is divided into two parts, humoral and cell-mediated immunity. These two types of adaptive immune response act in different ways to fight infection.
Humoral immunity focuses primarily on antibodies and the B cells from which they are derived. Cell mediated immune response refers to the direct actions of immune cells on each other, on cellular antigens, and on infected body cells, and thus focuses primarily on the action of T-cells.

The innate and adaptive immune responses collaborate by enhancing and regulating each other in many different ways. This collaboration results in a more effective response than either responses can achieve alone (figure 1).

A major subject of this thesis is the in vitro expression of recombinant antibodies specific to human immune deficiency virus (HIV-1). Accordingly the interaction between antibodies and HIV-1 will be discussed in more detail.

**Antibodies the tools and object of divers studies**

Antibodies are one of the most important molecules in mammals due to their crucial role in the defense against foreign substances like bacteria and viruses. Major advantages of antibodies are specificity and the ease of selection of antigen-specific binders, the characteristics that have made them invaluable laboratory tools in diagnostic testes, in
study of also gene products and as an important component of therapy for a variety of diseases.

**Structure and biology of antibodies**

Antibodies are multimeric serum proteins that are produced by the immune system during a process of natural selection. Typically, antibodies are composed of a basic unit, comprising two identical heavy chains and two identical light chains, which are covalently linked together by disulphide bonds. The light chains fold into two domains and the heavy chains into four or five. These domains are associated with separate antibody functions (Figure 2).

![Figure 2. Schematic drawing of a typical antibody molecule. As indicated, this protein is T or Y-shaped and has two identical binding sites for its antigen. The protein is composed of four polypeptide chains (two identical 50kDa heavy chains and two identical 25kDa light chains) held together by disulfide bonds and interaction between hydrophobes patches. Each chain is made up of several different domains. The antigen-binding site is formed where a heavy chain variable domain (VH) and a light chain variable domain (VL) come close together. These are the domains that differ most in their sequence and structure in different antibodies.](image)

Antibodies as a population have the ability to recognize a very wide array of molecules, but a single antibody has certain distinct specificity. Antigen binding is mediated by six hypervariable peptide loops, or complementarity determining regions (CDRs), that form a unique surface that specifically recognizes and binds a particular target molecule. The variable heavy chain domain (VH), located at the N-terminus of the heavy chain, provides the structural framework of the domain to display three of the CDRs, with the other three displayed on the corresponding variable domain of the light chain (VL). Moreover, when the VH and VL domains pair in the antibody molecule, the hypervariable loops from each domain are brought together, creating a single hypervariable site at the “tip” of the Fab fragment that forms the binding site for antigen. The other domains make up the constant (C) regions.

There are five classes of human immunoglobulin: IgA, IgM, IgD, IgG and IgE with four further subclasses of IgG and two of IgA. From a biological perspective, by far the most important class of antibodies is IgG.
Upon synthesis, antibodies are normally secreted into the extra cellular fluid or remain membrane bound on the B-cell surface as antigen receptors. The specific and high affinity-binding properties of antibodies have been used in biomedical sciences as in vitro tools for the identification of target antigens since the early 20th century.

Antibody engineering

The field of antibody engineering has changed rapidly in the past 20 years. Antibody engineering became possible with the development of hybridoma technology in 1975, relying on the fusion of a myeloma cell line with B-cells from an immunized animal, and the application of modern recombinant DNA technology on nucleic acids coding for immunoglobulin molecules.

Recent advances in antibody engineering allow de novo construction of antibodies and the selection of desired antibody specificities by the screening of phage display libraries. Many different antibody structures have been generated using standard expression technologies. These include full-length antibodies, antibody fragments (Fab or [Fab’]₂), and single chain Fv (scFv) (Figure 3).

These advances raised the possibility that antibodies can be directed to a selected cellular compartment by using classic intracellular-trafficking signals. Accordingly intracellular expression of both whole antibodies and antibody domains can be used to block a biological function. For example binding by an antibody may be used to block or stabilize macromolecular (e.g. protein-protein or protein-DNA) interactions.

To clone antibodies by genetic means from B lymphocytes, mRNA is isolated from hybridoma, spleen, or lymph cells reverse transcribed into DNA and antibody genes are amplified by PCR. This strategy requires oligonucleotide primers that can recognize most, if not all, antibody genes.

Figure 3. Schematic illustrations, showing various antibody fragments of biotechnical interest. Whole antibody Fig 1. A) Fab fragment. B) Fab variable domain (Fv). C) scFv fragment consists of the variable domains of the heavy and light chains of an antibody fused by a linker domain.

The smallest antibody-derived polypeptide that can bind antigen with reasonable affinity is a single \( V_H \) chain. \( V_H \) chains of most species are prone to aggregation in vivo because the largely hydrophobic area that normally forms the interface with the \( V_L \) domain is exposed to the solvent. In general, the presence of both the \( V_H \) and \( V_L \) chains is needed for high stability and antigen affinity of most mammalian antibodies. \( V_H \) and \( V_L \)
chains can be expressed as separate polypeptides in bacteria where they assemble into Fv fragments. However, in these dimeric proteins the two polypeptides chains are held together by noncovalent interactions and are therefore prone to dissociation and aggregation. These two chains can be covalently assembled, by engineering an interchain disulfide bond to give a dsFv antibody. This design is more stable than an Fv fragment, but is difficult to produce by fermentation, and the disulfide bond can be reduced under mild conditions. Recombinant DNA techniques can introduce a short polypeptide linker to fuse the V_H and V_L chains together into a single chain Fv (scFv) antibody fragment that retained the antigenic specificity of the original antibody (2). scFvs are relatively small (26–27 kDa), generally quite stable, and are encoded by a single gene, which simplify genetic manipulations. The most common linker in scFv is a flexible (Gly_4-Ser)_3 decapentapeptide (3). The two variable domains can be connected either as V_H-linker-V_L or V_L-linker-V_H, with the former being more common. The order of the two domains can affect expression efficiency, stability, and the tendency to form dimers in solution. If a scFv is found to have poor stability or low affinity compared with the parental antibody, engineering the linker sequence may improve the function of the antibody. A variety of linkers have been designed based on structural considerations, screening of combinatorial linker libraries, or natural linker sequences occurring in multi-domain polypeptides. In addition to scFvs, the other commonly used recombinant antibody fragments are Fabs (see Fig. 3). Fabs consist of two polypeptide chains, one containing the light chain variable and constant domains, V_L-κ or λ, the other a truncated heavy chain containing the variable domain and one constant domain, V_H-C_H1. Just as in intact IgG immunoglobulins, the two chains are linked together by a disulfide bond. The more extensive interface between the two chains and the presence of the disulfide bonds confer increased stability to denaturation. Although the expression of Fab requires the association of two chains, it is often achieved quite efficiently in bacteria (4 and 5). On the other hand, the presence of two chains somewhat complicates genetic manipulations, and the larger size of these proteins may limit their bioavailability for certain therapeutic applications.

**Hybridoma technology**

Since the mid 1970s it has been possible to fuse an activated lymphocyte with a suitable mutant myeloma to form a hybrid, immortalized, antibody-secreting cell line that expresses a distinct antibody type, a so-called hybridoma. Fusion can be achieved by incubating a suspension of two cell types with an inactivated enveloped virus called Sendai virus or with polyethylene glycol, both of which promote the fusion of plasma membranes. This is followed by selection of antigen-specific hybridomas (6) or myeloma cells that are deficient in enzymes essential for salvage nucleic acid synthesis pathways. Unfused lymph cells cannot exist in long-term culture and eventually die. In selective hypoxanthine-, aminopterin- and thymidine-containing (HAT) media, myeloma cells that have not found a lymph cell also die due to the presence of aminopterin which blocks normal nucleic acid synthesis. Only myeloma-lymph cell hybrids, which can use hypoxanthine and thymidine for DNA and RNA synthesis via salvage pathways, survive (7 and 8).
Figure 4. A mouse is immunized by injection of an antigen X to stimulate the production of antibodies targeted against the antigen X. The antibody forming cells are isolated from the mouse's spleen. Monoclonal antibodies are produced by fusing single antibody-forming cells to tumor cells grown in culture. The resulting cell is called a hybridoma. Each hybridoma produces relatively large quantities of identical antibody molecules. By allowing the hybridoma to multiply in culture, it is possible to produce a population of cells, each of which produces identical antibody molecules. These antibodies are called "monoclonal antibodies" because they are produced by the identical offspring of a single, cloned antibody producing cell.

**Combinatorial antibody libraries and phage display**

Combinatorial antibody libraries and phage display technique has proven to be a very powerful expression technology that allows affinity selection from combinatorial libraries of antibody fragments, in particular Fab or scFv, expressed on the surface of bacteriophages (9). The phage display library is a collection of independent clones, each carrying different foreign DNA insert in the phages genome or in the phagemid vector. Phage display has also been used for the discovery of novel ligands for peptide receptors, to define epitopes for monoclonal antibodies, select enzyme substrates (10), and screen cloned antibody repertoires (11). The fusion of a small-randomized peptide to the coat proteins of a filamentous phage allows the screening of peptides in binding assays without affecting phage infectivity. Furthermore, the amino acid sequence of the selected peptide can be determined by propagation and sequencing of the phage DNA.

**Isolation of the antibody encoding genes from immune donors:** cDNA is prepared from mRNA of blood cells obtained from white blood cells or other sources such as bone marrow, tonsils, etc. Heavy- and light chain gene fragments are amplified by the polymerase chain reaction (PCR) (Fig.5a).
Cloning of VL and VH genes into the plasmid and transformation of E. coli:
The heavy- and light-chains are cloned into the expression vector. The inserted gene will be expressed in fusion with one of three structural coat proteins of the phage, gene 3 protein (pIII), gene 6 protein (pVI) or gene 8 protein (pVIII) (Fig. 5b). This vector has been designed to contain a gene encoding one of the coat proteins, usually PIII, with specific restriction sites to enable insertion of the foreign DNA sequences and to facilitate the cloning procedure.

The genetic material encoding VL and VH antibody gene fragments is isolated from peripheral blood lymphocytes, amplified by PCR, and cloned into the phage display vector. The plasmids with both human VH and VL genes are then used to transform *Escherichia coli* (E. coli), and the bacteria is grown and infected with phage resulting in phagemid particles which carry Fab cDNA incorporated into genome and, the whole Fab protein displayed on the phage surface by fusion to the coat protein. In a process called panning, the phage populations of such Fab/phage display libraries are incubated with the antigen allowing Fab/phage bearing reactive specificities to bind to their cognate antigens. Unbound phage are washed away and the bound Fab/phage are eluted and are used to re-infect E. coli cultures for further propagation and eventually obtain human monoclonal Fabs that bind antigen (Figure 6).
When the phagemid carrying cDNA is introduced into E. coli, that subsequently infected with phage, the bacteria produces phage particles, which encapsulate the plasmid DNA encoding the antibody genes and displays the corresponding antibody fragments on the surface of the phage particle. In this way large libraries of antibody variable region genes can be prepared and further manipulated. Such libraries typically consist of between $10^6$ to $10^{10}$ different clones. From such libraries it is possible to isolate antigen-binding clones by simple affinity purification of phage particles on target antigen. This process, known as panning, creates sub-libraries enriched for antibody clones recognizing the target antigen.

The search for specific high affinity antibody or peptide fragments has resulted in the construction of libraries and phage selection methodologies.

Modification in library construction have sought to improve their functional size, increase the repertoire of immune and naive libraries and encode natural variation into the phage display system using in vivo technology (12 and 13).
Human immunodeficiency virus (HIV-1)

Some pathogens especially some viruses are able to escape the immune system, persist, cause chronic disease and become fatal when the immune system fails to function properly. The human immunodeficiency virus is the most well-known virus which induces a strong antiviral immune response, but at the same time establishes a chronic infection and disrupts the ability of the immune system to respond to new infections and antigens that ultimately will lead to immune deficiency of the cell mediated immune system.

The first cases of AIDS (Acquired Immune Deficiency Syndrome) were discovered in 1981 and the HIV, which causes AIDS, was identified two years later (14). The HIV is classified as a lentivirus, which is one of the three subfamilies of retroviruses. There are two isotypes of HIV referred to as HIV-1 and HIV-2 which resemble each other strikingly. However, they differ with regard to the molecular weight of their proteins, as well as having differences in their accessory genes and genomic organizations, but both serotypes of HIV are transmitted in a similar fashion and they both seem to cause clinically indistinguishable AIDS.

The structure and organization of the viral genome

As for all retroviruses, the genetic information of a retrovirus is RNA. This RNA is covered with a viral protein coat and together they make a core particle. The core particle is surrounded by a viral envelope, which contains membrane lipids and viral envelope protein. Each viral particle contains a number of glycoprotein complexes, which are integrated into lipid membrane, and are each, composed of trimers of an external glycoprotein gp120 and a transmembrane spanning protein gp41.

Figure 7. Schematic diagram of Human Immunodeficiency Virus (HIV). The virus envelope glycoproteins gp41 and gp120 together gp160, form the virus's spikes which are embedded in the plasma membrane of host cell origin. The core of the virus contains two kinds of protein subunits, p17 and p24 that enclose 2 copies of single-stranded RNA molecules and several molecules of reverse transcriptase, protease and integrase. The nucleocapsid p7 and p9 are associated with the genomic RNA.
The shape of the HIV is a sphere with a size of 100nm in diameter. The virus genome comprises 9,749 nucleotides, which is a comparatively small virus with other viruses (15). The viral envelope is derived from the infected host cell during the "budding" process. The structure of a HIV particle is shown in Figure 7.

The full HIV genome is encoded on each long strand of RNA (In a free virus particle, there are actually two identical separate strands of RNA) (Figure 8).

Figure 8. Genomic organization of HIV-1. A schematic view of the linear HIV-1 genome, the HIV genes depicted as open rectangles. The three major classes of viral RNA and the viral proteins that they encode are shown. The Gag, Pol, and Env genes are expressed as precursor polyproteins, which are then cleaved to yield mature viral proteins.

There are at least nine recognizable genes in the HIV virus. The three major genes in the HIV virus are the GAG, POL, and ENV.

The GAG genes are group specific antigens that make up the cone-shaped viral capsid. It contains around 1500 nucleotides (55kD), and encodes four separate proteins such as capsid protein (CA) p24, matrix protein (MA) p17, nucleocapsid protein (NC) p7 and p9. The loss of the GAG gene's function results in the loss of HIV's ability to "bud" out of a host cell (16).

The POL gene encodes four HIV enzymes. The most important is a polymerase called reverse transcriptase which is unique to retroviruses that copy the virus' RNA genome into DNA. The other three products of POL are protease, RNAse-H and Integrase. The protease cleaves proteins derived from GAG and POL preproteins into functional proteins. RNAse-H degrades the viral genome when it's been reverse transcribed to DNA. Integrase mediates the insertion of the HIV proviral DNA into the genomic DNA of an infected cell (17).

The ENV gene encodes a single preprotein, gp160 which during movement along the secretory passway to the cell surface is processed by cellular enzymes, into two envelope proteins, gp120 and gp41. The gp120 glycoprotein is responsible for binding of the virus to the cell CD4+ accessory receptors. gp41 is transmembrane and anchored to a gp120 through noncovalent interactions. During the infection the viral glycoproteins become embedded all over the host cell's membrane and this membrane becomes the
viral envelope when the virus buds out of cell during the virus maturation process. Envelop glycoproteins exist as trimers on the surface of infected cells and virion (18).

The other genes that are involved in the infection process as well as in regulating the expression of structural genes are called TAT, REV and NEF.

The TAT (trans-activator of transcription) is a regulatory gene that encode for the tat protein and is one of the first viral genes to be transcribed. This protein has a very crucial role in HIV replication. Tat acts principally to promote the elongation phase of HIV-transcription, so that full-length transcripts can be produced (19). Tat protein is also a toxin which released into the blood by HIV-infected cells (20 and 21).

The rev (regulator of virion) gene encodes for Rev, a 13kD sequence-specific RNA binding protein that facilitates the transport of unspliced and incompletely spliced viral mRNA from the nucleus to the cytoplasm (22). Rev regulates viral protein expression during transition from the early to late phase of HIV (23). It stimulates the production of HIV proteins, but suppresses the expression of HIV's regulatory genes. This means that it is responsible for deciding whether the virus will produce more regulatory proteins or more virion components (24).

HIV-1 contains four additional genes: nef, vif, vpr and vpu, encoding the so called accessory proteins.

The nef (negative regulatory factor) gene encodes for Nef, a protein that maintains in the cell cytoplasm next to the nuclear membrane and has multiple activities. It is believed this gene helps to make the host cell more capable of making new HIV virions. Its also believed that Nef accelerate AIDS progression by down regulation of the expression of several surface molecules of host cell that are important in immune function (25 and 26).

The vif (virion infectivity factor) gene codes for Vif, a 23 KD protein that increases the infectivity of the HIV particle. Vif is essential for HIV replication in PBC (peripheral blood cells) (27). Vif interferes and degrades one of the immune system's defense cellular proteins, which otherwise would make newly-formed virus particles non-productive.

The vpr (Viral Protein R) codes for a 10-kilodalton protein, Vpr that plays an important role in regulating nuclear import of the HIV-1 pre-integration complex, and it is required for virus replication in non-dividing cells (28). Vpr also induces cell cycle arrest in proliferating cells, which can result in immune dysfunction (29 and 30).

The vpu (Viral Protein U) codes for Vpu, a protein involved in viral budding, virion release from the cell and down regulation of CD4 (31).

The HIV genome also has a LTR, or long terminal repeats at each end of the HIV genome, which is not quite a gene but stretches of DNA sequence that are responsible for directing the enzymes of the host cell to copy or convert the integrated virus DNA into RNA. The LTRs are composed of three subregions designated U3, R and U5 (32). The 3’ end of U5 is defined by the location of a tRNA binding site, which acts as a primer for reverse transcription.
Cell entry and life-cycle of HIV-1

Human immunodeficiency virus type 1 (HIV-1) primarily infects human CD4+ T cells and macrophages (33-35), although other cells may also be infected (36-38).

The HIV-1 enters cells by binding to CD4 as well as chemokine coreceptors such as CCR5 or CXCR4 (39-43). The expression of coreceptors on CD4+ T-lymphocytes depends on their activation level. CXCR4 is mainly expressed on naive T-cells, whereas CCR5 is present on activated and effector/memory T-cells.

The first step of the entry of HIV into cells is the interaction of the gp120 envelope glycoprotein of the virus with the CD4 receptor on the host cell's membrane (44 and 45). This binding triggers conformational changes in gp120 that leads to interaction of membrane chemokine receptor with variable 3(V3) loop of gp120 (46-50). Different HIV-1 strains use different coreceptors to gain access to cells (41 and 43). The binding step is critical for membrane fusion and virus entry (51) but the precise mechanism for this binding is not clear yet. This binding is followed by membrane fusion which is mediated by gp41 component of the viral envelope allowing the injection of virus core into the host cell's cytoplasm (52 and 53).

The next process is reverse transcription of the HIV's genome from RNA into DNA by the viral enzyme reverse transcriptase (54 and 55). The viral DNA is then integrates into the cellular DNA by the viral Integrase enzyme (55). In this stage there are two possibilities for provirus (integrated virus DNA) to remain latent as viral reservoir (56), or to be transcribed along with the host cell's DNA.

The complete spliced virus transcripts are exported from the nucleus to the cytoplasm where viral proteins are synthesized. Alternative splicing of mRNA in cytoplasm leads to the formation of numerous proteins from the small genome of virus. Some of the synthesized structural components such as gp160 are exported to endoplasmic reticulum(ER) and Golgi complex for glycosylation and modifications (57-60). These are then assembled with the viral RNA and other viral polyproteins, and exit the cell through budding (Figure 9).

In order to complete its maturation process, which occur either in the forming bud or in the mature virion after it buds from the host cell, the polyproteins are cleaved to individual functional HIV proteins and enzymes (61 and 62).
HIV-1 and escape from immune system

HIV has a relatively long incubation period of about 2-3 weeks in infected individuals. The course of infection with HIV-1 in HIV-infected humans may vary dramatically, even if the primary infections arose from the same source (63). Once HIV has entered the body, macrophages and dendritic cells on the surface of mucous membranes bind virus and shuttle it into the lymph nodes, which contain high concentrations of T\textsubscript{H} cells (CD4+ T cell). The T\textsubscript{H} cells recognize foreign antigen bound to MHC proteins and initiate the activation of cells of both the humoral and cell mediated immune responses. Thus the infection with HIV in the majority of cases is associated with the production of virus specific antibodies and a strong virus specific CTLs in response to the virus. However, despite an immune system which is still active, the virus continues to replicate at quite high levels and a progressive decline in the number of CD4+ T cells occurs during this period. The answer to how HIV manages to survive such a strong antiviral immune response lies in a number of strategies that the virus uses to evade the host's immune response. One of the most important strategies is the ability to undergo "antigenic variation" which makes the virus able to mutate and change the key epitopes, which are recognized by the immune response. This has been shown for both antibody and T cell epitopes in HIV infection (64). In addition, and as mentioned before, HIV is able to exist as latent form within an infected cell as a provirus without
replicating, so that very few viral antigens are expressed in the cell. Permanent viral reservoirs, mainly in macrophages and latently infected CD4+ T-cells, are established in the early phase of infection and probably represent the major obstacle so far to successful eradication of HIV. Infections with lentiviruses typically show a chronic course of disease, a long period of clinical latency, persistent viral replication and involvement of the central nervous system.

HIV infects cells of the immune system, making these cells the target for attacks and damage by the immune systems’ own cells. Infected cells that express viral proteins on their surface are recognized and eliminated by CTLs. This reduces the number of T cells and contributes to the immune dysfunction and survival of the virus. During the process of budding, the virus may also incorporate different host proteins from the membrane of the host cell into its lipoprotein layer, such as MHC class I and II proteins, or adhesion proteins such as ICAM-1 that may facilitate adhesion to other target cells.

Cell-to-cell fusion, caused by HIV, also destructs the host cells. The fusion process start with just one cell that becomes infected and then can continue to fuse to other healthy T cells until a gigantic syncytium forms out of as many as 500 cells, causing the individual T cells to lose their immune function (65 and 66).

The bond between gp120 and gp41 is only loose and therefore gp120 may be shed spontaneously within the local environment. Glycoprotein gp120 may also be detected in the serum (67) as well as within the lymphatic tissue of HIV-infected patients (68). These free gp120 envelope proteins can bind to the CD4 receptor of non-infected cells. HIV infection can also directly cause cell death. As the virus moves out of the infected cell, during the budding process, holes are punctured in the cell membrane, causing the cell to swell up and die (69). The toxin Tat which released into the blood by HIV-infected cells is involved in T-cell apoptosis (21).

As was described previously, CD4+ T-cells play a central role in the host response to pathogens, and decline in numbers of these cells influences the regulation of the adaptive immune response dramatically. Thus HIV infection leads to disruptions of many aspects of the immune response, including antibody and T cell responses to new antigens as well as decreased in NK responses. As CD4+ T cell numbers decline, the defects become more marked, leading to a state of immunodeficiency which leaves the host susceptible to infection with a variety of common or opportunistic infections and even to certain types of tumors (70).

Neutralization of virus by antibody

Virus neutralization is the reduction in infectivity that results from the binding of antibodies to the virus particles (71 and 72). This definition includes the binding of antibodies to cellular antigens present on the surface of the virions, as has been described for primate-lentiviruses (73). The definition leaves open whether neutralization is restricted to viral infectivity of certain cell types, or blocks the entire cellular tropism of the virus (74 and 75), and by what mechanism it acts.

The process of virus neutralization in vivo can be indirect or direct (Fig. 10). Indirect neutralization requires secondary factor such as complement components to lyse
the virion, or cells which interact with the antibody-virus complex, leading in some cases, to destruction of the virion.

There are many mechanisms by which antibodies can directly neutralize virus infectivity, and every virus can be neutralized in a variety of different ways depending on a number of variables. Viruses have several antigenic (neutralization) sites, each consisting of a number of epitopes, and these may be present on one or more surface proteins. It is likely that the mechanism of neutralization will be influenced by the functional role of the epitope recognized by the antibody. In addition, properties of the antibody (e.g., paratope, isotype, number of molecules bound per virion) can also determine the mechanism of neutralization. Other factors, such as multivalent binding of antibody to virion, depend both on properties of the virion (epitopes spaced neither too close together nor too far apart) and of the neutralizing antibody (molecular interactions of epitope and paratope such that one arm of a bivalent antibody is oriented away from the virion). The situation is made more complex because any of the parameters mentioned may operate in combination. Finally the host and the target cell can determine qualitatively or quantitatively the extent of neutralization (72 and 76).

![Figure 10. Neutralization of virus:](image)

Neutralizing antibodies can be used to bind viral coat proteins and block viral pathogenesis. High-affinity human antiviral antibodies (e.g. for HIV-1, respiratory syncytial virus, and herpes simplex virus) have been selected from immune libraries using a variety of strategies. Small subsets of these antibodies show potent neutralization in vitro and antiviral efficacy in vivo in animal models (74, 77 and 78).
Neutralizing antibodies in HIV infection

As was mentioned before, infection with HIV results in a strong antibody response, which is directed to most structural proteins. Among these antibodies some have neutralizing capacity and some other participate in the lysis of infected cells by recruiting NK cells in antibody dependent cell-mediated cytotoxicity (ADCC). Unfortunately, these antibodies are not sufficient to prevent continued HIV infection as in most cases the disease progresses and eventually onset of AIDS occurs. This may be due to several factors such as the levels of antibodies that might be insufficient to block the spread of infectious virus. It can also depend on production of polyclonal antibodies against different parts of the virus, in which only some of antibodies have the potential to neutralize cell-free virus and subsequent viral infection in the body (79). Antigenic variation in envelope protein is another reason. It is now clear that the HIV has a high mutation rates for ENV gene that encode envelope proteins (70 and 81). It is believed that these mutations are created during reverse transcription of viral RNA to DNA (82). As was mentioned before surface glycoproteins are important in attaching of the HIV to the cell receptor. Thus, mutation in these proteins reduces recognition by antibodies. Changes in the HIV envelope proteins have been observed in virus isolated from different tissues in HIV infected individual (83-86).

How well antibodies can protect against HIV-1 infection is an issue under progression with important implications for vaccine design and the use of antibody to prevent infection after accidental exposure to the virus or to inhibit transmission of virus from mother to child.

The success of immunoprophylaxis in animal models using HIV-1 neutralizing monoclonal antibodies shed light on the difficulties of designing an effective vaccine based on neutralizing antibodies (74).

Due to location of HIV-1 envelop glycoproteins on the surface of the virus; these proteins are the major target for neutralizing antibodies. The immunodominant epitopes such as v3 loop of gp120, as well as CD4 binding site and the major epitope of HIV gp41 because of antibody accessibility are of more interest.

The HIV-1 envelop spikes are putative trimers of gp120-gp41 heterodimers and as described before, mediate binding to cell receptors and virus entry (44 and 45). The 3D structure of both gp41 (87-89) and gp120 (90-91) glycoproteins have been visualized through crystallography. The gp120 has a structure with inner and outer domains, named for their expected orientation in the oligomeric viral spike (90). The inner domain is believed to interact with the gp41, while the outer domain, which is quite variable and heavily glycosylated, is believed to be exposed on the assembled envelope glycoprotein trimer (91). The gp41 have a trimeric core consisting of parallel a-helices with heptad repeat sequences in N and C terminal. Before fusion, in the native conformation, much of the structure of gp41 is masked by gp120. Upon CD4 and coreceptor binding and conformational changes in gp120 that activate the fusion, some epitopes on gp41 which are important in fusion, transiently exposed and are accessible for antibodies (92 and 93).

Analysis of sequences important for coreceptor binding site of gp120 has shown that the V3 hypervariable region of the gp120 envelope protein carries the main determinants of both cellular tropism (94-96) and coreceptor use (40).
Expression of Anti-HIV-1 Ab in Bacteria & Eukaryotic Cells

V3 typically consists of 35 amino acids and plays a number of important biological roles (97). Not only it is critical for coreceptor binding, but it also determines which coreceptor CXCR4 or CCR5 will be used for entry (96). In addition V3 may interact with other elements in the viral spike to control the overall sensitivity of the neutralization (98). Finally, immunization with HIV-1 envelope glycoproteins often elicits neutralizing responses directed primarily against V3 (99 and 100).

The goal of finding antibodies that can neutralize multiple isolates of HIV-1 has been very difficult to achieve. However, several Env-specific human monoclonal antibodies (hmAbs) have been found (101) to exhibit neutralizing activity to primary isolates from different clades including the anti-gp120 antibodies b12 (102 and 103), 2G12 (104-106), m14 (107), m18 (108), F105 (109), 447-52D (110) and Fab X5 (111), and the anti-gp41 antibodies 2F5 (112), 4E10 (113 and 114), Fab Z13 (114) and m48 (115).

mAb b12 binds to the recessed CD4 binding site (116), whereas mAb 2G12 recognizes a unique cluster of oligomannose sugars on outer domain (117). mAb 4E10 and 2F5 recognize adjacent and highly conserved contiguous epitopes in the C-terminal, membrane proximal region of gp41, indicating that gp41 is not completely masked by gp120. The 2F5 epitope is centered on the sequence ELDKWA (112 and 118) whereas 4E10 recognizes an epitope containing the sequence NW F(D/N) IT (114, 119 and 120) in C-terminal to the 2F5 epitope.

The conserved C-terminal region of the gp41 extracellular domain that encompasses the 4E10 and 2F5 epitopes is critical for env-mediated membrane fusion and virus infectivity (121 and 122).

An interesting case in point is antibody to the constrained cysteine loop CSGKLC (residues 603–609) (123 and 124). This epitope is recognized by 90–100% of the HIV-infected individuals (125 and 126).
Numerous human mAbs have been isolated for this epitope and its vicinity (127-132). Human mAbs 1B8.env (128), 41-7 (130), clone 3 (131) and 2F11 (132) all bind this epitope, but 41-7 and 1B8.env are not neutralizing.

Identification and characterization of HIV-1-neutralizing antibodies to the conserved structures has also been investigated. The problem is that conserved structures in envelope glycoprotein that are important for replication (79 and 133, and 134) are hidden by variable loops, extensive glycosylation, transient exposure, occlusion within the oligomer, and conformational masking; thus elicitation of neutralizing antibodies to this part are rare and usually occurs after relatively long periods of maturation (101 and 135).
Comments on Experimental procedures

Plasmid constructs

In the present study, the cloned antibody fragments were expressed as Fab, IgG and scFv-κ format in nonlymphatic cell lines.

In the first paper, in order to express Fab fragment the heavy (HC) and the light (LC) chains obtained from a combinatorial antibody library, using the pComb3 system, were transferred to a modified eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA) and a set of eukaryotic expression vectors (pcHC, pcLC, pcLCHC and pcHCLC) were constructed. pcDNA3 has a very strong CMV promoter which allows high expression level of the gene of interest in mammalian cells.

To generate pclgG1, pcHCLC was modified by engineering a Fc fragment of a human γ1 antibody between the HC and LC fragments.

In the second paper the gene encode scFv-κ was cloned downstream of the regulatory element of TRE plasmid which include tetracycline-controlled gene expression system in order to control expression of scFv-κ.

In paper III genes encode Fab from a number of anti HIV-1 and HCV clones were transferred to the vectors adapted for expression of IgG in drosophila S2 cells.

In paper IV a number of Fab molecules reactive against a conserved HIV-1 gp41 were selected and transferred to vectors. Same methodologies as paper I were used. Moreover, antibody coding gene of two clones were transferred to vector for IgG expression in S2 cells. Schematic representation of the mainly used plasmid vectors generated in present study is shown in figure 12.
Expression of recombinant antibodies

Cell lines and expression systems

There are many different expression systems that can be used for the production of whole recombinant antibodies and antibody fragments. These include bacterial, yeast, insect or mammalian cell culture systems and transgenic animals or plants. The expression system of choice is partially dependent upon the intended use of the antibody, as well as the antibody yield derived from each system.

Antibody expression in prokaryotic cells (E. coli)

Escherichia coli (E.coli) was discovered by Theodor Escherich in 1885 and is one of the main species of bacteria that live in the lower intestines of mammals known as the gut flora. E.coli has been used as a model for bacteria in general and has a long history of laboratory culturing and manipulation. Its structure is much simpler than eukaryotic cells, which has made it a target for studies of life science. Since bacteria do not have a nucleus, the genome of E-coli, which consists of a single circular DNA chromosome, exists within the cytoplasm of the cell anchored to the cell wall. Indeed they lack all of the intracellular organelles such as Golgi apparatus, endoplasmic reticulum, lysozome, mitochondria, which are characteristic of eukaryotic cells. However they are generally capable of “independent life” and they possess all the biosynthetic machinery that is needed for this, including 70S ribosomes, which are distributed throughout the cytoplasm. The most complex region of the cell is often the cell wall. E. coli plays an important role in modern biological engineering: to synthesize DNA and/or proteins which can be produced in large quantities.

Bacterial systems are suitable for the expression of scFvs and Fabs. Because bacteria lack the cellular machinery to glycosylate proteins, only aglycosylated antibody fragments are produced from bacterial fermentation. Important strategies for the production of recombinant proteins are intracellular accumulation, or secretion into the periplasmic space of E. coli. Because this compartment has an oxidizing milieu allowing the formation of disulfide bounds. The product of scFvs in bacterial expression systems is usually low, primarily as a result of incorrectly folded protein retained either at the inner cell membrane or as an insoluble aggregate in the cytoplasm. Although the insoluble protein can be extracted and refolded, high expression can cause toxicity and cell death of E. coli (136 and 137).

Antibody expression in eukaryotic cells

Mammalian cells are of great importance for the production of recombinant human protein therapeutics, including blood clotting factors, growth factors, and monoclonal antibodies.
COS cells are African green monkey kidney cells that have been transformed with an origin-defective mutant of simian virus-40 (SV-40). COS cells have been used to transiently express a large number of heterologous gene products. Expression of functionally active antibodies in COS cells was first reported in 1987 (138) and has now become almost routine (139-143, 144 and 145). COS cells constitutively express SV-40 T-antigen therefore support replication of expression plasmids containing the SV40 origin of replication, amplifying the introduced expression cassettes. Theoretically this result in high expression yields. Although these cells normally are grown in serum containing medium, after transfection they can be grown in serum-free medium to facilitate purification and analyses. Expression levels of 0.1-1.5 µg/ml are generally obtained (146). COS cells usually are well suited for use in the rapid expression of monoclonal antibodies (mAbs). As they are eukaryotic cells, one can express full-length biologically active mAbs that contain the proper post-translational modification, processing and disulfide bond formations. Typically, COS cells are used to quickly express and characterize the multiple mAbs generated from phage display combinatorial libraries (146).

CHO (Chinese hamster ovary) cell line is one of the most widely used mammalian cell lines as it is known for its high stability of chromosomally integrated heterologous transgenes (147). These cells are relatively easy to cultivate at large scale, are readily transfectable, are capable of growth at high densities and are able to grow in suspension and in serum- and protein-free medium. CHO cells have proved to be successful for the high level expression of a large number of foreign proteins (148-151) such as mAb (143, 152, 153 and 149).

CHO cells also have a well established track record for expressing heterologous gene products other than mAbs, and have proven successful in the co-expression of foreign genes in the past (138, 144, 145, 148-157). These cells also offer the use of co-amplification with a number of different selectable genes, such as those encoding glutamine synthetase (GS), di-hydrofolate reductase (DHFR) and adenosine deaminase (ADA) (158). Certain CHO cell lines have been certified for production of proteins to be used as therapeutics in humans.

HeLa cells are human cervical cancer cells that have been maintained in continuous culture since February 1951 and have been cultured in laboratories around the world. HeLa cells proliferate indefinitely in culture and have the same features as CHO cells regarding gene expression.

S2 (Schneider's 2) cells are *Drosophila melanogaster* embryo cells regarded to have macrophage like characteristics. S2 cells have shown to be easily transfected, and multiple copy numbers of the transgene can be stably integrated to genome of these cells in a single event. They grow in suspension, in serum free media and can grow at room temperature without any need of a controlled atmosphere, though their optimal growth conditions are at 28°C. S2 cells have been used to express fully functional antibodies (159).
Tetracycline-inducible expression system

Several inducible systems for expression of recombinant genes, based on eukaryotic as well as prokaryotic regulatory mechanisms, have been described. Many, however, have been disappointing because of poor inducibility, pleiotropic inducer effects, or both. The tetracycline-inducible gene expression system developed by Gossen and Bujard has proven useful for inducing or repressing the expression of a particular gene in mammalian cells, because the external repressive agent tetracycline does not interfere with the overall metabolism of mammalian cells (160) and release from tetracycline-mediated repression is a fast process (161). A tetracycline-controlled trans-activator protein (tTA), composed of the repressor of the tetracycline-resistance operon (tet from E. coli transposon 10 (Tn10) and the activating domain of viral protein (VP) 16 of herpes simplex virus, induces transcription from a minimal CMV promoter [P (hCMV-1)], fused to seven tet operator (tet O) sequences in the absence of tetracycline but not in its presence. Because tetracycline turns off the expression, this system is often referred to as "tet-off" system. As a complement to this approach, a "tet-on" system was developed by mutating tTA to generate a reverse tTA (rtTA) that only binds to tet O, and thereby activates transcription in the presence of tet (figure 13). Both tet systems offer the advantage of using an inducer, tetracycline, with well-characterized pharmacokinetics at a subtoxic level. Because the affinity of the tetR for tetracycline is very high, low to moderate amounts of the antibiotic or its analogs are sufficient to modulate gene expression. Levels of induction with tTA is as high as $10^5$-fold over background have been observed, whereas with rtTA, induction of target gene expression up to $10^3$-fold has been reported (161).

Figure 13. Schematic image of gene regulation in the tet-on gene expression system. The reverse tet-responsive transcriptional activator (rtTA) binds TRE and activates transcription in the presence of doxycycline.
Transfection and experimental design

There are a number of different transfection strategies to transfer DNA into cells. The choice of the transfection method as well as the requirements on the quality of the DNA to be transfected varies with cell type. Therefore it is necessary to optimize the transfection methodology and DNA concentration for each cell line. Two widely used transfection methods, which we also have used in our experiments are described below.

Lipofection (liposome transfection) is a technique used to transfer genetic material into a cell by means of liposomes which form vesicles that can easily fused with the phospholipid bilayer of the cell membrane. The DNA to be transfected is incubated with an appropriate concentration of lipids in an appropriate solution for 30 to 45 minutes allowing the liposomes to form. The liposome/DNA complex is then added to the cell culture which is incubated for 2-5 hours (figure 14).

![Figure 14. Schematic image of lipofection strategy. The liposome/DNA complexes fuse with the cell membrane and diffuse through the intracellular membranes. The liposomes break down and the DNA traverse to nucleus, where they either integrate or will be degraded. (The mechanisms of liposome degradation and DNA transport to nucleus are not clear).](image)

Electroporation (electropermeabilization) is a transformation/transfection method by which plasmid DNA can be transferred to eukaryotic as well as prokaryotic cells. This method is based on making pores in cell membrane by an electrical pulse of about several hundred volts across a distance of several millimeters of two aluminum electrodes in two sides of a plastic cuvette and for a short period of time. Electroporation occurs as a result of the reorientation of lipid molecules of the membrane to form hydrophilic pores in the membrane (162). Cell membrane permeability is increased that give extracellular compounds a chance to enter into the cell. Cells to be transfected are mixed with DNA in water or other low salt solution. Afterwards, the cells are handled carefully until they are recovered and are able to divide.
Purification and concentration of IgG antibodies

The supernatant of antibody secreting cells in culture usually contain many other molecules such as extracellular proteins, salt, debris of death cells, which may influence the folding and antigen binding capacity of the antibodies. In addition the concentration of antibodies in supernatant is usually low. Antibodies can be purified and concentrated by passing the antibody solution through a fast phase containing antibody ligands such as protein G or protein A (figure 15A). Protein G is a cell wall protein from group C and G Streptococci that binds strongly to the Fc region of IgG. Protein G binds to a wide range of immunoglobulins and the binding buffer does not need to have a high ionic strength. Protein G also tends to have a higher antibody binding affinity than Protein A. However, a lower elution pH is required to desorb the immunoglobulin from a Protein G agarose column. Consequently, most researchers currently use recombinant Protein G for purifying immunoglobulins.

The solubility and/or concentration of purified antibodies can be increased by dialysis through a semipermeable membrane, such as a cellulose membrane with pores. Smaller molecules and ions traverse the pore whereas antibodies, which have dimensions significantly greater than the pore diameter, are retained inside the dialysis bag. This effect called salting-out which also is useful for concentrating diluted solution of antibodies. The dialysis bag containing antibody solution is placed in a buffer with high salt concentration. Water molecule traverse the pore outside the dialysis bag and the antibodies inside become concentrated (figure 15B).

Figure 15. A) Purification of IgG antibodies by protein G column. Medium from cell culture expressing IgG antibodies are passed down a column containing protein G coupled sepharose beads. The antibodies bind to G protein through Fc fragments while all other molecules in medium such as extracellular proteins are eluted in the unbound fraction (I). The IgG antibodies are then eluted in almost pure form by addition of an acidic buffer with low pH (III). B) Concentration and salting-out. The purified protein is transferred to dialysis bag, which is placed in a low salt concentration buffer.
Immunoprecipitation and western blot analysis

Immunoprecipitation (IP) is one of the most widely used immunochemical techniques by which peptides or proteins that react specifically with an antibody are removed from solution by addition of an insoluble form of an antibody binding protein such as Protein A, Protein G or second antibody. In this method the same antibody binding strategy similar to antibody purification is used. Also protein G coupled sepharose or magnetic beads that facilitate the isolation of antigen/antibody complex out of the solution are used. Analysis of the immunoprecipitated molecules are usually performed by separation in SDS-PAGE gels followed by immunoblotting, by which the quantity or physical characteristic such as molecular weights of antigens can be determined (figure 16). The IP technique also enables the detection of rare proteins which otherwise would be difficult to detect since they can be concentrated up to 10,000-fold by immunoprecipitation. The antigen can also be labeled by radioactive isotopes before the immunoprecipitation procedure; either by culturing cells with radioactive precursor or by labeling the molecule after synthesis has been completed.

Figure 16. Schematic representation of the principle of immunoprecipitation. I) the antibody is added to a mixture of proteins in the cell lysate that specifically binds to its antigen. II) antibody-antigen complex is absorbed from solution through the addition of an immobilized antibody binding protein such as Protein G-magnetic beads. III) Upon applying the antibody-antigen-protein G magnetic beads complex to a magnetic field, the beads are pulled to the side of the tube. Subsequent liberation of the antigen can be achieved by boiling the sample in the presence of SDS. IV) immunoprecipitated molecules are analyzed by gel electrophoresis followed by immunoblotting.


**In vitro neutralization of HIV-1**

The anti-viral activities of an antibody specific to virus surface antigens, for some viruses can be analyzed by neutralization assays. In this method the antibody mediated reduction in titers of infectious virus is measured. Cells or cell lines are grown together with virus (e.g. HIV-IIIB) in the presence or absence of anti-virus sera or monoclonal antibodies (figure 17).

For HIV-1, extracellular viral protein such as p24 antigen or reverse transcriptase in the culture supernatant is measured, often by ELISA (enzyme-linked immunosorbent assay) (163). The viral protein concentration is directly correlated to the virus titer. These assays require several rounds of virus replication before expressed proteins can be quantified, and thus only indirectly measure the actual number of target cells infected.

![Figure 17. Mechanism of virus neutralization by antibody. a) Antibody binding to envelope glycoproteins may block virus attachment to the surface of target cells. b) Binding of antibody to virus trigger internalization by endocytosis c) the virion may thus ultimately be destroyed through lysosomal degradation.](image)

**Immunostaining and Fluorescence Microscopy**

The purpose of immunofluorescence is to detect both the presence and the subcellular localization of an antigen. The key to this entire process is the ability of specific antibody to recognize and bind to its cognate antigen. In immunofluorescence staining of cells most commonly two sets of antibodies are used. Primary antibody binds to the antigen of interest that can subsequently be detected by a secondary, fluorescent
dye-coupled antibody. Fluorescent dyes make localization of the antibody antigen more readily visible when looking through a fluorescence microscope. When a light illuminates the fluorescent dye, it absorbs the light and emits a different color light which is visible to the eyes and can be photographed.

The first step of immunofluorescence staining of cells is to fix and permeabilize the cells, to ensure free access of the antibody to its antigen. The second step of cell staining involves incubation of cell preparations with antibody. Unbound antibodies are removed by washing, and the bound antibodies are detected either directly (if the primary antibody is labeled) or, indirectly using a fluorochrome-labeled secondary reagent.
Results and Discussion

Technological advances made during the past decade, such as phage display (11), ribosome display (164), the protein fragment complementation assay (165), and the availability of antibody libraries (166) has greatly facilitated the problem of obtaining recombinant antibodies with the desired specificity. To produce these antibodies as full-length molecules and as fragments, several expression systems utilizing either prokaryotic or eukaryotic organisms have been developed. Insect cell culture (S2) as well as mammalian cell culture, both lymphoid (myeloma) and non-lymphoid (COS, HeLa and CHO cells), are optimal choice for the expression of biologically active full-length, recombinant antibodies. Although mAb fragments can be expressed in bacteria, especially E. coli, not all clones may be properly assembled and/or correctly folded, and all are non-glycosylated. Bacterial systems lack the cellular machinery to glycosylate proteins and therefore they are not suitable for the expression of full-length antibody.

Expression of correctly folded antibody molecules is crucial for their binding affinity (and neutralization of virus in vitro). Working with anti-HIV-1 antibody fragments expressed in the periplasm of E.coli, we noted different results from different batches of the same clone, findings that may have been related to misfolding of the proteins occurring to varied degrees in different batches.

The experiments described in this thesis were mainly designed to demonstrate the feasibility of expressing functional antibodies against HIV-1 in mammalian cells, originally isolated by phage display.

In paper I we investigated how different expression hosts could affect the functional properties of anti-viral antibodies. We also showed that such antibodies selected from phage display libraries could be expressed in the mammalian culture system with even improved neutralizing capacity.

A set of eukaryotic expression vectors was constructed with cloning sites matching those in the phagemid vector pComb3: pcHCLC and pcIgG1 were designed for expression of Fab and IgG1, respectively. We evaluated the system on three human anti-HIV-1 Fab clones that were isolated from a combinatorial library. The Fab fragments were specific for two epitopes of the envelope glycoproteins of HIV-1 (gp120 and gp41) and blocked virus infectivity in vitro (167). By transferring the genes of isolated Fab fragments to the appropriate vectors and subsequently expressing the antibodies in COS and CHO cells, we could perform a comparison of functional activity between Fab fragments expressed in bacterial and mammalian cells as well as Fab and whole IgG1. Fab fragments expressed in mammalian cells showed increased virus neutralizing activity compared to the same Fab clones expressed in E.coli, very likely as a result of incorrectly folded protein retained either at the inner cell membrane, or as an insoluble aggregate in the cytoplasm, in the bacteria (136). HIV-1 neutralizing activity was, however, remarkably similar between Fab and whole IgG1 reagents expressed in CHO cells. Thus according to our results, full-length, bivalent IgG do not always confer improved neutralizing capacity compared to the monovalent Fab molecule. Neutralizing capacity of
the purified mAbs expressed transiently by COS-7 cells, and stably by CHO cells was also compared, and no significant differences were noted (Figure 18).

In paper II we took advantage of the tetracycline-regulated gene expression system to allow inducible expression of scFv-κ antibody against envelope glycoprotein of HIV-1 in HeLa and CHO cells.

A major breakthrough in the technology of antibody engineering was the derivation of single chain molecules which were obtained by joining the VH and VL domain from a mAb with a flexible linker which allowed the reconstitution of the original VH/VL association. In this way an antibody fragment was produced in form of a single-chain molecule, which retained the antigenic specificity of the original antibody (2).

In the mid 1980, the use of antibodies as discovery tools and as gene therapeutic agents has been greatly enhanced through studies of their intracellular expression as intrabodies, where they have been shown to be capable of inhibiting the expression of specific microbial, viral and cellular genes and in some cases even supplying gain of function properties (168 and 169).

In order to control expression of the gene of interest, we utilized two plasmids prtTA (reverse tetracycline transactivator) and pTRE (tetracycline response element) based on the tetracycline-regulated gene expression system developed by Bujard and Gossen. CHO or HeLa cells were transfected separately with prtTA vector for constitutive expression of rtTA protein, driven by the human CMV promoter that also

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**Figure 18.** Neutralization of HIV-1SF2 and HIV-1LAI by clones 3, 17 and 108 expressed as Fab in E.coli and CHO cells, and as IgG1 in CHO cells. Inhibition of virus infectivity was measured as reduction of p24 production on day 7 post-infection.
carry regions for the neomycin resistance gene, and neomycin resistant clones were isolated. The genes coding for several scFv-κ clones against gp41, gp120 of HIV-1 and tetanus toxoid were inserted into pTRE vector. To generate stable cell lines with tet-regulated expression of the single chain antibody genes, CHO/HeLa –rtTA cells were co-transfected with each of pTRE-scFvκ and pTK-Hyg vectors. Hygromycin resistance clones were isolated and assayed by ELISA for scFvκ expression. The populations of resistant cells were used for experiments demonstrating Doxycycline induction, which was added to culture medium at a concentration range of 0.001 to 10 µg/ml. The highest induction was obtained in majority of the clones when 100ng/ml Doxycycline was added to culture media. Antibody expression was up to 300ng/ml in induced state. A low level of antibody expression of about 10ng/ml in the absence of Doxycycline was also detected. Expression level of antibodies decreased rapidly by the time down to 30ng/ml. Due to relatively inefficient inducibility by Doxycycline and instability of gene expression further experiment with this system was not promising.

Another round of transfection, cell selection and expression of scFvκ antibodies was performed using the second generation of prtTA called prtTA2s-M2. To be able to select transfected cell, a Hygromycin gene was inserted to the plasmid encoded the prtTA2s-M2. Moreover, the genes encode TRE-scFvκ were transferred to pcDNA3 (z+) encoded zeocin resistance gene. HeLa cells were transfected with the prtTA2s-M2 and cells were subsequently selected for Hygromycin resistance clones. These stably rtTA2s-M2 protein expressing cells were transfected with different TRE- scFvκ (z+) clones and were selected for zeocin resistance cells. Clonal selection of transfected cells resulted in a number of cell lines with high expression level upon induction with 100ng/ml doxycycline (figure 19). Basal expression of antibodies in the absence of doxycycline was detected.

![Figure 19](image_url)

**Figure 19.** Induction of scFvκ antibody expression in HeLa tet on cells analysed in absence (-) and presence (+) of doxycycline. Lane A and B: scFvκ clon3 and lane C and D: scFvκ TT.

The results from two independent experiments demonstrated a higher gene expression level in presence of doxycycline in transfected cells when the second generation of reverse tetracycline transactivator (rtTA2s-M2) was used.
The background expression was detected in both experiments, which reflected the activity of minimal MCV promoter. The reason for this can be that transactivator binds to operator in inactive state in absence of doxycycline. Another reason can be the presence of tetracycline in culture medium. In fetal calf serum, which is used as complement to cell culture medium small concentrations of tetracycline may be traced, this can activate the transactivator. It has been shown that doxycycline binds to the cell and extracellular matrix and can not be removed properly by multiple washing steps (170).

In both experiment the induction level of gene expression varied in different clones that might be due to variations in the site of integration or number of copies integrated to the cell genome. Some of the cell lines exhibited greater that 50-fold induction of gene expression in the presence of Doxycycline, still considerably less than the $10^3$-fold increase of HeLa cells expression reported by Bujard and Gossen (161).

By inserting a resistance gene to the pTRE plasmid the frequency of transfected cells was increased compared to cotransfection with Hygromycin according to our experience. To have a resistance gene in same vector as TRE gene didn’t influence the background gene expression.

Generally, application of the tetracycline expression system for regulation of scFvκ was a demanding laboratory process due to repeated selection and cloning stages. The tetracycline regulated system may not be perfect but is the best existing system for regulation of gene expression in our hands.

Modification of the vectors for inducible antibody expression resulted in a more desired regulatable gene expression and indicated the possibility of improving the system for additional laboratory and in vivo applications.

In paper III S2 cells have been used to express fully functional antibodies (159). The heat shock cognate 3 (HSC3) protein in the S2 cells is a homologue to immunoglobulin heavy chain binding protein (Bip) localize to the ER of eukaryotic cells. It is believed that Bip functions as a chaperone which support proper protein folding and protein translocation into the ER lumen (171). The aim of this study was to evolve stably IgG expressing S2 cell lines that fulfill the need of efficiently production of correctly folded IgG antibodies in large scale.

A series of vectors for inducible expression of human Ig heavy (HC) and light chains (LC) were constructed, on both separate plasmids and in combination constructs. These vectors were adapted for expression of human IgG1 against HCV and HIV-1 in Drosophila S2 cells. The S2 cells were co-transfected either with pMThIgG1, pMThIgG1-p1, pMThLC, or pMThHC and selection vector pCoBlast. In addition, pMThLC, pMThHC and pCoBlast were co-transfected. Antibody expression was induced by the addition of CuSO$_4$. Cell supernatant was collected and screened for transient expression of antibody by ELISA. The result demonstrated higher expression of antibodies in cells transfected with the construct with two promoter pMThIgG1 than with single promoter pMThIgG1-p1. Stable cell lines expressing the IgG1 anti-HCV and -HIV clones resistant to Blasticidin were established within 2-4 weeks. The cell culture supernatant was collected after an expression period of 10-14 days followed subsequent purification of IgG antibodies qualitative and quantitative analysis of these antibodies compared to the same clone expressed in (mammalian) CHO cells were performed using ELSA, western blot, immunofluorescence microscopy and neutralization test in vitro.
Milligram quantities of antibodies could be obtained during 4-5 weeks post initial transfection from these cells.

Data from neutralization of HIV-1 primary isolate demonstrated that antibodies expressed in S2 cells bound the antigen with equal specificity and affinity as antibodies produced in CHO cells (figure 20), that is in agreement with Gutteri et al. published reports (172).

In transient expression of antibody, levels of 0.1-1.5 µg/ml in COS cells (146 and 155) and 1-2 µg/ml in CHO cells (173) have been reached which are almost at same level as the antibody expression level in S2 cell.

Stably expression of antibody in other insect cell has resulted in 0.06mg/l (172) which is much lower than 5-35 mg/l that have been obtained in this study.

In conclusion, the S2 expression system was easy to set up and allowed faster establishment of high-level expression of stable cell lines compared to mammalian cell lines. This characteristic make S2 cells ideal for quick production of large amount of antibodies from several clones of antibodies to allow thorough characterization and testing.
In paper IV the HIV-1 neutralization potency of Fab antibodies against gp41 of HIV-1 was studied. In the mature trimeric spike of HIV-1, much of transmembrane gp41 glycoprotein is masked by gp120. After CD4 and coreceptor binding, gp120 undergoes conformational changes that expose gp41 and activate the fusion machinery. The fusion process takes only minutes, during which the transiently exposed epitopes on gp41 is perhaps accessible for inhibitors and antibodies (88, 89 and 174). Both the N- and C-terminal portions of the external domain of gp41 contain heptad repeat (HR) sequences. C-HR and N-HR together form a six-helix bundle, positioning the fusion peptides for insertion into the host-cell membrane. In principle, antibodies that could bind to the N-HR inner helical core after receptor activation block entry of HIV-1 (175). The broadly neutralizing antibodies recognize gp41 epitopes that are close to the viral membrane but accessible during fusion (93 and 176).

Of the large number of mAbs that have been generated against gp41 Env protein only a few numbers of mAbs have been identified that exhibit broad and potent HIV-1 neutralizing activity. Among these few but invaluable mAbs 4E10 and 2F5 recognize adjacent and highly conserved contiguous epitopes in the C-terminal, membrane proximal region of gp41. The 2F5 epitope is centered round the sequence ELDKWA (112 and 118) whereas 4E10 recognizes an epitope containing the sequence NWF (D/N) IT (114, 119 and 120) in C-terminal to the 2F5 epitope.

The amino acid sequence ELDKWA is highly conserved linear epitope in HIV-1 gp41 envelope glycoproteins (177, 178 and 112).

In this study a number of Fab molecules from a human Fab γ1/κ library, reactive against the neutralizing peptide epitope ELDKWA within the gp41 of HIV-1, were selected using phage display technology. For the selection, equimolar concentrations of three overlapping synthetic peptides covering a conserved HIV-1 neutralizing epitope, a.a. 653-677 were used. Fab molecule directed to this epitope, have previously shown to be important in the fusion of HIV-1 to host cells membrane. Peptide binding specificity of the isolated clones, were tested in an inhibition assays, where the gp160 binding of Fabs in the presence of the three mentioned peptides were blocked. All selected clones were inhibited specifically by p251, which contained 15 amino acid located in the C-terminal of the selected domain. Same result was obtained, when the serum from bone marrow donor (at the same time point of donation) was used in another inhibition assay. The inhibition pattern of the peptide specific Fab molecules reflected the peptide reactivity of the donor serum. Such a data previously have been reported, in which the close correspondence between the donor and isolated antibodies from combinatorial libraries have been seen (179 and 180).

DNA sequencing of the selected clones showed that the selected Fabs all had distinctly different clonal origins of their heavy chains and they all were specific for a particular peptide, and thus possibly for a single epitope. Their molecular differences combined with different biofunctional properties may provide tools and bases for further elucidation of the process of virus neutralization by this region of gp41.

5 of the 7-peptide specific clones expressed in E.coli were neutralized HIV-1LAI. The Fab clones were also assayed for neutralization of five primary viruses isolated of Swedish donors. 4 of 5 isolates were neutralized in vitro by one of the clones; other Fab clones neutralized 2-3 isolates each (table 1).
Tabble 1. In vitro neutralization data (p24 assay) for seven of the peptide specific Fab clones and two non peptide specific (*) ones. For positive reactions, IC$_{50}$ is given in µg/ml; >2 µg/ml was considered negative. nt = not tested. Primary isolates begin with "A", the infectious dose used in the assay is indicated below the designation of the isolate.

The genes coding for several of the clones was transferred to eukaryotic vectors and were expressed in mammalian and Drosophila S2 cells.

COS and CHO cells were used for transient and stable expression of Fab antibodies, respectively. Two of the anti-HIV gp41 clones were expressed as IgG1 in S2 cells at high level. The secreted antibodies were purified and tested against primary HIV-1 isolate by virus neutralization assay in vitro. Improved neutralization capacities of clones were found when Fab/ IgG were expressed in COS, CHO and Drosophila S2 cells respectively compared to the same clones expressed in E.coli. Neutralization of HIV-1 IIIB isolate by IgG1 of clone 101 and 108 expressed in S2 cells is shown in figure 21.

![summary of HIV-1 IIIB NT activity](image)

**Figure 21.** Virus neutralization by IgG expressed in S2 cells. Neutralization of HIV-1 IIIB isolate by IgG1 of clone 101 and 108.
The ability of antibodies to bind the gp41 \textit{in vitro} was evaluated by immunoprecipitation studies. Cell lysate containing HIV-1 envelope glycoprotein was precipitated with purified clone 101 and 108 anti gp41 antibodies expressed in S2 cells. The antigen/antibody complex was isolated from solution using protein G- magnetic beads and subsequently was loaded on a gel prior to Western blot analysis. As expected, the antibodies precipitated gp41 in cell lysate, which confirmed the specificity of these antibodies to the gp41 (figure 22).

In conclusion, many HIV-1-infected individuals initially do not develop neutralizing antibodies against HIV-1. Only a handful of broadly neutralizing antibodies against envelope glycoproteins have been identified. In addition and because of viral diversity, it has been difficult to induce neutralizing antibody responses against diverse primary HIV type 1 strains.

The identification of broadly cross-reactive HIV-1 neutralizing mAbs indicates that such antibodies could play an even more important role in future than it says today.

Taking advantage of recent recombinant DNA- and phage display technologies isolation and engineering of antigen specific antibodies against any antigen has been remarkably facilitated (181 and 182). Based on these technologies, different research groups have successfully isolated neutralizing mAbs and antibodies in different formats such as Fab scFv or whole IgG molecule have been constructed (2, 7 and 9). The ability of none lymphatic eukaryotic cells, such as tumor cells or \textit{in vitro} manipulated cells in expressing high levels of competent antibodies have facilitated analysis of neutralizing mAbs antibodies in vitro. Synergistic or additive neutralization of HIV-1 by such mAbs
have been shown by different research groups (183-185).

In most of the cases the monoclonal antibodies have been tested on substantial neutralizing activity against HIV-1 primary or laboratory isolates with high binding affinity. In several studies anti HIV-1 mAbs have protected against virus challenge with laboratory isolates, when they have been used for passive immunization of laboratory animals (186 and 187).

In conclusion, human antibodies isolated as Fab fragments could be expressed in eukaryotic cells as Fab or IgG. Several of our clones were improved in their antiviral activities by the expression in mammalian or insect cells compared to protein of the same clone produced in E. coli. The anti-HIV1 antibodies had promising in vitro HIV-1 neutralizing data and could be further explored as molecular tools for understanding the detailed mechanisms of virus neutralization via the epitopes involved. This is of considerable interest for vaccine design, and may hold promise of the development of novel therapeutic modalities for HIV-1 prophylaxis or after accidental exposure to the virus. Thus, our studies may serve as a basis for future trials involving the use of mAbs in preventing of HIV-1 infection through passive immunotherapy, and possibly in the development of new strategies in inhibition of virus entry into cells including vaccine design.
Concluding remarks

The development of the techniques to produce monoclonal antibodies has revolutionized the approach to study and understand the pattern of infection during the course of the HIV-1 infection as well as other viral diseases. *In vitro* expression of the antibodies have played important role in these studies not only by providing sufficient amount of antibodies or even viral proteins but also in designing of the effective anti HIV-1 vaccine.

In this study we have utilized mAbs against HIV-1 glycoproteins gp120 and gp41 as a tool to investigate the competency of in vitro produced antibodies. Bacterial E.coli as well as eukaryotic cells has been used as host cells.

The quality and quantity of *in vitro* produced protein depends of many factors which should be optimized as a practical way of making the expression more efficient. Here in we discuss the most important factors related to our experiments.

One of the most important factors which influence the expression level of the transgene is the vector used to transfer the gene of interest. The expression levels of the introduced gene depend mostly on the strength of transcriptional regulatory elements such as promoter (188). There are several highly efficient viral promoters which can be used for transgene expression in bacteria or eukaryotic cells. Generally only a few are considered as ubiquitously strong elements and widely used thus far. Cytomegalovirus (CMV) immediate early promoter has been found to be one of the strongest viral promoters (189). This promoter has been widely used in various vectors for high expression level of proteins in mammalian cells. In our study the CMV promoter was used in vectors adapted for regulation of transgene expression in different mammalian cell lines.

However, the choice of promoter depends on the circumstances and to what purpose the expressed protein is used. For example SV40 promoter has been used rather than the CMV promoter to drive transiently expression of antigens that induce cell death upon overexpression of transgene. The SV40 viral promoter is suitable for physiologic control for reporter assays compared to CMV, but for gene expression where high expression level of a gene of interest is required; the CMV promoter is more usable (190).

Another important element in a vector is the poly A sequence which is needed for transcriptional termination and is known to vary between the different genes in vivo. The process of transcriptional termination seems to influences the rate of expression (191). The SV40 PolyA sequences are used in most of vectors, as well as in vectors in our experiments.

Bacterial gene regulatory elements have been used to construct vectors suitable for transgene expression in prokaryotic cells.

The sequence and structural biology of the gene to be expressed is also considered important. Post transcriptional modification like glycosylation play important role in folding and with that the biological function of the proteins such as full length IgG molecule. Of this reason they can not be expressed in prokaryotic cells, which do not have the glycosylation machinery. In addition, all eukaryotic cells are not either suitable for expression of transgene. Most mammalian cell types demand special conditions for
growth, such as special nutrition or even presence of other cells. Usually using such cells for a purpose like experimental expression of proteins is either impossible or very expensive. Generally, cell types with high growth rate and no need of special treatment are preferable for such transgene expression in vitro. Some cancer cell line like HeLa (cervical epithelial cancer cell)-, mouse myeloma- as well as COS-, CHO- and S2 -cells have been shown to be suitable for in vitro expression of antibodies and other proteins. Of course the grow condition for these cells also should be optimized.

When establishing stable cell lines, the position where the transgene incorporates to the host cell genome is also important in expression of the gene product. To obtain the best result it’s necessary to isolate cells with high level expressing by clonal selection.

Further more the methods which are used to introduce or transfer the transgene to host cell play important roll in transfection efficacy. Though plasmid vectors can be transferred into the cell by physical or chemical methods, e.g. electroporation (192), particle bombardment (193), lipofection (194), viral vectors and can even be injected directly into the cell. Some methods can have relatively higher transfection efficiency than others but also can have toxicity which can result in cell death and loss of transfected cells. One simple and practical solution to such problems is to optimise the appropriate transfection method for each cell line.

As described in the present thesis, we have used several antibody clones against gp120 and gp41, selected from a phage display library derived from a HIV-1 infected individual. These antibodies were expressed in scFv, Fab or IgG formats and in a variety of cells such as E. coli, COS-, HeLa-, CHO- and S2- cell lines. The quantity of these antibodies were analysed by different methods like ELISA and western blotting. The potential of these antibodies to bind the cognate antigen were also analyzed by immunoflourescence staining, immunoprecipitation and virus neutralization assays. To reach best result of both high -expression level and -binding specifity, each moment of the experiments demanded carefully optimization of several components, such as i) the quality and quantity of the plasmid DNA baring the antibody gene needed for transfection of each cell line, ii) the transformation/ transfection strategy suitable for bacteria or cell lines, and iii) the quality and optimal concentration of antibody fragments for different assays, etc.

Long-term stability of plasmid DNA in transfected cells was important for continuously production of mAbs antibodies in our study. Still, most of our stably transfected cells were not able to keep the transgene expression for longer than 30 passages. This could partly depend on methylation of the promoter (such as CMV) regulating the expression of the transgene. Methylation has shown to silence the promoter or decrease the level of expression (195). In general, cell lines dividing at a high rate such as cancer cells have a relatively unstable genome, which also may give rise to loss of the transgene during each replication event. However, the mechanism behind reduction of expression in our cell lines has not been studied yet.

Undoubtedly, there are more methods which can be used to show the interaction between antibodies and antigen, but the methods we have used here to show such an interaction have been promising and sensitive enough, but could still benefit from additional optimization.
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