Antibody Engineering as Opportunity for Selection and Optimization of Anti- HIV Therapeutic Agents

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Abstract: Since among patients who receive care for HIV-1 infection, the prevalence of antiretroviral drug resistance is 50%, clearly, new approaches to control viral replication are needed. A hundred years later the first proposal of a magic bullet by Paul Ehrlich antibody –based therapeutics currently enjoys unprecedented success. The methodologies developed in these years such as antibody library, phage display, single domain antibody and the possibility to modify the already well-studied antibodies in a way to render them more suitable for the specific purpose, make antibody a different and valid alternative therapy. Four human\humanized antibodies for anti HIV treatment are currently in clinical trial with some promising results. They target different antigens: the gp120 of the viral coat and the receptor or co-receptor of the virus. The development of therapeutic antibody in HIV field is progressing slowly but today, the improvement in the knowledge of antibody structure and function together with advances in protein engineering can be used to reshape antibody molecules and devise strategies to obtain more suitable antibody for therapy.

Keywords: HIV, antibody engineering, phage display.

1. INTRODUCTION

1.1. Clinical Need of New Therapeutic Approaches

Human Immunodeficiency virus type 1 (HIV-1) is the causal agent of the acquired immunodeficiency syndrome (AIDS) and currently, approximately 40 million individuals worldwide are infected with HIV. The introduction of highly active antiretroviral therapy (HAART), has greatly improved the quality of life of many infected individuals and mortality for AIDS have dropped dramatically. However, currently available antiretroviral drugs have three major shortfalls: 1) the drugs have relatively high toxicities that cause undesirable side effects, including myocardial infarction; 2) the drug activity of the Reverse Transcriptase inhibitors is cell dependent, since they are active post-infection; and 3) HIV has a high mutation rate leading to the rapid development of drug-resistant viral variants.

For these reasons many patients treated with HAART regimens still fail to achieve or maintain optimal control of the infection and failure of the currently available antiretroviral agents leads to the development of HIV-1 drug resistance. The prevalence of drug-resistance HIV-1 among newly infected persons increased from 3.4% during the period 1995-1998 to 12.4% during the period 1999-2000. Among patients who receive care for HIV-1 infection, the prevalence of antiretroviral drug resistance is 50% [1-6]. Clearly, new approaches to control viral replication are needed.

Drugs that target the interactions between the HIV envelope and the cellular receptor complex are a `new entry' into the scenario of HIV therapy, and have recently raised great interest because of their activity against multi-drug resistant viruses [7].

1.2. Mechanism of HIV Entry Into Host Cell

The first step in virus entry is binding of the viral envelope to its primary receptor, CD4, on the surface of macrophages or T-helper lymphocytes. Binding to CD4 is mediated by gp120, the surface subunit of the envelope. In its native form, the envelope glycoprotein (Env) is a heteromultimer composed of three gp120 molecules and three gp41 molecules, which remain attached through noncovalent interactions. Conformational changes in gp120 triggered by CD4 binding expose structural elements that engage one of two chemokine receptors (CCR5 and CXCR4). Co-receptor binding allows the hydrophobic N-terminus of the gp41 ectodomain, also named "fusion peptide", to insert into the target cell membrane. The antiparallel association of two helically coiled heptad repeats (HR-1 and HR-2) in the gp41 ectodomain to form a six-helix bundle leads to the close approximation of the cell and virus membranes, resulting in their fusion [8].

A novel class of anti-HIV drugs, the entry inhibitors, is currently in clinical development [9] and progressing toward the market and promises to protect the cells from infection [7]. Learning from Nature, an alternative and powerful way to inhibit HIV entry could be represented by antibody therapy.

2. IMMUNE RESPONSE

2.1. Humoral Immune Response to Infections

The mammalian immune system has evolved to provide protection against exogenous agents, such as viruses, bacteria and toxins, developing barriers, soluble molecules and

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cells able to counteract their effects with an immediate and broad and a delayed and more specific response, respectively named innate and acquired immunity. Here we will focus on the more versatile component of the acquired immunity, i.e. the antibody molecule that has evolved to bind specifically and with high affinity to a wide range of antigens.

Antibodies are considered as natural therapeutic agents for their ability to neutralize toxins and viruses either by binding alone or, more frequently, by triggering other components of the immune system such as the complement system and NK cells or macrophages.

2.2. Humoral Immune Response to HIV

The immune response to HIV-1 infection has been extensively studied in human infected individuals in the attempt to design protective vaccines or to identify the most efficient way to induce the production of anti-HIV neutralizing antibodies. Neutralizing antibodies are defined by their ability to block, or neutralize, the infectivity of virus. Neutralizing antibodies can act by preventing the virus from binding to its receptor or by blocking entry events subsequent to receptor binding. The targets of HIV-1 neutralizing antibodies have been more extensively studied than those of any other lentivirus. As Env mediates HIV entry and is the only viral surface protein exposed to the surrounding environment, it is a major target for neutralizing antibodies and a potent immunogen. Neutralizing antibodies are believed to act, at least in part, by binding to the exposed Env surface and obstructing the initial interaction between a trimeric array of gp120 molecules on the virion surface and receptor molecules on the target cell [10-12].

Env-specific antibodies are generated as early as a few weeks after productive infection or immunization. Such antibodies are isolate-specific and lack broad neutralizing activity. In fact, HIV permanently escapes from neutralizing antibody responses of the host by continuous recombination and mutations, by epitope masking or by gp120 shedding. Therefore neutralizing antibodies responses of the host are chasing behind a rapidly evolving virus and mainly non-neutralizing antibodies are present in the host.

Studies of the isolated neutralizing antibodies found by different groups have unusual long hydrophobic VHCDR3 regions (see 3.1 chapter) [13,14].

3. ANTIBODY THERAPY

3.1. Antibody Structure

Antibodies are divided into five major classes: IgM, IgG, IgA, IgD, and IgE (divided on the basis of differences in the structure of their constant regions), also called isotypes, de-

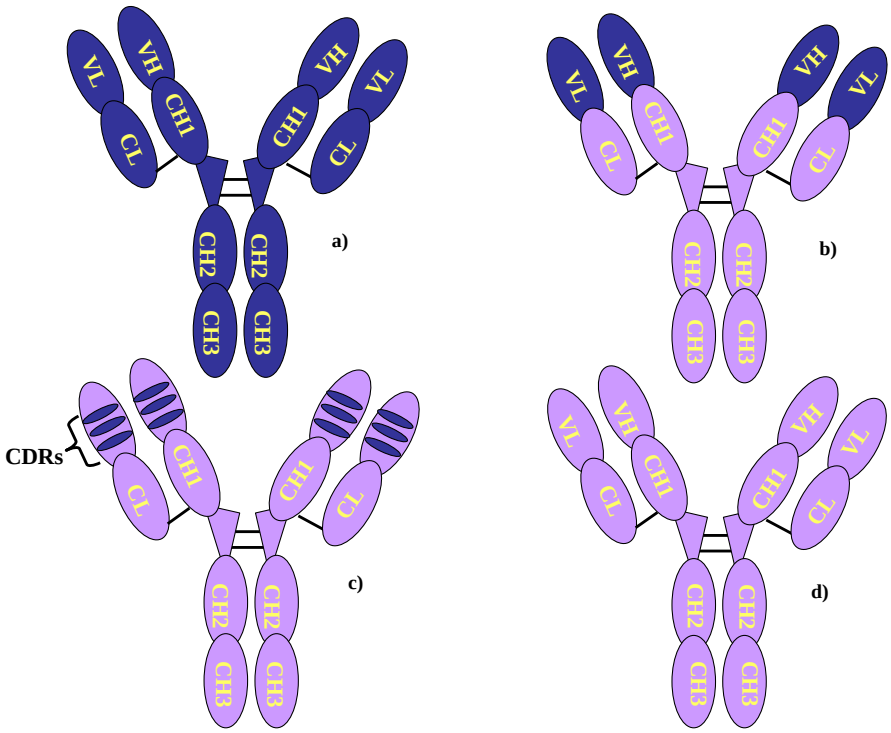

Fig. (1). Antibodies in clinical development and their nomenclature.

Genetic engineering allows the generation of chimeric, humanized and human antibodies starting from mouse monoclonal antibodies and conventions has been established to provide a format for naming of mAbs. Panel **a**): Mouse antibody (….momab), fully mouse antibody generated by hybridoma technology (all domains blue); panel **b**): Chimeric antibody (….ximab), that contains antigen-binding variable regions from the immunized animal (blue domains) grafted onto human constant regions (violet domains); panel **c**): Humanized antibody (….zumab) contains only the mouse CDRs (blue) of the original immunoglobulin grafted onto an extensive human antibody framework and all human constant regions (violet); panel **d**): Human antibody (….umab), completely human antibody generated by phage display technology or from transgenic animals (all domains violet).

pending of which heavy chain they contain (α, β, ε, γ, μ); there are also two classes, or isotypes, of light chains called κ and λ which are distinguished by their carboxy-terminal costant regions.

The model structure of the immunoglobulin, having as prototypic molecule IgG (see Fig. **1a**), is a tetrameric molecule of ~ 150 kDa with a symmetric structure composed of two identical heavy (H) chains and two identical light (L) chains joined by disulphide bonds to form a "Y" shaped molecule. The length of each heavy chain is about 440 amino acid with a molecular weight of 55 kDa, instead the two light polypeptidic chains are 220 amino acid in length and weight 24 kDa.

The heavy chains contain a variable (V) domain, so named because it contains the regions of variability in amino acid sequence, called VH domain and three constant (C) domains numbered sequentially from amino terminus to carboxyl terminus (CH1, CH2 and CH3 domains); the light chains contain a V domain, called VL domain, and a single C domain, called CL domain.

Each variable domain shows three hypervariable regions in sequence called complementarity determining regions (CDRs, highlighted in Fig. **1c**). They differ in length and sequence among the different antibodies and are mainly responsible for the specificity (recognition) and affinity (binding strength) of the antibodies to the antigen. These regions called CDR1, CDR2 and CDR3 are spaced by less variable regions called framework regions (FR: FR1, FR2, FR3, FR4), which support CDR regions. The tridimensional structure indicates that the CDRs are displayed on the antibody surface creating a pocket to receive the antigen.

Pivotal proteolysis experiments have demonstrated that the antigen recognition functions and the effectors domains of the antibodies are spatially segregated. The amino-terminal variable regions of both heavy and light chains participates in antigen recognition, in particular the third hypervariable region (CDR3) has the most extensive contact with bound antigen, instead the C region of the heavy chain mediates effectors functions.

3.2. Antibody Fragments

The modular domain architecture of immunoglobulins has been exploited by protein engineering to create a wide variety of functional antigen-binding fragments who differ in molecular-weight (from 12 to 150 kDa) and in valency (n), from monomeric (n=1) to tetrameric (n=4) and possibly higher. The Fab format (fragment antigen binding), achievable either by proteolytic cleavage or protein engineering, contains two polypeptidic chains composed of two domains, VH + CH1 and VL + CL with an interchain disulfide bond at the terminal of each constant domain (molecular weight around 60 kDa). At present the building block that is most frequently used to create novel antibody formats is the single-chain variable (V)-domain (scFv) consisting of a single polypeptide (30 kDa) formed by the variable regions of the heavy and light chains joined by a peptide linker of up to ~15 amino acid residues. In alternative the variable heavy and light domains can be assembled as Fv, without a linker peptide, exploiting their natural hydrophobic interaction. Examples of possible antibody formats are showed in Fig. (**2**).

3.3. Evolution of Antibody Therapy

Antibody therapy was first proposed by Paul Ehrlich in 1900 as a magic bullet, but it takes more than 100 years (1997) before the first antibody had been approved by Food and Drug Administration (FDA) for human use. At present, more than 20 antibodies are FDA approved and hundreds of new antibodies and antibody-derived reagents are in clinical trials.

We can recognize at least 4 biotechnological steps that make these results possible: Hybridoma Technology, Polymerase Chain Reaction (PCR) discovery, Transgenic Mice generation and Phage Display Antibody Library.

In 1975 Köhler and Milstein invented the hybridoma technology, that allows for the production of large amounts of pure antibodies with a predetermined specificity [15,16], and for this invention they received the Nobel Prize in 1985. Mouse hybridomas, generated from the stable fusion of immortalized myeloma cells with B cells from immunized mice, can grow indefinitely in culture and produce antibodies with predetermined specificity called monoclonal antibodies (mAbs). The somatic fusion technique to produce mAbs *in vitro* gives rise to a plethora of utilizations in different fields. Monoclonal antibodies can be used as tools in research laboratory and from diagnostic to therapeutic reagents in clinic. The discovery of mAbs represents a milestone in the history of medicine.

However initial clinical trials with these reagents have indicated some disappointing results: first, the need of large amounts of mouse antibody because of the relative short half-life (1-2 days) of these antibodies in circulation compared to human immunoglobulins (2-3 weeks); second, induction of a human anti-mouse immune response (HAMA) that cause in patients an enhanced clearance of the injected antibodies from the serum, allergic reactions and formation of immune complexes in circulation blocking their therapeutic effect. A single administration of a mouse mAb induces a HAMA response in about 50% of patients, and repeated administrations lead to a HAMA response in more than 90% of patients.

In 1983 the second biotechnologic advance was obtained by Kary Mullis who discovered Polymerase Chain Reaction (PCR) a technique that was able to revolutionize molecular biology and whose discovery was recognized by the Noble Price in 1993.

The modular arrangement of immunoglobulin domains associated with PCR technique facilitated the engineering of antibodies. The gene segments encoding the domains of interest are isolated from the mRNA of a culture of hybridoma cells, amplified by using PCR and cloned into expression vectors which contain genes encoding the human constant domains. In this way, by transplanting the domains of interest of a mouse antibody into a human antibody backbone, it was possible to build chimeric and humanized antibodies.

The development of genetic engineering has been central to the clinical use of antibodies. This technology has allowed the conversion of existing mouse mAbs into mouse-human chimeric antibodies greatly refining and expanding the therapeutic potential of the modality of treatment. Chimeric antibodies (Fig. **1b**) are obtained by joining the antigen-

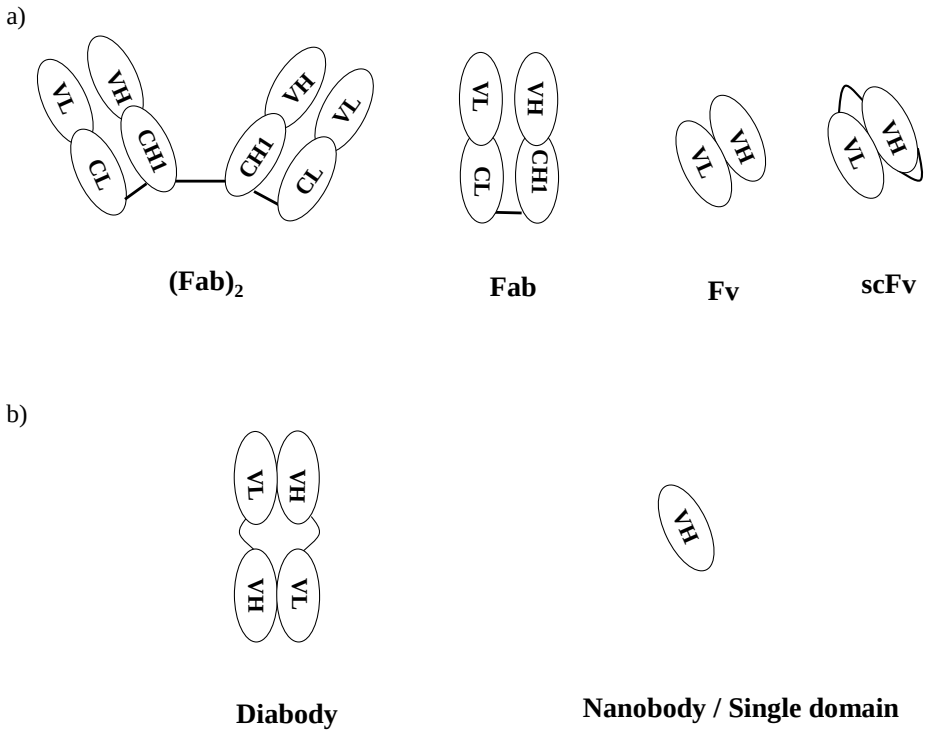

Fig. (2). Schematic representation of antibody formats.

The scheme shows a variety of antibody fragments generated by enzymatic cleavage\antibody engineering (Fab and Fab_2) and by molecular biology techniques (Fab, scFv, Fv, diabody and nanobody). Since the depicted antibody fragments could be generated by antibody of any species, the domains are not color field.

binding variable domains of a mouse mAb to human constant domains: mouse VL to human CL and mouse VH to human CH1-CH2-CH3 for light and heavy chain, respectively [17,18].

Humanized antibodies (Fig. **1c**) are created by grafting the antigen-binding loops, (CDRs), from a mouse mAb into a human IgG. The generation of high-affinity humanized antibodies generally requires the transfer of one or more additional residues from the FRs of the mouse parental mAb. Several variants of the humanization technology have been developed [19].

These genetically engineered construct showed large and overlapping variability in their rate of clearance from the circulation in humans, beta half-lives ranging from 4 to 15 days for chimeric antibodies and from 3 to 24 days for humanized antibodies.

Chimeric and humanized mAbs used in human clinical trials indicated a variable degree of immunogenicity: chimeric mAbs in general show better pharmacokinetics and less immunogenicity than mouse mAbs but patients eventually tend to develop levels of HAMA comparable with those observed with mouse mAbs, indicating that the immunogenicity of chimeric mAbs can vary greatly in a way not yet predictable and can be directed against the mouse V region with characteristic of an anti-idiotypic response. At present, no antibody response has been detected against the few CDR-grafted antibodies entered in the clinics; however, it should be taken in consideration that patients entered in those clinical trials had impaired immune responsiveness. The ideal reagent for human therapy could be a completely human antibody (Fig. **1d**).

4. HUMAN ANTIBODIES FOR THERAPY

4.1. Human Antibodies Generation

Human antibodies have been generated by a combined polyethylene glycol/electrofusion method. Using this method have been generated the human neutralizing antibodies 2G12 (directed against gp120) and 4E10 and 2F5 (directed against gp41) [20]. A cocktail of this antibodies has been used on a small number of patients showing in some cases a delay of HIV rebound [21]. Still there are considerable difficulties in making stable and high producing human hybridomas secreting human mAbs of the required specificity, but recent technologic advances [22a] allowed the production of new monoclonal antibodies [22b]. Moreover, alternative approaches based on transgenic mice or in phage display were developed.

4.2. Human Antibodies from Transgenic Mice

Thanks to the increasing ability in manipulating the mouse genome and in cloning large sized DNA fragments, it became feasible to exploit the natural strategies of the immune system, i.e. the natural recombination and the affinity maturation processes, recapitulating in mice the human antibody repertoire [23]. The creation of transgenic mice expressing human immunoglobulin genes required two major genetic manipulations, the inactivation of the mouse antibody production machinery and the introduction of human immunoglobulin loci in their germline configuration. This

complex process required the separated generation of mouse strains with inactivated mouse immunoglobulin genes or newly introduced human immunoglobulin loci and their successive crossbreeding. At the end of the process, it was possible to obtain mouse strains with a large and diverse V gene repertoire that, in a full immunocompetent context and upon immunization, are able to produce high affinity human IgGk and IgGλ antibodies. Furthermore, the hybridoma technology, well-established in mice, can be easy applied to the B cells obtained from these animals enabling to efficiently and rapidly select monoclonal antibodies. However, still we can envisage some limitations to the full exploitation of this strategy of human antibody generation and among them we can cite: the incomplete representation of the human antibody repertoire; the need of an immunization process; the difference in glycosylation between mice and humans (see chapter 5.2 for the role of antibody glycosylation and glycoengineering) [24-27].

4.3. Human Antibodies by Phage Display

A new way of making fully human antibodies has been demonstrated in 1990 by John Mc Cafferty, *et al*. using phage display technology [28]. Bacteriophages, or simply phages, are members of viruses of Ff group (strain fd, f1 and M13) that infect male Gram-negative bacteria cells, such as *Escherichia coli*, using pilus as receptor, inducing a state in which the infected bacteria produce phage particles without undergoing lysis.

The structure of the virions consists of an outer protein capsid enclosing a single-stranded DNA molecule. For display technology the two viral proteins of the envelope used are: pVIII protein, expressed by the gene-8 (major coat protein) in 2700 copies and generally used for peptide display; pIII protein, necessary for host cell recognition and infection, expressed by the gene-3 (minor coat protein) in 3 to 5 copies and generally used for display of antibody fragments.

Antibody phage display technology consists in the selection of antibody fragments from combinatorial libraries displayed on the surface of filamentous phage. The principle is to generate antibody by cloning immunoglobulin variable genes using recombinant DNA technology into phage genome (Fig. **3**).

This methodology is becoming an important tool in biotechnology for the generation of diagnostic and therapeutic molecules and for the study of natural immune responses [29].

This technique allows to display on the surface of bacteriophages the antibody fragment of interest in a scFv or Fab fragment format. Antibody phage-display libraries can overcome all the problems associated with conventional hybridoma technology along with immunization methods for obtaining mAbs and, importantly, can be derived from the human antibody repertoire; in this way it is possible to generate human antibodies from diverse human antibody libraries against a variety of antigens, including those that are difficult to raise by immunization, for example against self-antigens or protein of the lumen of the endoplasmic reticulum [30].

The immunoglobulin repertoire (antibody library) can be derived from the antibody genes expressed in a variety of B cells (PBL, Bone marrow or spleen). Large libraries of Fab fragments or ScFv, used for the selection of binders to a desired target can be constructed both from non-immunized than immunized individuals. “Immunized” phage libraries can be assembled from the V genes expressed by the B cells of an animal or a patient known to have mounted a particular immune response as a result of immunization or exposure to an infectious agent. Each source of repertoire has advantages and disadvantages; thus, the choice must be done considering the target and the future use of the reagent. In particular the few HIV broadly neutralizing monoclonal antibodies available today (see also Table **1**) have all been isolated from B cells of infected individuals. The B cells repertoire in the lymphoid organs of these individuals is enriched for antigen-specific B and plasma cells that have undergone clonal expansion and antigen-affinity maturation, thus increasing the likelihood of selecting high-affinity antibody fragment even when the library size is not so large (i.e. 10^7 clones) but with an affinity in the range of 10^6 M^{-1} to 10^8 M^{-1} (typical of the secondary immune response).

Libraries can also be constructed from the V region expressed by the B cells from “non immunized” individuals in the attempt to recruit all the diversity generated by the natural immune system. Importantly, it should be noted that the B cells from immunized or infected individuals, have been recruited, through positive and negative selection forces, in primary and secondary lymphoid organs. The net effect of these processes is a circulating B-cell repertoire that is less diverse than potentially attainable, based on rearrangement of Ig-gene segments, N-region insertions and somatic hypermutation. This *in vivo* antibody–repertoire selection is mirrored in the eventual “naïve” phage library (Fig. **3**) [31].

Alternatively repertoires could be derived from germ line antibody genes or created artificially.

The genes for the library are amplified from mRNA using primers (see V BASE directory website: http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html) for the

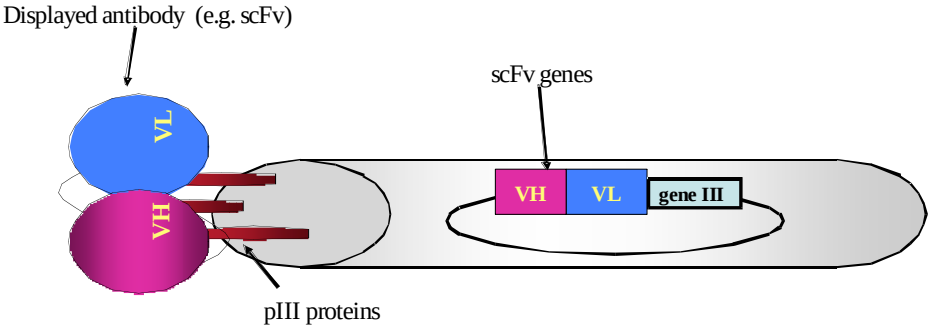

Fig. (3). ScFv expression on the surface of the filamentous phage.

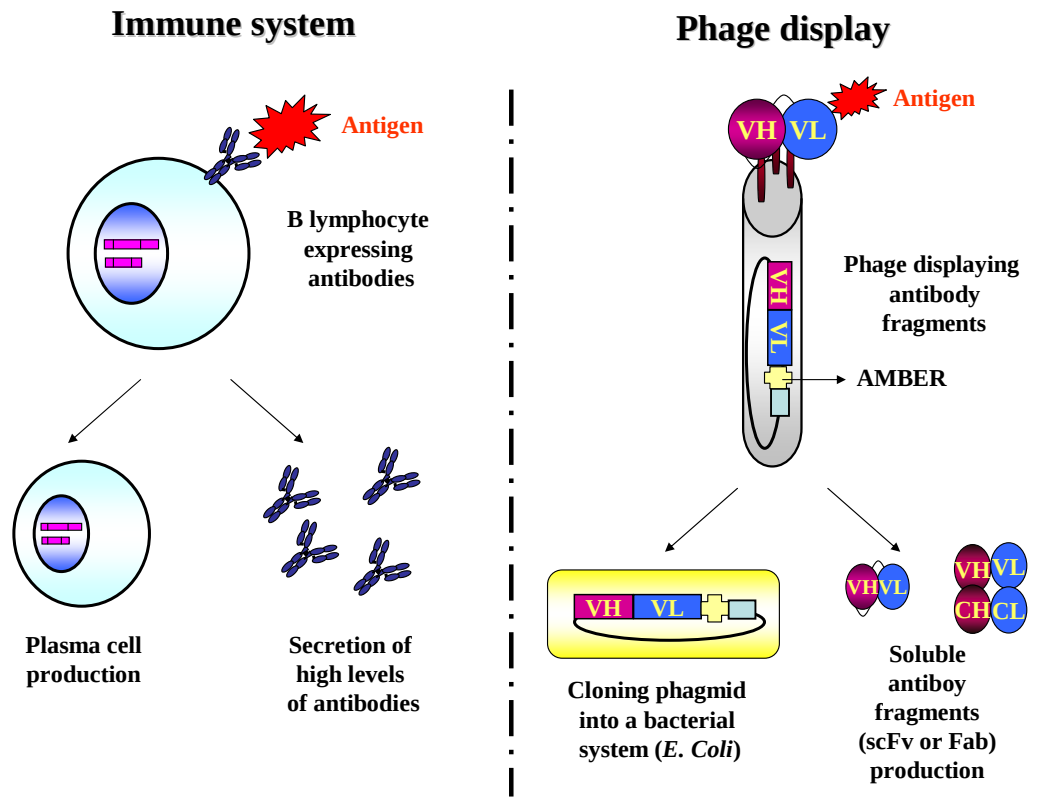

Fig. (4). Mimicking the immune system *in vitro* using phage display technology.

In the immune system (**left panel**) B lymphocyte express antibodies after binding with the antigen and differentiate in plasma cell able to produce high levels of antibodies; in a similar manner, mimicking the strategies of immune system, phages (**right panel**) display antibody fragments selected on binding with the antigen and the replication of phages in *E. coli* permits the production of antibody fragment, as fusion protein on phage surface in non-suppressor strain (e.g. TG1) or in soluble form, when the infection occurs in suppressor bacterial strain, such as HB2151.

conserved regions of the antibody gene family using the polymerase chain reaction and cloning them into expression vectors.

Phagemids, the more popular vectors for display, are plasmids which contain DNA sequences that allow packaging into phage particles, in particular are designed to contain the origins of replications for both the phage and bacterium in addition to gene III, appropriate multiple cloning sites and an antibiotic-resistance gene. Furthermore the inclusion of an amber stop codon between the displayed-protein gene and the phage-gene to which it is fused has been used to provide a vector that doubles for both phage display and protein expression. Phage display technology allows mimicking, *in vitro*, several aspects of the natural antibody response (Fig. **4**).

A crucial advantage of this technology is the linkage of displayed antibody phenotype with its encapsulated genotype which allows the evolution of the selected binders into optimized molecules and permits the rapid determination of the amino acid sequence of the specific binding peptide or protein molecule by DNA sequencing of the specific insert in the phage genome.

One of the most powerful applications of phage display has been the isolation of recombinant antibodies with a unique specificity. In this regard, phage display technology can be used to: (i) generate human mAbs or humanize mouse antibodies, significant for cancer immunotherapy, (ii) isolate human antibodies from patients exposed to certain viral pathogens to better understand the immune response during infection and how protective antibodies are generated, and (iii) elucidate the specificity of autoimmune antibodies [32] or infected patients. In fact, Antibody Phage Library has been built to study the molecular profile of HIV antibody response [33,34]. In these studies the heavy chain CDR3 regions of the antibodies show a remarkably conserved extended length. This data confirm that human antibodies to viral pathogens seem to have longer than average heavy chain CDR3 regions [35,36]. In nature has been demonstrated that camel and shark antibody repertoire has CDR3 with an unusual length that confer to this antibody stability and very good affinity. Theoretically these long hydrophilic CDR3s are important in determining the polyreactive capacity of the molecule.

Antibodies are selected by subjecting the phage library to antigen affinity selection (Fig. **5**). Standard procedure is based on the use of: i) antigens coated directly or indirectly (trough biotin-avidin interaction) on plastic surface (panning) or on different solid matrix (affinity chromatography); ii) antigens which are biotinylated and coupled to streptavidin-coated paramagnetic beads (magnetic selection). Depending on the availability of the antigen, and the type of

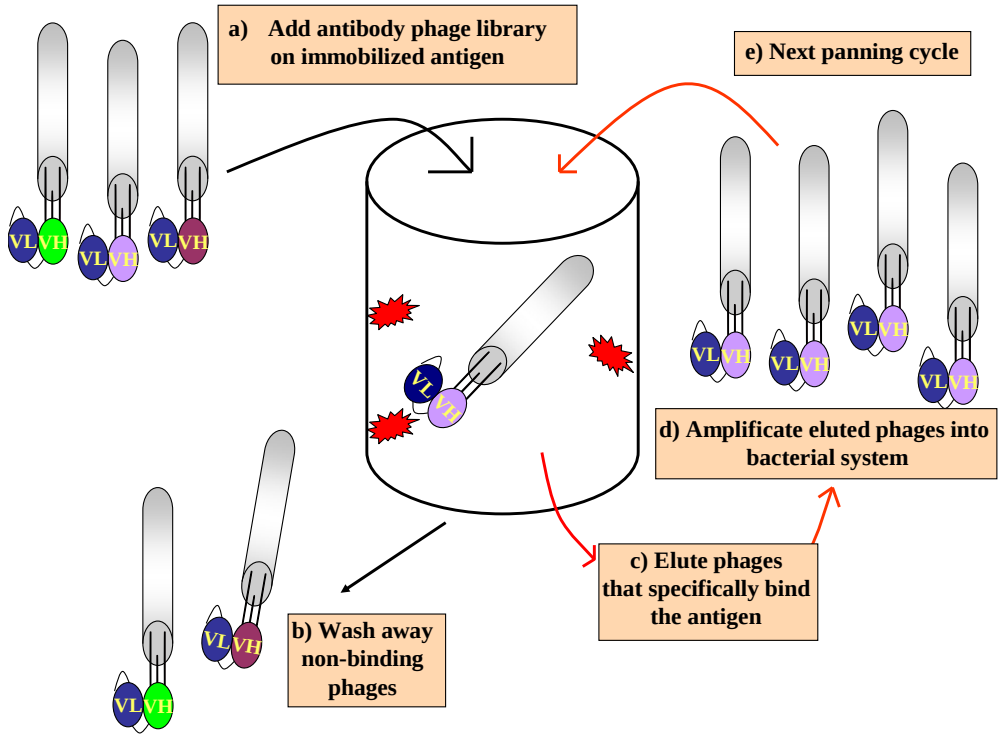

Fig. (5). Schematic representation of antibody selection from phage display libraries.

Selection of phages could be performed using antigens in solution, directly on cells or *in vivo* applying a multi-step procedure: **a**) addition of the initial antibody phage library on the chosen matrix to allow the binding of phages to the target antigen; **b**) removal of nonspecifically bound phages by washing; **c**) elution of specifically bound phages by soluble apten [42], alkali [43] or acid [44]; **d**) amplification of library by infection and propagation of eluted phages in *Escherichia coli;* **e**) new panning cycle of eluted phages starting such as step a). This procedure that allows the enrichment of phage library for members that bind an immobilized target is called *panning*. The first panning round is the most critical since selection for any abnormalities or mistakes at this point will be amplified during further panning.

desired binder, in principle, any target that is suitable for affinity interaction with phage can be used. Thus, selection has also been successfully carried out with whole cells [37-40] and even with live organisms [41].

4.4. Guided Selection

When the physiological status and correct conformation of the relevant cell surface molecule have to be retained, the use of whole live cells as a direct source of target antigen is recommended [37-39,41,45]. However the selection of phage antibodies against unpurified cell surface markers by panning on whole cells has proved very difficult due to the huge number of different antigens with different expression levels. Thus, a general methodology, originally called epitope imprinting selection and now defined as “guided selection” [46-48] was developed involving the use of a mouse template antibody chain (either light or heavy chain) to drive selection of a human antibody with corresponding specificity from a pre-assembled human antibody repertoire. This methodology is derived from the chain shuffling approach, a technique set up to improve the affinity of a "primary" antibody by sequentially replacing the heavy and light chain variable (V) region genes with repertoires of V-genes [49].

By this methodology, the antigen- as well as epitope-specificity of a mAb with desired properties is retained. The procedure has been tailored to specific needs, such as the derivation of an antibody against a predefined epitope on a cell surface antigen for which a mouse mAb is available but the purified antigen is not. The combined use of guided selection and panning on whole cells might be particularly relevant for the target antigens for which a mouse mAb have already demonstrated clinical utility. In addition to the theoretical advantages of the method, it also avoids the need for cumbersome purification of antigens.

Guided selection uses one of the variable chains of an available mouse mAb directed against the target antigen to drive selection of a corresponding-specificity human antibody from a pre-assembled repertoire of genes encoding the variable domains of human antibody heavy and light chains. The different steps of the procedure are described in Fig. (**6**). Starting from a mouse antibody as a template guided selection generates, *de novo*, human antibodies; this contrasts with CDR grafting where residual mouse framework residues and hypervariable loops have to be retained to recreate the antigen binding site. The risk of any potential immunogenicity is therefore minimized using guided selection as there are no mouse residues in the final therapeutic product. The specificity and functionality of the starting mouse mAb is broadly retained in antibodies generated through guided selection; this is due to each mouse template chain providing a framework for the generation of the antigen-binding site with a complementary human chain that can make similar contacts to the antigen [50, 51].

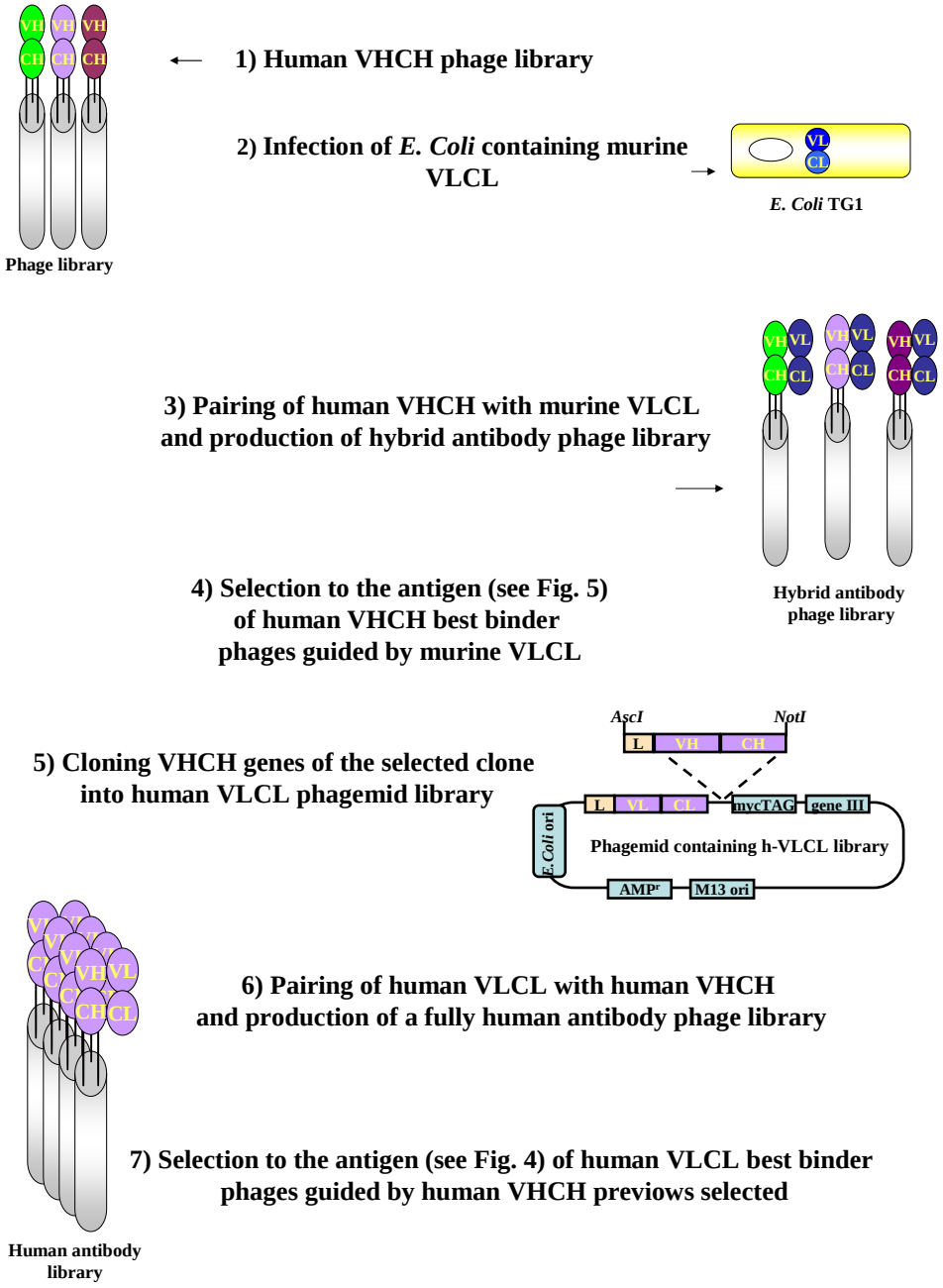

Fig. (6). Guided selection.

The VLCL chain of the starting mouse antibody is cloned into a chosen plasmid using appropriate restriction enzymes, then the vector is used to transform *E. coli* by electroporation. Follows the infection of bacteria using a repertoire of human VHCH library obtained, for example, from human donor peripheral blood lymphocyte mRNA (step **1-2**). The mouse variable light chain, secreted in the periplasm, pairs with a human VHCH chain forming a hybrid human/mouse Fab (step **3**). These hybrid phages allow the binding of the antibody to the same epitopic region on the antigen as the original mouse antibody. The hybrid mouse-human Fab phage repertoire is selected on immobilized antigen (step **4**) as described in Fig. (**5**).

After screening and identification of the best human VHCH follows the second shuffle. The selected VHCH is amplified by PCR and cloned into the phagemid library containing pre-cloned repertoires of human VLCL genes (step **5**). The resulting completely human Fab phagemids obtained by pairing with the repertoire of human VLCL domains (step **6**) are rescued by infection with helper phage and are again selected on antigen (step **7**) as described in Fig. (**5**). The endpoint of this sequential chain shuffling procedure is a set of completely human antibodies, binding with high probability to the same epitope on the antigen as the original mouse antibody. Finally the selected phage antibodies are further characterized (step **8**).

5. OPTIMIZATION OF MAB STRUCTURE

5.1. Protein Engineering

Today, protein engineering can be used to reshape antibody molecules and devise strategies to obtain suitable antibody fragments. The very high cost of conventional antibodies is already becoming a major burden for the health care budgets in many countries; thus, the possibility to use different and less expensive expression system is very attracting. Thanks to advances in molecular biology now it is possible to produce recombinant antibodies using bacterial cells (in particular *Escherichia coli*).

ScFv can be engineered into a diabody format, i.e. a scFv dimer, by using a linker of 5 aminoacids, too short to allow the pairing between chains of the same polypeptide. In the diabody, VH and VL pairing occurs between complementary domains of two different chains creating a stable non covalent bivalent antibody (Fig. **2b**).

The smallest antibody-derived binding structure is the so called single domain (also named nanobody or VHH in camelids and V-NAR in sharks). The idea to engineering antibodies in this format derives from the discovery that certain types of organisms (camelids and cartilaginous fishes) have high affinity single V-like domains mounted on a Fc equivalent domain structure as part of their immune system [52,53].

The V-like domains display long surface loops. Thanks to their loops, V-like domains directed to target antigens which are critical for the viral life cycle, are able to penetrate cavities that have a conformation evolved to escape naturally occurring entire antibodies.

Importantly, the cloned and isolated VHH domain is a perfectly stable polypeptide harboring the full antigen-binding capacity of the original heavy-chain antibody. Isolated VH domains from other mammals can be stabilized by masking hydrophobic surface patches [54]. Noteworthy, the neutralizing anti HIV response and repertoires from HIV infected patients present mainly antibodies with this feature [13,14].

Other antibody manipulations consist in: affinity maturation using different methodologies such as sequential CDR optimization [55] chain shuffling [42,49,56], or random point mutation [57] obtained both by error prone polymerase or a mutator strain; engineering of monovalent structures (Fab, scFv, single domain) in multivalent structures to increase functional affinity (avidity).

If required, scFv or Fab fragments can be grafted onto on Fc [58-60], thus it is possible to reconstitute an entire antibody molecule and chose the isotype more suitable for the desired purpose. Moreover during the last decades the knowledge of antibody structure is improved so much that today it is possible to use antibody engineering to improve or decrease antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) and recycling of the molecule acting on the residues of the antibody molecule that react with the FcγRs and FcRns complex. The engineering of the Fc of a mAb to tailor antibody Fc property is extensively reviewed in [61] and [62].

The protein engineering enables also the choice of the antibody isotype tailored according to the expected effector functions. For example the constant regions of the IgG4 isotype are generally chosen when a poor recruiting of ADCC and CDC is required as in the case of anti-CD4 or CD28 antibodies. On the bases of the identification of IgA neutralizing anti-HIV antibody in protected individuals who are highly sexually exposed to HIV-1 but remain persistently IgG seronegative the use of Mucosal immunoglobulin A (IgA isotype) can be envisaged [63].

5.2. Glycoengineering

The imunoglobulins, in their native state, contain a variable number of glycosylation sites located in their constant regions. The prototype IgG molecule has two asparagine N-linked oligosaccharide chains, whose inner core is constant while the length and composition of the external branching is variable and depends on the species of origin, being that of different mammalian expression systems similar but not identical, the cellular context and the manufacturing conditions [62,64].

Although the IgG glycans represent only an average of 3% of the total mass of the molecule, the presence or absence of fucose and sialic acid moieties in the oligosaccharide chains was found to greatly affect antibody interaction with immune effector cells, dramatically changing the activation of ADCC.

Glycoengineering of antibody producing host cells to express mAbs with enhanced effector functions has been therefore attempted with success. Chinese hamster ovary (CHO) cells manipulated to knockout a fucosyltransferase or other enzymes involved in IgG fucosylation resulted in an ADCC many-fold higher [65,66], and glycoengineering of the yeast *Pichia pastoris* allowed the production of recombinant human antibodies with glycan composition optimized for antibody-mediated effector functions [67].

6. ANTI-HIV ANTIBODIES CURRENTLY IN CLINICAL TRIAL

The major characteristics of the antibodies already entered in clinical trials as HIV entry inhibitors are summarized in Table **1**.

Table 1. Antibodies who have Completed Clinical Trials for HIV

Antibody name	Target Ag	Origin	Isotype	Trial status	Reference
Ibalizumab	CD4	Humanized mAb	IgG4	II	[68,69]
PRO542	gp120	Human fusion protein	IgG2	II	[73,74]
PRO140	CCR5	Humanized mAb	IgG4	I	[77,78]
HGS004	CCR5	Human mAb	IgG4	I	[77]

The monoclonal antibody Ibalizumab, formerly named TNX-355 and Hu5A8, is a humanized IgG4 mAb that binds to the second (C2) domain of CD4. In contrast to attachment inhibitors, Ibalizumab does not prevent gp120 binding to CD4, but is thought to decrease the flexibility of CD4, thereby hindering the access of the CD4-gp120 complex to the co-receptors CCR5 and CXCR4. This mAb is a potent inhibitor of HIV-1 *in vitro*, and shows synergy when combined with other anti-HIV drugs or the fusion inhibitor enfuvirtide [68,69]. Ibalizumab does not appear to interfere with immunological functions that involve antigen presentation [70,71]. Phase 1 studies of Ibalizumab showed promising activity, with up to a 1.5-log10 reduction in plasma HIV-1 RNA levels 14–21 days after a single dose [9], but resistance emerged after administration for 9 weeks [72]. A phase 2 study of Ibalizumab was successfully completed in 2009 and showed that this mAb at an optimized background regimen resulted in significantly greater reductions in plasma HIV-1 RNA compared with the background regimen alone.

The PRO542 is a tetravalent CD4-immunoglobulin fusion protein that contains the D1 and D2 domains of human CD4 fused to the heavy and light chain constant regions of human IgG2,k [73,74] that binds to gp120. Viral load reduction was initially observed in adults with HAART failure and in antiretroviral-naïve children [74,75] but modest reductions in plasma HIV-1 RNA levels were recorded in a phase 1–2 trial in patients with advanced HIV disease [76]. No additional studies of the antibody are ongoing at this time (www.clinicaltrials.gov).

The monoclonal antibody PRO140 is a humanized IgG4 mAb targeted against the CCR5 receptor found on T lymphocytes of the human immune system interfering with HIV's ability to enter the cell. In May 2007 the researcher announced results from the phase I clinical trial of the drug [77,78] showing a dose dependent decrease in viral load in a small number of HIV-infected patients with CCR5-tropic virus.

The monoclonal antibody HGS004 is a fully human IgG4 mAb that specifically binds to the second extracellular loop of CCR5 with robust *in vitro* activity against a diverse panel of CCR5-tropic HIV-1isolates, thereby inhibiting HIV envelope–dependent cell-cell fusion and blocking viral entry. *In vitro* studies demonstrated that HGS004 acts synergistically with all currently approved classes of antiretroviral agents such as zidovudine and lamivudine [77]. The results of a phase 1 clinical study in patients with HIV-1 infection suggests that limited exposure to a wide dose range of HGS004, is safe and well tolerated, although several questions were raised pertaining to the clinical pharmacology of the compound [79].

7. CONCLUDING REMARKS

Genetic engineering can be used to harness and to reformat individual antibody obtained from hybridoma, transgenic mouse or phage selection. Today 22 antibodies are approved by FDA and the application ranges from cancer, inflammation and autoimmune diseases sometimes also with excellent results and more than 200 antibodies are currently in clinical development [80]. All of the approved antibodies are full IgG antibodies, but three which are Fabs. Small fragments may have the advantage to better penetrate the tissue and in the case of HIV to better make contact to the envelope glycoprotein of the virus accessible only by small molecules.

Even if the development of therapeutic antibody in the HIV field is progressing slowly we can anticipate that the improvement in the knowledge of antibody structure and function together with advances in protein engineering will lead to optimization of suitable antibody based reagents for HIV infection control.

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