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Review

Antibody biotechnology

Abdelaziz Benjouad

Laboratory of Biochemistry and Immunology, University Mohammed V-Agdal, Faculty of Sciences and CNRST - Rabat - Morocco. E-mail: benjouad@fsr.ac.ma or benjouad@cnrst.ma

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Antibodies are the support of adaptive humoral immunity and they are characterized by their high diversity, clonality and specificity. The extremely diverse fields of application of monoclonal antibodies (mAbs) have continuously stimulated the development of antibody engineering especially after the discovery hybridoma by Köhler and Milstein (1975). This review summarize the main antibody biotechnology approaches that have lead to the development of murine mAbs, chimeric mAbs, humanized mAbs , combinatorial antibody libraries and entirely human mAbs.

Key words: Antibodies, immunoglobulin, chimeric, human, murine.

INTRODUCTION

Antibodies were identified at the end of 19th century following the work of Pasteur as the support of specific humoral immunity (adaptive). The pioneering work of von Behring (Nobel Prize in 1901), and Kitasato (1883-1885) into the ability of antitoxins to neutralize toxins lead to the extraordinary development of serotherapy in Europe. However, it was Ehrlich (Nobel Prize in 1908) through his prophetic visions, the side chain theory of immunity and the magic bullets who holds the therapeutic potential of antibodies.

Another milestone in the history of antibodies was the work of Porter and Edelman (1959-1969) on the structure of immunoglobulin (Nobel Prize in 1972). During the 1970s, attempts were made to understand the mechanisms of synthesis and the association of the heavy and light chains of immunoglobulins (antibodies) by applying the method of production and selection of cell hybrids that was developed earlier in the beginning of the 1960s by geneticists. It was Köhler and Milstein in 1975 that succeeded in obtaining hybridomas by the fusion of B cells from mice immunized by a predefined antigen with mouse myeloma cells, and selection of clones producing specific antibodies (Köhler and Milstein, 1975). This discovery marked the beginning of the monoclonal antibody era. This major advance in science allowed Köhler and Milstein to win the Nobel Prize in 1984. This Nobel Prize was shared with Jerne for his theory of clonal selection of lymphocytes, which that he had proposed earlier in 1955 with Burnet, and thus corroborated, by the work of Köhler and Milstein. With the development of molecular biology, Tonegawa and Leder groups (1976) solved immunoglobulin gene organization, work that also allowed Tonegawa to win the Nobel Prize in 1987. By showing the existence of several genes (genetic segments) at the locus of immunoglobulins, a major contribution to resolving a central topic in immunology that is the diversity of antibodies, which results to a great extent from somatic recombination between variable genes, was made. This topic has preoccupied immunologists including Ehrlich (side chain theory), Jerne and Burnet (clonal selection theory) and others for a long time. In addition, the demonstration that one protein (immunoglobulin) is encoded by the rearrangement of separate genes has overturned the paradigm: one gene encodes one protein.

The discovery of hybridomas by Köhler and Milstein marked the beginning of the era of monoclonal antibody biotechnology and opened the way for rapid development of therapeutic monoclonal antibodies. However, the use of murine monoclonal antibodies in therapeutic approaches in humans was rapidly shown to be limited by their immunogenicity. In fact, these antibodies induce anti-mouse antibodies in humans, a phenomenon known as "HAMA (Human Anti-Mouse Antibody), which hampered their therapeutic effects (Schroff et al., 1985).

To overcome this major drawback of the immunogenicity of murine antibodies, genetic engineering experiments were undertaken earlier in the 1980s to manipulate monoclonal antibody encoding genes. These studies have lead to the development of chimeric and humanized antibodies (Morrison et al., 1984; Takeda et al., 1985; Co and Queen, 1991; Jones et al., 1986; Queen et al., 1989). More recently, there has been great success in

the development of fully humanized antibodies using transgenic animals (Lonberg et al., 1994; Green et al., 1994) or recombinant libraries built with fragments of human immunoglobulin chains (McCafferty et al., 1990; Clackson et al., 1991; Huse et al., 1989). These combinatorial libraries mimic the diversity of the human antibody repertoire and have the advantage of engendering entirely human antibodies without the need for animal immunization (Hoogenboom and Winter, 1992).

HYBRIDOMA TECHNOLOGY

The success of murine monoclonal antibodies obtained by the hybridoma methodolgy developed by Kohler and Milstein (Köhler and Milstein, 1975) resides in the advantages that these antibodies have compared to polyclonal antibodies. Indeed, monoclonal antibodies are produced by a single lymphocyte clone and are characterized by their specificity in respect of to a given antigen (a monoclonal antibody recognizes a single epitope). In addition the hybridoma technology is easily reproducible and automated, the hybrid cells can be stored for many years in liquid nitrogen and antibodies production is homogeneous. The hybridoma method involves several steps (Galfré et al., 1977; Köhler and Milstein, 1975; Pontecorvo, 1975; Fazekas de St Groth, 1980): immunization of animals, fusion of B cells from immunized animals with myeloma cells, selection, screening and cloning of hybrid cells secreting the desired antibody and the production in large amounts of antibodies from the selected clone. The widely used animal is the mouse (or rat), the antigen can be used either purified or not (cell extracts, membranes and particles etc). After immunization of the animal, splenocytes are isolated and then fused *in vitro* with myeloma cells using polyethylene glycol (PEG). The myeloma cells used for fusion are deficient in one of the key enzymes involved in the biosynthesis of nucleotides, mainly hypoxanthine-guanine phosphoribosyl transferase (HGPRT).

After fusion, cells are grown in a selective medium (HAT: Hypoxanthine Aminopterin Thymidine). The aminopterin blocks the endogenous synthesis of nucleotides in myeloma cells (HGPRT-/-). The hypoxanthine may generate IMP (inosine monophosphate), a precursor for AMP and GMP in purine biosynthesis and thymidine overcoming the block in thymidilate synthetase by aminopterin. Thus, through the complementation, hybrids cells may survive in the selective medium (HAT) and become immortal. Myeloma cells that are HGRPT-/- can-not divide and splenocytes that are primary cells cannot survive for a long time. Fusion allows the obtainment of immortalized cell lines secreting antibody of predefined specificity as described by Milstein and Köhler. The next steps consist in the selection and cloning of cell hybrids secreting an antibody of interest. The large production of antibodies from a selected clone may be achieved by cell culture in a bioreactor or by inducing ascites in mice. The latter practice raises serious ethical problems related to animal testing and it is less defendable.

RECOMBINANT ANTIBODY TECHNOLOGY (CHIMERIC AND HUMANIZED ANTIBODIES)

The first generation of recombinant antibodies was obtained by the fusion of genes encoding variable regions of heavy (VH) and light (VL) chains (which determine the specificity of antibodies) of a murine monoclonal antibody with the invariable regions of a human immunoglobulin. These antibodies that are about 70% of human source are named "chimeric antibodies" (Morrison et al., 1984; Takeda et al., 1985) (Figure 1). The immunogeneicity of these anti-bodies in humans is significantly diminished, and their specificity and affinity are generally not affected compared to the parental murine antibody. The method of preparing chimeric antibodies consists of the isolation of DNA encoding the VH and VL domains of a murine monoclonal antibody and its linkage to DNA encoding the invariable parts of human immunoglobulin chains. The choice of constants regions depends on the isotype looked-for, but the most commonly used are the gamma1 IgG heavy chains and the constant domain of the kappa light chain.

In order to further reduce the immunogenicity of chimeric antibodies, scientists have proceeded to increase the portion of human antibody by taking into account the fine mapping of antigen recognition sites (paratope). Thus, the six complementarity determining regions (CDR) of heavy and light chains of parental murine antibodies have been grafted *in vitro* onto the framework regions of VH and VL of the human Ig and then recombined with the human heavy and light constant regions of human Ig (Co and Queen, 1991; Queen et al., 1989). These antibodies consisting of more than 90% of human sequences are named humanized antibodies (Figure 1).

Yet, humanized antibodies have considerably decreased immunogenicity, and problems of specificity and affinity were observed, especially with the first generation of humanized antibodies (Torres et al., 2005). This was due to the fact that the amino acid residues located at the framework regions were sometimes critical to the interaction of the paratope with the epitope. The mapping of variable regions of mouse antibodies and their comparison (alignment) with their human counterparts has identified critical amino acids that were successfully introduced by directed mutagenesis into the human variables regions. Other changes (site of glycosylation for example) are sometimes needed to improve the properties of the humanized antibody.

Obviously, efforts were pursued to obtain fully human antibodies. To this end, many technologies have been developed and the best known are transgenic animals

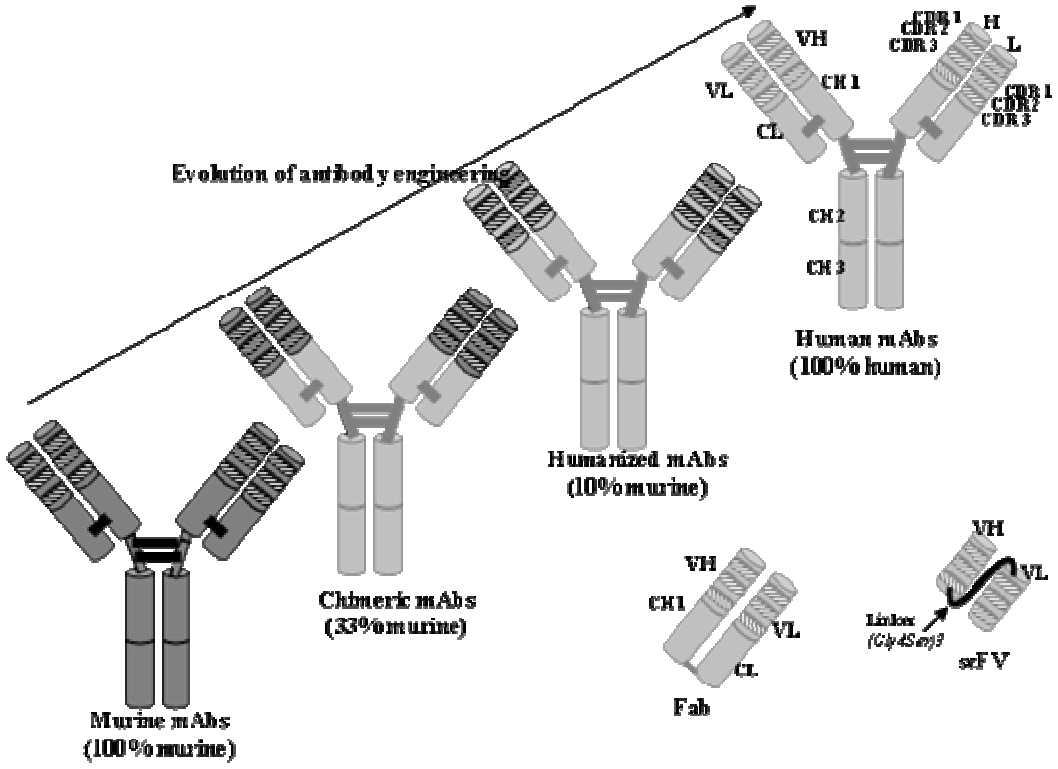

Figure 1. General scheme of antibody engineering. The figure shows a *general structure* of antibodies (immunoglobulins) and the evolution of antibody engineering from murine monoclonal antibodies (mAbs) to fully human mAbs. Also, are presented the structure of chimeric and humanized antibodies and that of scFv (single chain variable fragment) and Fab (antigen binding fragment fragments). H: Heavy chain, L: light chain, VH: variable domain of the heavy chain, VL: variable domain of light chain, CH1, CH2, CH3: constant domains of Heavy chain, CL: constant domain of light chain, CDR: Complementarity determining region.

(Lonberg et al., 1994; Green et al., 1994) and recombinant combinatorial antibody libraries (McCafferty et al., 1990; Clackson et al., 1991; Huse et al., 1989). The later approach has the advantages of generating antibodies without the need for immunization, to surpass immune constraints that are system related to immunization and to mimic the exceptionally large antibody diversity created *in vivo* by the immune system.

HUMAN ANTIBODIES DERIVED FROM ANTIBODY LIBRARIES

Fully human antibodies have been developed by means of an innovative recombinant antibody library technology. Several technology platforms were developed in order to create and to screen human recombinant antibodies libraries, that is phage display (McCafferty et al., 1990; Clackson et al., 1991; Huse et al., 1989; Winter et al., 1994), yeast display (Boder et al., 2000; Boder and Wittrup, 1997) and ribosome display (Lipovsek and Pluckthun, 2004; Hanes et al., 2000).

However, the most popular method is the phage display technology (Winter et al., 1994; Hoogenboom, 2002). This technique consists in the expression of recombinant immunoglobulin fragments on the surface of filamentous phage and their expression and amplification in *Escherichia coli* followed by extensive screening by biopanning.

The most common fragments used in recombinant antibody libraries are scFv (single chain variable fragment) and Fab (antigen binding fragment) (Bird et al., 1988; Ward et al., 1989; McCafferty et al., 1990, Clackson et al., 1991; Huse et al., 1989; Huston et al., 1988; Skerra and Plückthun, 1988) (Figure 1). scFv consists of a link between the VH and VL regions in the same polypeptide and Fab consists of an association of the light chain with VHCH1 domains. To make the scFv or the Fab libraries, mRNA from B cells is reverse transcribed to produce cDNA encoding variable domains by using a set of PCR primers corresponding to variable gene families and fashioned in a manner so as to encode scFv or Fab fragments. The DNAs are introduced into a phage-display vector by fusion to a coat protein gene pIII of the phage. This procedure generates phage-displayed antibody repertoires. Selection of phage pools bearing the anti-

body specificity is made by screening against the requisite immobilized antigen. Usually, several rounds of amplification by infection of host bacteria (*E. coli*) and selection are needed to obtain specific clones.

By means of these technologies, different types of highly diverse libraries (10^7-10^{10}) can be constructed that is immune antibody libraries, natural naïve libraries, synthetic naïve and semi-synthetic libraries.

Immune antibody libraries

These libraries are constructed with VH (VDJ) and VL (VJ) gene pools that are cloned from B cells of immunized human donors. These libraries illustrate the antibody diversity existing in immunized individuals and they are biased against certain antigens (Burton et al., 1991; Clackson et al., 1991; Lee et al., 2006).

Natural naïve libraries built from natural, unimmunized individuals

These antibody libraries are derived by harvesting VH (VDJ) and VL (VJ) genes from naive B cells. These libraries mimic the diversity of the antibodies in non-immunized individuals that is donors that have never encountered the antigen of interest against which antibody fragments should be selected (Vaughan et al., 1996; Sheets et al., 1998; de Haard et al., 1999; Little et al., 1999).

Synthetic naïve and semi-synthetic libraries

Synthetic libraries are built entirely *in vitro* on the basis of structural analysis of the antigen binding sites of antibodies by using designed synthetic oligonucleotides (Knappik et al., 2000; Cobaugh et al., 2008; Fellouse et al., 2004; Persson et al., 2006). The approach consists of introducing defined diversity into the CDR regions. The synthetic CDR repertoires are then incorporated into defined VH and VL framework genes. Semi-synthetic libraries are fashioned by combinations of natural and synthetic diversity and they are designed to increase diversity of natural libraries (Hoet et al., 2005).

Whatever the platform used to develop and to screen antibodies and the type of libraries chosen, the recovery of antibodies with improved binding affinity is still the main objective (Hoogenboom 2005). The efficient binding of these antibodies to their target is of importance for both therapeutic and diagnostic use. To this end, mutagenesis and selection strategies have been used to make and to isolate a leading antibody (Hoogenboom and Winter, 1992; Ho et al., 2005; Wu et al., 1998).

Antibody fragments obtained from antibody libraries have a smaller size, lack the Fc domain and have a very short half-live. These antibody fragments need to be converted to full-length human IgGs (Jostock et al., 2004; Sarantopoulos, 1994) for therapeutic applications as was the case for chimeric and humanized antibodies. Antibody fragments may be tailored for therapeutic purposes; they may be modified to vehicle active molecules such as radio-isotopes, toxins, cytokines, enzyme etc. In these cases, the therapeutic effect is due to molecules shared with the antibody. Also, antibody fragments may be generated as bispecific molecules to recognize two different antigens.

FIELDS OF ANTIBODY APPLICATION

The fields of application of monoclonal antibodies and their derivatives are extremely diverse and the discussion on this aspect is beyond the scope of this review. However, it is important to note that the extraordinary development of antibodies technology was continuously stimulated by the increasing interest in antibodies as therapeutic products for treatment of cancer, pathogenic infections, transplantation, autoimmune disease and others pathologies or as tools for clinical investigation, diagnosis, experimental analysis and research (Waldmann, 2003; Fun, 2007; Orlandi et al., 1989; Pijpers and Belsey, 2006; Ortho Multicenter Transplant Study Group, 1985; Milstein and Cuello, 1983; Faulds et al., 1994).

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