Human Antibodies 18 (2009) 1–10
DOI 10.3233/HAB-2009-0196
IOS Press

Three decades of human monoclonal antibodies: Past, present and future developments

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Abstract. The hybridoma technique has been shown to be a most reproducible method for producing rodent monoclonal antibodies but poor results were obtained when it was used for generating human monoclonal antibodies. For immunotherapy, murine monoclonal antibodies are inadequate, whereas human monoclonal antibodies are virtually indispensable. Cellular, chemical, genetic and molecular methods to generate human monoclonal antibodies have been developed. Most often, the monoclonal antibodies for therapy are selected after deliberate vaccination, according to their high affinity towards an arbitrarily-chosen epitope of a pathogen or cellular antigen and therefore the selection is obviously skewed. A major hindrance of the production of therapeutic human monoclonal antibodies is the lack of an appropriate strategy to define and select the antibodies that would be effective *in vivo*. In contrast to antibodies induced by vaccination, there has been only a marginal interest in monoclonal antibodies which reflect antibodies of the innate immunity. In the future, human monoclonal antibodies that resemble antibodies that are ubiquitously present in sera of healthy individuals might serve as novel therapies in diseases such as Alzheimer's disease, where no other therapy exists.

Keywords: Chimeric antibodies, Epstein-Barr virus, immunotherapy, human, humanized antibodies, hybridoma, molecular engineering, monoclonal antibody, phage display, transgenic mice

1. Introduction

In 1975 Köhler and Milstein succeeded in producing monoclonal antibodies *in vitro* [1]. This dramatic achievement could not have been accomplished without the extensive scientific and technical progress made earlier by many laboratories worldwide. Although the original hybridoma technique has proved to be extremely reproducible, new strategies were introduced to improve the production of monoclonal antibodies in general and of human monoclonal antibodies in particular. Alternative techniques have also been developed to create native and even non-native, newly composed antibodies. Because of the enormous clinical potential initially ascribed to monoclonal antibodies, there have been continuous attempts to construct human therapeutic monoclonal antibodies. Today, the pharmacological industries are intensely involved in these developments. The current approaches to production of human monoclonal antibodies are still inadequate, as attested to by the continuous attempts to improve the techniques. In addition, the methods offered today to test the *in vivo* efficacy and effectiveness of human monoclonal antibodies are not satisfactory. Finally, the fact that today only very few completely-humanized monoclonal antibodies are used in the clinical setting indicates that the field is in its initial phase.

This review presents the main technical developments, starting with the initial hybridoma method and ending with the current approaches for engendering therapeutic human monoclonal antibodies.

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ISSN 1093-2607/09/$17.00

2. Köhler and Milstein's original method (Fig. 1)

According to the classical hybridoma method, mice are immunized with a mixture of antigens, their spleen cells are fused with an immunoglobulin non-secreting, drug-sensitive cell-line and cloned in microwell plates. Supernatants derived from the microwells are analyzed for specific antibodies and cells from positive wells are further grown and cloned. Unlimited amounts of monoclonal antibodies can thus be produced *in vitro*. The method is based on three principles: a) each B lymphocyte produces only one antibody (i.e., an antibody with specific H and L chain-derived variable regions), b) the lymphocytes used for fusion are derived from donors that were sensitized with specific immunogens, and c) B cells can be immortalized into immunoglobulin-secreting, continuously growing *in vitro* cell lines.

Köller and Milstein's ingenious hybridoma technique has been widely used to fulfill both scientific and practical/medical objectives. The incredible success of the method, which guaranteed the production of murine antibodies with seemingly all possible specificities, has caused some of the inherent limitations related to the application of these antibodies in the clinic to be overlooked. First, the antibodies are produced by murine cells and thus differ from proteins made by human cells. Second, the specificity of the monoclonal antibodies, i.e., the molecule epitope recognized by the antibodies and their biological function, resembles those of the rodent immune system. Thus, murine monoclonal antibodies bind only to the molecular epitopes which the mouse identifies as immunogenic. Moreover, the lymphocytes used to make the hybridomas are usually B cells from a mouse immunized by a selected molecule and via a specific route. Hence, the monoclonal antibodies may not resemble those protective antibodies which are induced under natural circumstances. Although generally ignored, these limitations are critical when certain therapeutic requirements are assigned to the antibodies.

A major disadvantage of the murine monoclonal antibodies when used for therapeutic purposes is that they are recognized in the patients as allogeneic proteins. Indeed, the human anti-mouse antibody (HAMA) response following treatment with mouse monoclonal antibodies rapidly inactivates and eliminates the latter.

The hybridoma method, which was so efficiently exploited in rodents, failed when adopted for the production of human monoclonal antibodies. The failure and inadequacy of the method to create human hybridomas were due to some obvious and some unexpected difficulties. First, there was no appropriate drug-resistant myeloma cell line for immortalization of human B cells. Second, antigen-sensitized human splenocytes are not as readily accessible as those of rodents. Third, in contrast to rodents, the availability of antigen-sensitized lymphocytes is extremely limited.

In a recent publication, Adekar et al. [2] presented a modification of the hybridoma method which sought to overcome some of the limitations in making human monoclonal antibodies. They introduced *in vitro* antigen-specific sensitization of human B cells before hybridization with an appropriate myeloma cell partner and successfully produced IgG anti-botulinum neurotoxin antibodies. It is not clear whether the method is reproducible for other antigens too.

3. The Epstein-Barr virus method (Fig. 2)

In the early seventies, Dr. George Klein of the Karolinska Institutet, Stockholm, studied the role played by Epstein-Barr virus (EBV) in Burkitt's lymphoma. At that time EBV was shown to efficiently immortalize *in vitro* human immunoglobulin surface-positive B cells into immortalized lymphoblastoid cell lines (LCL's) [3], provided anti-EBV cytotoxic T cells were first removed or inactivated. The emerging cell lines which express nine virus-induced proteins, preserve the characteristics of the initially virus-infected cell. Indeed, the LCL's secrete and even express cell-surface immunoglobulin. At the end of 1974, I was a post doctoral student with George Klein who put forward the idea that immortalization with EBV of antigen-committed B cells would probably establish cell lines that secrete human antibodies.

Obviously, immortalization of peripheral blood lymphocytes (PBL) of an immunized human donor creates a polyclonal cell culture. Because of the extremely low frequency of antigen-specific B cells among peripheral blood lymphocytes, there is hardly any chance of detecting these cells in the emerging immortalized cell culture. We, therefore, first enriched for specific antigen-committed cells and then infected the culture with EBV. Enrichment of the specific cells was carried out by selection of cells that expressed on their cell-surface the corresponding antibody. Indeed, selection of antigen-binding cells before the viral infection enabled us for the first time to establish a variety of human monoclonal antibody secreting cell lines [4]. This original method was applied to produce IgM, IgG and IgA human monoclonal antibodies against a vari-

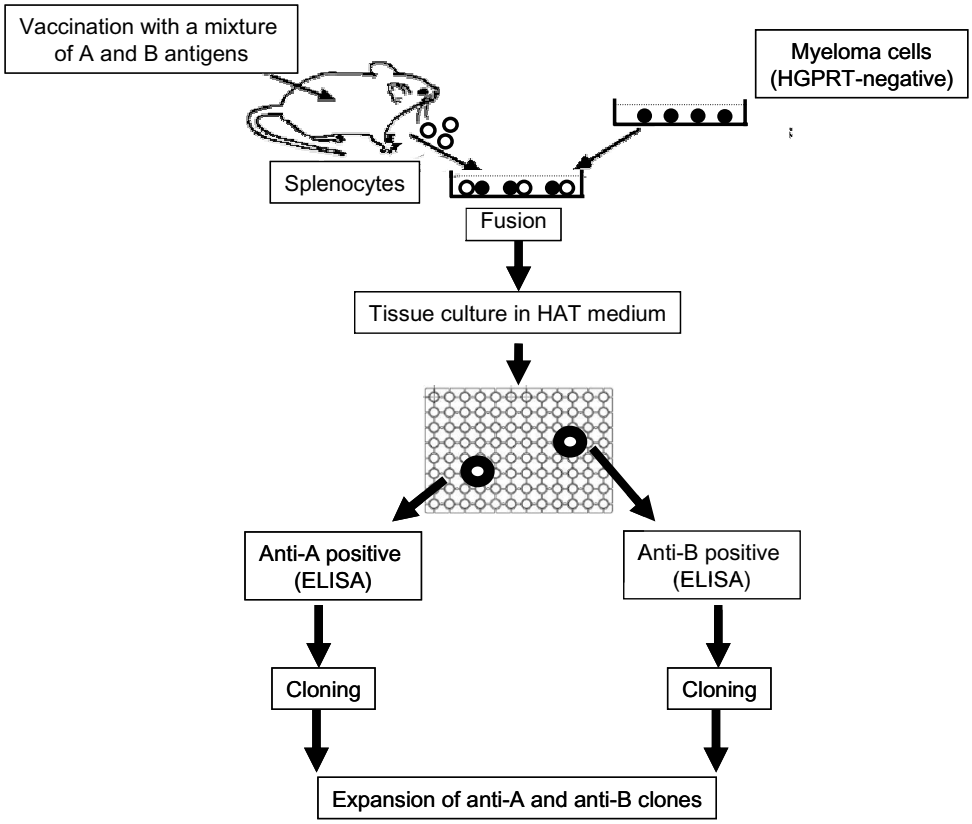

Fig. 1. Hybridoma method for making murine monoclonal antibodies.

ety of antigens. Greater stability and increased amount of secreted antibody were obtained by fusion of the EBV-immortalized antibody secreting cells with an appropriate heterohybridoma [5]. Recently, the EBV method was further improved by using CpG to stimulate the peripheral blood lymphocytes simultaneously with infection with EBV [6]. The stimulation with CpG extends the target B-cell population which is EBV-immortalizable so that it consists virtually of all the B cells within the PBL, including memory CD27 positive cells but excluding plasma cells. The frequency of antigen-committed B cells in PBL even in the blood of an immunized individual is very low and therefore it is crucial to immortalize as many B cells as possible. The efficiency of EBV-induced immortalization is significantly heightened if the cells are infected with an excess of virus [7].

Vaccination of healthy individuals to generate antigen-committed B cells for making monoclonal antibodies is not permitted but there is a plausible alternative strategy to prepare these cells by antigen-specific sensitization of PBL *in vitro* prior to EBV immortalization [8]. However, this approach has so far not proved successful.

Recently we, together with Dr. R. Laskov from the Hebrew University, Hadassah Medical School, found that the occasional loss of antibody activity occurring during prolonged *in vitro* tissue culture of LCL's is due, at least partly, to induction of a specific enzyme, namely activation-induced cytidine deaminase (AID) [9,10]. This enzyme, which plays an important role in somatic hypermutation (SHM) and isotype switching in B cells [11], is upregulated in EBV infected cells. The outcome of SHM in the *in vivo* maturation of B cells is the selection of antibodies with a higher affinity, whereas *in vitro* such focused selection does not occur. Thus, mutations may induce a decrease or an increase in antibody affinity during prolonged LCL culture.

4. Chemical and molecular methods: Chimeric monoclonal antibodies

The facility with which murine monoclonal antibodies can be produced using Köhler and Milstein's

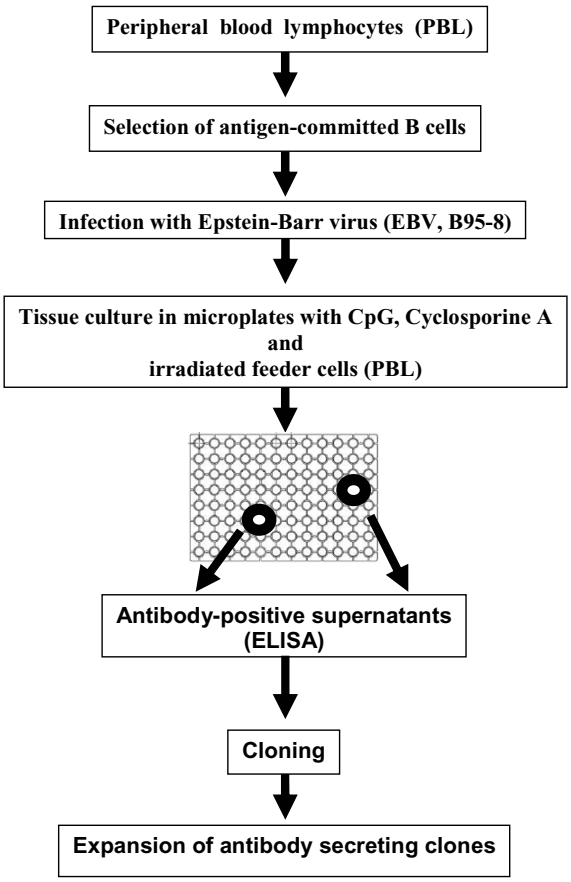

Fig. 2. Outline of Epstein-Barr virus method to engender human monoclonal antibodies.

method and the serious constraints these antibodies impose upon their use in the clinical setting led to new approaches. Methods were developed to convert, at least partly, the readily available rodent monoclonal antibodies into antibodies with predominantly human immunoglobulin chains, preserving those parts of the murine antibody which correspond to the antigen-binding sites.

Initially, the Fc portion of the antibody molecule, which dictates the functions of the antibody, was chemically exchanged with a human constant portion [12], giving rise to chimeric monoclonal antibodies. The antigen specificity of the chimeric antibodies is identical to that of the initial mouse monoclonal antibody, whereas the functions are determined solely by the human Fc domain. In comparison to the original mouse monoclonal antibodies, the chimeric molecules are less "murine" and they therefore induce a significantly decreased HAMA response in human recipients. However, the remaining immunogenicity renders even these antibodies non-tolerable.

5. Humanized monoclonal antibodies

The molecular methods developed and improved in the past two decades and the greater comprehension of the structure and function of the different antibody domains led to novel revolutionary approaches to the production of monoclonal antibodies. Whereas the hybridoma and EBV methods facilitate immortalization of specific antibody-committed B cells, the molecular techniques focus on immortalization of genes corresponding to specific antibodies. Molecular techniques were used to further eliminate those portions in the murine immunoglobulin chains that are not involved in the binding of antigen and to replace them with the corresponding human sequences. Complementarity-determining regions (CDR's) within the variable regions of both the heavy and light chains play a prominent role in the binding specificity of the antibody. DNA fragments that correspond to the CDR's were grafted into the framework of human immunoglobulin genes using molecular methods [13]. In addition, replacement of some amino acid residues in the constant regions of the "recipient" human immunoglobulin with the corresponding amino acids of the mouse "parental" monoclonal antibody proved advantageous [14].

Thus, humanized antibodies retain the specificity and binding affinity of the "parental" murine monoclonal antibody, they exhibit reduced immunogenicity in humans and they acquire biological functions of choice.

6. Molecularly-engineered, completely human monoclonal antibodies (Fig. 3)

Development of the PCR method made it possible to amplify the entire immunoglobulin genes or some of their components. Subsequently, these genes could be introduced into a variety of cells to produce the corresponding antibodies. Methods were applied to prepare libraries of plasmids with the cDNA's of heavy and light chains from PBL's and even from single cells derived from naïve and immunized human donors. The combinatorial libraries were used to transfect bacteria which, in turn, were seeded on appropriate drug-supplemented agar medium. Colonies producing active antibodies were thus detected and isolated. This method facilitates the formation even of antibodies which have never been made *in vivo* [15]. The main disadvantage of the method is that it does not provide appropriate ready-to-use tools for the selection and isolation of the desired high affinity specific antibodies.

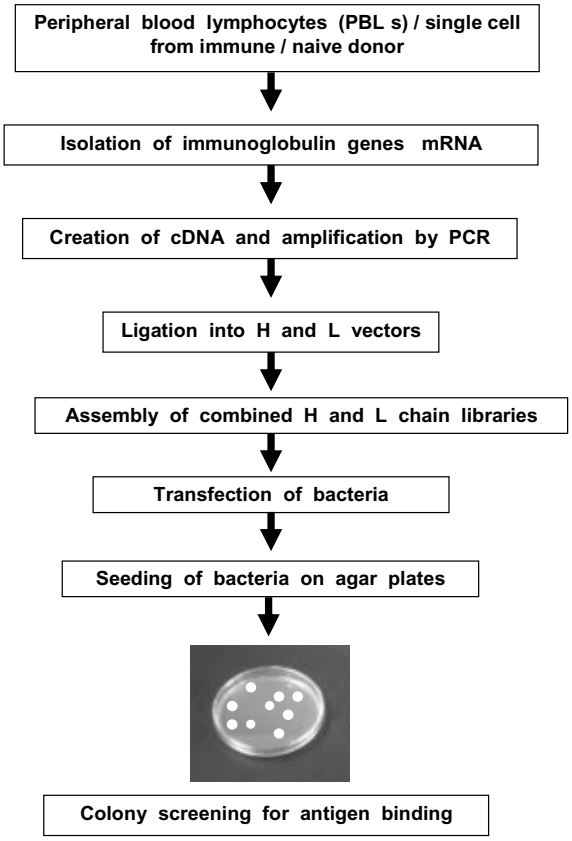

Fig. 3. Molecularly engineered monoclonal antibodies made by combined H and L chain libraries.

Jespers et al. [16] used molecular methods to produce fully human monoclonal antibodies by pairing the human V_L chain with the V_H of a mouse monoclonal antibody and then pairing the "successful" human V_L chain with a repertoire of human V_H chains. It was thus possible to come up with a human antibody with the specificity of the original murine antibody.

Molecular engineering of antibodies enables their production in cells derived from a variety of sources, including bacteria [17] and plants [18]. The glycosylation pattern of proteins, which is cell-dependent, is crucial for therapeutic antibodies and therefore the type of cells in which they are generated is of major importance.

7. Phage display method (Fig. 4)

Phage display technology [19] is an ingenious approach which provides the tools for creating and efficiently isolating high affinity recombinant native and non-native monoclonal antibodies. A recombinant library is constructed from diverse variable regions of the immunoglobulin genes (i.e., single chain of joined heavy and light variable fragments (scFv), Fab fragments or single V_H or V_L domains). Each library is fashioned from cDNA derived from immune or naive B cells. The DNA library is ligated into a surface protein gene (gene III) of a bacteriophage. The bacteriophages thus display on their surface the antibody constructs fused with the surface protein. Phages expressing the required specificities are readily isolated and enriched, using antigen-conjugated affinity binding columns. Eluted phages, similarly re-selected, are used to infect Escherichia coli to produce the monoclonal antibody construct. Alternately, the genes of the specific antibody are excised and cloned into whole human IgG expression vectors and subsequently transfected into appropriate cells to produce fully human monoclonal antibodies. Since this combinatory library randomly matches the V regions of the heavy and light chains, the resulting products include not only combinations that were expressed by B cells *in vivo* but even novel combinations ("non-native") which never existed before. The libraries prepared from both naïve and immunized human donors enable the selection of high affinity antibodies. The antibody products are skewed as they depend on the specific antigen/epitope and enrichment methods used to isolate the bacteriophages.

The phage display technique has the drawback that it does not facilitate isolation of the genes corresponding to the full-length IgG molecule. Mazor and coworkers [20] constructed a single plasmid which in bacteria can express both light and heavy full-length chains. The assembled antibody produced by the bacteria is bound by an Fc-binding protein that is anchored in the membrane of the bacteria. Thus, bacteria expressing an antibody with a required specificity are readily isolated by affinity chromatography using solid phase-conjugated antigen.

8. The transgenic mouse approach (Fig. 5)

Köhler and Milstein's classical hybridoma technique in rodents has proved to be an extremely reproducible, straightforward and problem-free method but has been rather disappointing in the human context. The fact that production of human monoclonal antibodies by the available techniques has been by far more complicated, inspired the development of a novel technique exploiting transgenic mice [21]. Accordingly, the genes of the heavy and light chains of human immunoglobulins

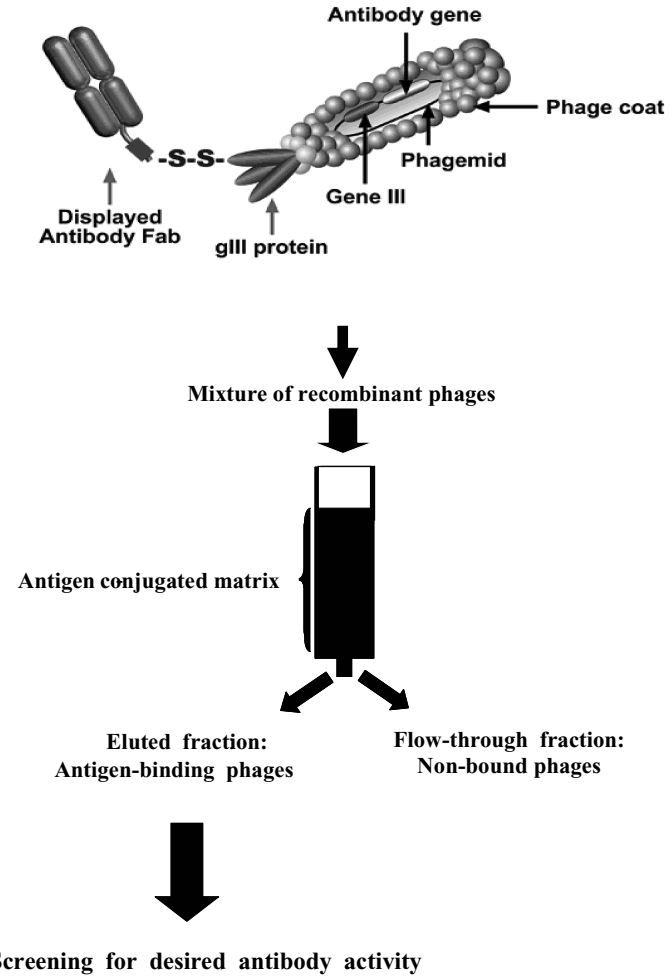

Fig. 4. Outline of phage display method for producing monoclonal antibodies. The schematic (top) depicts the engineered bacteriophage (with Enco's permission).

replaced those of the mouse genes. Upon vaccination, these knockout/knockin mice produced human antibodies and their spleens were used to make human monoclonal antibodies, applying the conventional hybridoma technique. Similarly, a specific TransChromo technology was developed whereby human chromosomes 14 and 2 were introduced into heavy and light chain gene-deficient knockout mice [22]. At the moment some problems related to the technology remain unresolved. First, the human immunoglobulin genes transferred into the knockout mice are incomplete. Second, the Ig-"humanized" transgenic mice possess murine T cells and therefore their humoral response is not purely human-specific. Third, in this system glycosylation of the human antibodies is mouse-specific. Thus, if the antibodies are intended for immunotherapy they will be recognized by anti-Galα 1-3Gal antibodies [23] normally present in human serum. Fourth, the durability of the foreign human chromosomal material is of major concern. A disturbing drawback is that biological industries are the proprietors of the knockout/knockin mice, which are, therefore, not freely available to the scientific community.

9. Choice of therapeutic human monoclonal antibodies

Since monoclonal antibodies resemble but a very small fraction of the antibody repertoire induced by any antigenic challenge, the criteria used for their selection are of major significance. Needless to say, the generation and, even more so, the preferred choice of human therapeutic monoclonal antibodies or the correspond-

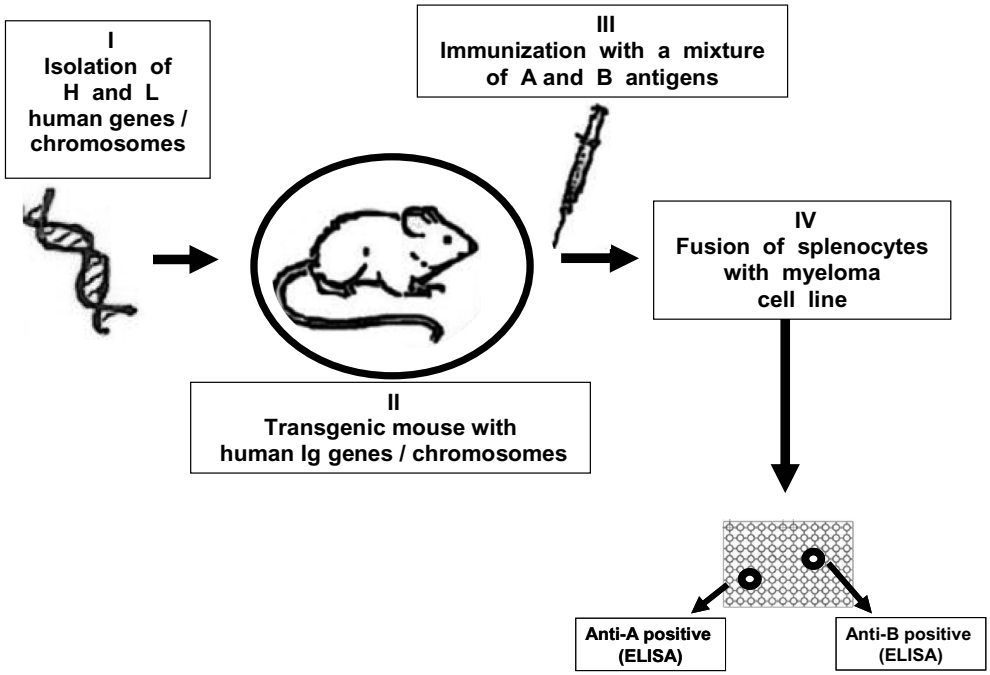

Fig. 5. Production of human monoclonal antibodies by transgenic mice.

ing genes, depend on specific considerations and criteria which are only partly shared with those applied in the context of murine monoclonal antibodies. Generally, murine monoclonal antibodies are evaluated primarily by the molecular epitope of their specific antigen and by their affinity. These principles may be valid for human therapeutic monoclonal antibodies, yet it is their biological functions *in vivo* which are of major importance. Indeed, selection of monoclonal antibodies according to their specificity might be misleading. For example, in sera of HIV patients some antibodies against HIV antagonize other anti-virus protective antibodies [24].

Natural antibodies against a variety of pathogens and altered cells are present in the sera of normal humans. The antibodies against bacteria and viruses are either natural (i.e., innate immunity) or induced after an earlier encounter with the specific pathogen (i.e., acquired immunity). Protective anti-pathogen or anti-altered cell antibodies have been tailored for millions of years to serve as a safeguard against common pathogens and transformed cells. The specificity and the affinity of these antibodies are not necessarily identical to the high-affinity and epitope-selected monoclonal antibodies which are made so efficiently *in vitro*. This holds true for a variety of antibodies, as for example anti-influenza antibodies [25], anti-HCV protective antibodies [26] and HIV-neutralizing antibodies [27].

Innate immunity-related antibodies play a role also in the removal of cellular waste, and modified and transformed cells. Vollmar and Brandlein [28] showed that the natural anti-tumor antibodies in normal serum are germ-line-coded natural IgM antibodies. These IgMs preferentially bind to carbohydrate epitopes on post-transcriptionally modified surface receptors and induce cancer-specific apoptosis.

B cells that produce innate immunity-related antibodies might be a promising source for generating therapeutic monoclonal antibodies.

The PBL of individuals who are deliberately vaccinated against specific pathogens are used as a source of B cells to make anti-pathogen protective monoclonal antibodies. However, since there is no comprehensive analysis on a single cell level, the choice of the "appropriate" B cells for immortalization is uncertain. Wrammert et al. [29] showed that vaccination against influenza induces a transient IgG anti-influenza response which has a distinct clonality and kinetics. They also found that in influenza-vaccinated humans the response of memory B cells and antibody-secreting plasma cells (ASC) is organ-specific. Wrammer and coworkers who successfully prepared and analyzed recombinant anti-influenza fully human antibodies derived from single ASC's, showed that the anti-influenza vaccine-induced response has a limited antibody reper-

toire [29]. The apparent restricted response, as reflected in the emerging monoclonal antibodies [29], is probably skewed and differs from that of the authentic *in vivo* response. Indeed, in contrast to these results, a much broader repertoire of human anti-influenza antibodies was obtained using the Symplex technology [30]. This technology, which applies both cellular and molecular procedures, allows identification of high-affinity antibodies from immunized or naturally-immunized individuals and enables rapid direct cloning of the immunoglobulin genes from single antibody-producing cells.

In a recent elaborate multistep study, Kurosawa et al. tried to outline a method for the selection of anti-tumor protective human monoclonal antibodies [31]. First, a phage antibody display library was constructed from a healthy individual to produce a repertoire of recombinant antibodies. Antibodies binding to a variety of tumor cell lines were then identified. Second, further selection allowed the isolation of antibodies that stained a variety of malignant cells. Finally, functional assays *in vitro* (anti-tumor ADCC assay) and *in vivo* (anti-tumor reaction in athymic mice) were used to choose antibodies of possible therapeutic potential. This laborious procedure which integrated molecular technology, cell selection and functional assays, outlines a comprehensive straightforward procedure for isolation of human therapeutic antibodies.

Human monoclonal antibodies that resemble *in vivo* genuine antibodies are of importance when considered as candidates for therapeutic antibodies and they are indispensable for basic research on autoimmune diseases. The *in vitro* produced RF-AN monoclonal antibody (autoimmune IgM anti-IgG antibody, i.e., rheumatoid factor) was established in my laboratory by an EBV-immortalized B cell derived from a rheumatoid arthritis patient [32]. This antibody, which was the first autoimmune antibody produced *in vitro*, has been studied intensively. The sole authentic source for pathogenic autoimmune antibodies would be B cells derived directly from non-treated patients. In contrast, autoimmune monoclonal antibodies produced using molecular techniques do not necessarily resemble the authentic pathogenic antibodies.

10. Future developments

Human monoclonal antibodies have entered the clinic primarily as a passive vaccination in malignancies and inflammatory diseases. Only few of these antibodies are entirely of a human source. The monoclonal antibodies used today in the clinic are prepared from vaccinated donors and reflect an acquired humoral immune response. Today, there is only a vague understanding of the role played by "natural" antibodies in the homeostasis of healthy individuals. The majority of the "natural" antibodies in the serum are induced by unknown and non-defined antigenic challenges. Even if the molecular targets of the antibodies are known, their function is obscure. It is anticipated that some of the antibodies play a role in the homeostasis of normal molecules and their breakdown products, in removal of waste and aged normal and transformed cells. Obviously, the source of the cells to be used to make monoclonal antibodies resembling "natural", possibly protective antibodies, is B lymphocytes from non-vaccinated healthy individuals. The frequency of these B cells in normal individuals can be readily deduced from the titre of the corresponding antibodies in intravenous immunoglobulin (IVIG) preparations, the commercially-available pooled human IgG. In the future, human monoclonal antibodies resembling widespread "natural" antibodies might offer promising novel reagents for immunotherapy.

Novel developments in clinical research point to additional areas where human monoclonal antibodies might be applied as passive vaccination, such as diseases of the central nervous system (i.e., Alzheimer's disease (AD)) and autoimmune diseases (i.e., systemic lupus erythematosus). In a murine model of AD it has been shown that passive and active immunotherapy a) eliminates the typical amyloid beta (Aβ) plaques which characterize also the brain of AD patients and b) improves the cognitive behavior of the sick mice [33]. The presence of anti-Aβ IgG antibodies in the serum of all individuals [34] is an indication that these antibodies play an important role in the homeostasis of the amyloid precursor protein (APP). This assumption is backed by the finding that there is an improvement in the cognitive behavior of AD patients who are treated with IVIG [35,36].

We established human monoclonal anti-Aβ antibody producing LCL's by EBV-infection of B cells derived from healthy blood donors [37]. These antibodies represent genuine antibodies that presumably participate in the homeostasis of certain form(s) of the amyloid and APP in healthy individuals. The anti-Aβ monoclonal antibodies bind to the N-terminal of the 43 amino acid-long Aβ amyloid. *In vitro* they bind to Aβ and not to any other type of amyloid and they specifically stain amyloid plaques in brain sections derived from AD pa-

tients. If the clinical trials will convincingly confirm the encouraging results of the murine model of AD, human therapeutic monoclonal antibodies derived from healthy individuals may prove to be promising reagents for future therapy.

Lupus is an autoimmune disease characterized by the presence of a variety of autoimmune antibodies. Some of the patients develop a psychiatric syndrome that is associated with a high serum titre of anti-ribosomal proteins (i.e.,anti-P antibodies). Recently Matus et al. [38] showed that anti-P antibodies recognize an integral membrane molecule present on neurons. This protein is preferentially distributed in the brain in areas involved in memory, cognition, and emotion. In tissue culture, the anti-P antibodies induce apoptosis of neural cells whereas astrocytes, which do not express the protein, are not affected by the antibodies. The finding that IVIG has a high titre of anti-P IgG antibodies (Steinitz, unpublished results) points to a possible inhibitory or blocking role which they might play in the homeostasis of healthy individuals. If certain anti-P antibodies can indeed antagonize the effect of the pathogenic anti-P antibodies in lupus patients, production of the former might provide an attractive reagent for novel immunotherapy. Towards this end we have applied the EBV method to produce anti-P antibody secreting LCL's.

The humoral immune system in higher vertebrates is unique in its ability to generate highly diverse antibody responses against pathogens, as well as against certain malignancies. The likelihood of discovering a single "appropriate" monoclonal antibody is probably far fetched. In nature, an efficient immune response against pathogens is never monoclonal, probably because the polyclonal response is by far more effective. Thus, we may expect that in the future immunotherapy will involve a mixture of selected monoclonal antibodies with a variety of specificities.

Acknowledgements

This study was supported by the Ernst and Lili Rosenhain Fund, Bruxelles, Belgium. We thank Dr. Alexandra Mahler for her editorial assistance.

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