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Review Article

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Evaluation of the Progression of Hybridoma Technology: Methods, Applications, Advantages and Drawbacks

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Abstract

Many recombinant technologies have been used to produce antibodies in a number of laboratories. Since 1975, hybridoma technology has become the most widely used technique to generate antigen specific monoclonal antibodies (MAbs). Here, we review the techniques, applications, benefits and challenges of hybridoma-based generation ofMAbs. In this technique hybrid cells are obtained by fusing B cells, with formerly prepared myeloma cells. The fused cells are thus called hybridoma cells (hybridomas). Hybridomas, which are able to secrete specific antibodies, directed to a target antigen, are selected and sub-cloned to a large medium for mass production. A large quantity of MAbs generated through hybridoma technology has been extensively employed to detect the target molecules in research and clinical laboratories. Additionally, MAbs have long been used for prevention and treatment of a variety of diseases. Although, hybridoma technology is capable of producing a large quantity of desired MAbs, it still has its short-comings.

Keywords: Monoclonal antibodies; Hybridoma technology; B lymphocyte; Myeloma cells; Antigen

Introduction

Antibodies are a type of glycoprotein, also called Immunoglobulins (Igs), secreted by B lymphocytes, and they are major components of an acquired immune system [1,2]. An animal exposed to foreign molecules (antigens), for significant periods of time, produce mixtures of variety of antibodies (polyclonal antibodies) from many different lineages of B lymphocytes. Polyclonal antibodies are rarely used to diagnose an epitope of a target antigen, because they often have lower specificity and higher cross-reactivity, and this turns out to be false positive results [3]. However, these days, it is not difficult to generate many copies of antibodies with higher degree of specificity to a certain antigenic determinant of a target antigen. These are termed as MAbs [4,5]. MAbs can be generated *in vitro* and *vivo* by phage display methodologies and transgenic mice respectively, as well as by other numerous recombinant techniques [6]. In this review we focus on the widely used method, murine hybridoma [7].

Hybridoma technology is a technique which was developed by Koheler and Milstein in 1970's [8]. Hybridomas are generated by somatic fusion of an immunized splenic B cell against an antigen of interest, with a transformed myeloma cell that can grow and divide indefinitely [9,10]. Antibodies produced by this technique are derived from the same lineage of B Lymphocytes that possess identical specificity to the target antigen [11]. Hybridoma technology generates a bulk quantity of MAbs which are routinely used to detect molecules of interest for diagnostic and research purposes [12,13].

The importance of therapeutic proteins has grown rapidly since the emergence of the biotechnology industry more than 30 years ago [14]. Today, MAbs are important classes of biopharmaceutical products [15]. Over thirty different kinds of therapeutic MAbs products have been approved for use in the United States and Europe by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA), and hundreds are currently undergoing clinical trial [8,16]. However, although the recent growth in using MAbs for therapeutic application, they are actually even more effective in preventing variety of diseases [17].

Methods

Hybridoma-derived MAbs are produced from immortalized plasma B cells *in vitro* or *vivo* [4,5]. Depending on the design of the experiment, investigators utilize several recombinant approaches to generate functional MAbs. There are a number of methods that have emerged, over the past 30 years, for producing MAbs derived from immunized rodents, and cloning of human Ig genes, for instance, single B cell analysis, human-human hybridoma, transgenic mice, yeast display, phage display, humanized antibodies, chimeric antibodies, and hybridoma technology [7,18,19]. Besides the previously mentioned methods, a novel fluorescence-based method has been recently used to produce MAbs. In this technique, the source of MAbs is an antigen specific memory plasma cells (derived from immunized animal's bone marrow) that express Surface IgG 8. Despite the advancements of several new recombinant techniques to produce MAbs, the process of MAb production is skewed to hybridoma technology [20]. We, therefore, explain hybridoma technology through four main steps in the subsequent pages (Tables 1-3).

Technology	Description	Advantage	Limitation
Fluorescent foci method	Direct identification and isolation of antigen-specific IgG-secreting cells from immunized animal bone marrow using standard microscope slides and micro manipulator device	Avoid the inefficient hybridoma fusion stage a able to produce diverse panels of functional recombinant MAbs	

		very fast method, incubation and isolation of single cell is carried out within 2 hours	
Single B cell analysis	Memory B cells or plasma cells expressing surface IgG have been employed to generate MAbs using flow cytometry and micro engraved array chips	Get rid of the inefficient hybridoma fusion stage and combinatorial display	Restricted to larger animals, and require a combination of antibody reagents so that it is difficult to apply other than human species. Unable to screen antigen-specific plasma cells and memory B cells
Human-Human Hybridoma	Fusion of human B cells (from tumor patients) with Karpas 707 H myeloma cells	Hybrid cells grow and secrete human Igs continuously for several months	Insufficient human B cells expression desired antibodies
Transgenic mice	Innate mouse antibody genes are suppressed and replaced by human antibody genes	Simple, avoid antibody maturation step, and depends on the most commonly used techniques	Produce weaker immune response thus high number of immunization required
Yeast display	Antigens of interest is displayed as a fusion to the Aga2p protein on the surface of yeast	Eukaryotic expression and processing	Libraries are constructed by combining antibody variable region genes randomly, Small size mutagenic library and differential glycosylation compared to mammalian cells
Phage display	Bacteriophages are engineered to display wide human antibody gene libraries	Large size phage display libraries that increase the probability of isolating the desired antibody	Libraries are constructed by combining antibody variable region genes randomly, and Antibody maturation required
Humanized Antibodies	Genetic recombinant of mouse variable region sequences to human constant region sequence (≈ 1:12)	Trigger lower immune response than chimeric and murine antibodies.	Expensive, time consuming , and reduce binding affinity
Chimeric antibodies	Genetic recombination of mouse and human antibody genes, (≈ 1:2)	Low immunogenicity reaction and effective target selection	Trigger immunogenicity reaction though in lower level. Expensive, time consuming , and reduce binding affinity
Hybridoma technology	Fusion rodent B cells with cancer myeloma cells.	Relatively simple to obtain immortalized hybrid cell lines	High immunogenicity reaction, inadequate cytotoxic activities, and hybridoma screening lack diversity

Table 1: Advancements of several MAbs producing technologies, and their advantages and drawbacks [7,8].

Immunization

Triggering immune system of animals by administering a target antigen is the first stage of hybridoma technology [5,21]. Substances such as, proteins, carbohydrates, multiple antigenic synthetic peptides, complex lipids, DNA, haptens, whole cells, and cell extracts are able to stimulate immune responses when they are properly delivered into the body of animals such rodents (mouse, rat and hamster) and rabbit [22,23]. These molecules are often foreign to the individual animal and are collectively termed as immunogens [24].

Normally, a laboratory animal is injected with a mixture of target antigen and agent that boost immune response (adjuvant) in multiple times over a course of several weeks, until the target antibody titer is achieved [25]. The efficacy of immunization is mainly determined by inoculation routes, selection of proper adjuvants, dosages, and injection intervals [20,21]. The most frequently used method to convey the antigen of interest is injection into the peritoneum of the host animal 5. Each and every series of antigen injection is followed by external electric field (electroporation). The role of electroporation is not only to boost a significant non-specific immune response, but also encourages immunoglobulin class to switch and produce high affinity immunoglobulin. The main purpose of the immunization processes is thus to select an individual animal which shows the best response to the antigen of interest [25].

The intensity of immune response and the production of target antibodies are assessed by taking blood sample from the animal tail blood capillaries, at a fixed distance from the tip [26-29]. Ultimately, aseptic dissociation and isolation of target B cells and cultivation of myeloma cells are carried out from spleen and bone marrow, respectively. Even though *in vivo* immunization has been the first choice for decades in hybridoma based generation of monoclonal antibodies; *in vitro* immunization is also possible such that a small amount of antigen can trigger immune response to culture splenic cells [25].

Fusion and selection

Once the host animal spleen is confirmed of producing antibody of interest, the second step of hybridoma technology is fusion of membrane myeloma cells (derived from the bone marrow) and B cells (extracted from immunized animal spleen) [30]. Hybridomas, unlike the unfused parent cells comprise two distinctive characteristics. First, hybridomas generate the desired antibodies, a feature contributed by B lymphocytes. Second as a result of the fusion with myeloma cells, hybridomas are immortal. Currently, there are a number of suitable mouse myeloma cells for fusion which are available at the market for biomedical research, such as SP2/0.Ag14 (SP2), P3/X63/Ag8.653 (NS0/U), P3/NS1/1.Ag4.1 (NS1), F0, and PAI0; of all NS1 and SP2 are the most well-known myeloma cells. On the other hand, 210-RCY3-Ag1,2,3 (Y3), IR983F (983) and YB2-0 (YB2) are rat myeloma cells appropriate for fusion formation with respective B Cells [31-33].

Myeloma cells lacking hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme are selected prior to the fusion process [34]. Myeloma cells lacking HGPRT cannot use the purine salvage pathway to synthesize purine nucleotides from intermediates; therefore they are completely depending on de novo pathway to synthesize nucleotides for survival [21]. Fusions of the two distinct types of cells are performed using several approaches including by adding chemical (polyethylene glycol), viral (Sendai virus), and electrofusion [24,35].

Once hybridomas are formed, cells are seeded in a special and selective hypoxanthine aminopterin thymidine (HAT) containing medium aimed at getting rid of unfused cells [21]. Immediately, after plating, free B cells die out because they cannot survive in culture medium, leaving only hybridoma and free myeloma cells [5]. HAT medium only supports the growth myeloma-splenic B cells, but inhibits the growth of free myeloma cells because of the presence of aminopterin ingredient that blocks the de novo pathway, the only available nucleotides biosynthesis in HGPRT negative unfused myeloma cells [25]. For this reason free myeloma cells have a short life span. Fused myeloma cells however, survive as B lymphocytes partner which supply the important enzyme (HGPRT) and are able to carry out purine salvage pathway. It is important to note that normal cells attain two chief nucleotide biosynthesis pathways; de novo and salvage pathway [36]. When de novo pathway (cardinal nucleic acid synthesis pathway of all normal cells) is blocked, normal cells switch to salvage pathway nucleotide biosynthesis for survival. Salvage pathway synthesize purine and pyrimidine nucleotides from intermediate compounds, generated from nucleic acid degradation [37].

Some fused myeloma cells return to a former state and become free myeloma cells. Therefore, continuous scanning for the viable hybridoma cells is indispensable. After determining viable hybridomas of interest, credible hybridoma cells are harvested, diluted and sub-cultured in a separate hypoxanthine thymidine (HT) medium plate. Each culture well will eventually have monoclonal hybridomas [25].

Screening and sub-cloning

Screening is the third stage of hybridoma technology, which is the process of identifying and separating hybridomas, secreting the desire antibodies, from non-specific hybridomas [21]. Not all hybridomas secrete target antibodies; it is thus crucial to perform primary screening test to each visible colony. In general, it is advisable to carry out screening test when the confluence of the hybridomas culture reaches minimum 75%. This is because the growth pattern of newly formed hybridoma cells is quite different. Each hybridoma cell requires different optimal growth conditions. Some slow growing hybridoma cells can emerge around 25 days after successful fusion of myeloma cells and splenic B cells. So, it is unpractical to control the growth of hybridomas by counting in each cell [38]. Rapid screening of specific hybridomas is often done using Immunoassay such as ELISA and immunocytochemistry [39,40]. For instance, ELISA can be used to analyze hundreds of supernatant samples within a couple of hours. It is utilized to wash off majority of non-specific hybridomas [31].

Eventually, pure and stable hybridoma cells can be grown into a large culture medium for generating adequate amount of target MAbs 21. Mass culturing of pure hybridoma cells has two main advantages. First, it maintains the quality of the hybridoma, and second, it provides enough cells for cryopreservation. Depending on the intention hybridomas can be stored at a variety of temperatures. Generally, hybridoma cells are preserved at -20°C or -80°C [38].

Figure 1: Main stages of hybridoma-based production of monoclonal antibodies.

Characterization of Antibody

Pure hybridomas that generate MAbs can be employed for different purposes [21]. However, a slight difference in the specificity and reactivity of MAbs can confound results. Thus, characterization of antibodies, before utilization as a diagnostic and therapeutic reagent is necessary. Characterization of MAbs focuses on the specificity, reactivity and cross-reactivity, biological activity, purity, and quantity [41-43]. Likewise, accurate and specific identification of heavy and light chain of antibodies 37 helps in many purposes such as defining the immunoglobulin class or sub class, identifying the presence of a single isotope (e.g. IgG1) 42 and dictating the most appropriate column purification technique for a culture supernatant [44].

The detection ability of MAbs is greatly influenced by assay system treatment. A variety of assay systems provide different environment to the MAbs. Consequently, they accomplish their job tremendously well in some systems and little or none in other systems [45]. In other words, the environment within the assay system determines how antibodies identify and bind to the target antigenic determinant. Some techniques within the assay system can denature, mask and prevent accessibility of the target epitope. MAbs characterization serves as a great deal opportunity to test against similar antigen determinant (Figure 1) [35,46].

It is important to note that some antigen binding site of MAbs can interact with more than one antibody binding site of an antigen that have similar chemical composition. The exposure of a specific antigenic determinant to its corresponding antigenic combining site of the particular antibody can also be altered by a slight artificial change

in the investigative procedure. For this reason, it is crucial to prepare epitope mapping by meticulous evaluation of the target antigen and a given antibody. Epitope mapping helps to characterize the amino acid residues of antigenic determinant directly as they interact with given antibodies [47].

Application

MAbs have proven to be instrumental in the advancement of research, diagnosis, vaccination, and therapeutic applications [24]. For instance, monoclonal antibodies can differentiate subsets of B cells and T cells, which is supportive in identifying different types of leukemia. In addition to this, specific monoclonal antibodies have been used for cell surface markers' definition on white blood cells and other cell types. This has led to realization of Cluster of Differentiation (CD) markerswhich provides and insight to several hundred different cell surface components of cells by, for example, fluorescence-activated cell sorting (FACS). Following are three major applications of MAbs in prevention, diagnosis and treatment of diverse diseases.

Prevention

Systematic administration of MAbs plays a vital role in treating antibiotic resistant microorganisms and incurable infectious diseases [48,49]. Nevertheless, MAbs are more efficient in prophylaxis than treatment of various diseases 17. For many years, it has been known that natural and artificial passive immunization with antibodies, prevent diverse diseases. Hybridoma technology, opened novel approaches for passive immunization of several transmissible diseases, such as mucosal transmission infectious diseases. Similarly, human MAbs prevent several serious diseases such as venereal, respiratory and diarrheal diseases [50].

MAbs produced by hybridoma technology have served as a platform for distinguishing normal cells from abnormal or cancerous cells by developing biomarkers. Recently, MAbs linked with anti-cancer agents are used to chase antigens in occult cancer cells and prevent cancer metastasis 35. Moreover, MAbs against complement activation play a vital role in preventing disease. For example, MAbs targeted to the complement component 5 (C5) inhibit the complement cascade by blocking the production of complement components 5a and 5b-9 and thereby prevent the development of collagen-induced arthritis as well as improve already established arthritis [51].

Diagnosis

Monoclonal antibodies are widely used not only in the diagnosis of diseases but also in the experimental diagnosis such as that of Cancer biology, Microbiology and Immunology researches.Firstly in the diagnosis of disease, there are a number of applications of which the range is from that of hybridoma-derived monoclonal antibodies to the diagnosis of parasitic diseases, for instance, diagnostic tests and utilization of MAbs; these have included radioimmunoassay (RIA) and ELISA techniques which are there to detect parasite antigen in host tissues and body fluids and circulating host antiparasite antibody. From a broader-spectrum, functions of monoclonal-derived reagents have significantly increased especially the specificity of diagnosis by completely removing the cross-reactions between adjacent parasitic species, without suffering any great loss of sensitivity [45,52]. Monoclonal antibody-secreting hybridomas are normally identified by ELISA protocol, immunoelectron microscopy, Western blot as well as immunofluorescence utilizing Encephalitozoon species from fresh and also utilization of extracted fixed samples from patients and from *in vitro* cultures [53]. In the performance of immunofluorescence assay, monoclonal antibody can strongly be recognized by Encephalitozoon species such as E. intestinalis, E. hellem, and E. cuniculi. Whereas inimmunoelectron microscopy assays, monoclonal antibody can vault to the exospore of this Encephalitozoon species, while in Western blot assays, it can be recognized by three to seven antigens which normally possess molecular masses ranging from 34 to 117 kDa. In addition to this, a new gizmo for identifying spores in bodily fluids and a biopsy sample which is an efficient diagnostic test have been developed; it is a sensitive and specific monoclonal antibody-based immunoassay which is largely diagnosing common microsporidian infections, predominantly with Encephalitozoon species [54].

Significantly, monoclonal antibodies help organs and tissues to be categorized depending on certain expression markers that define them as well as providing a reflection of cellular or tissue genesis [55]. There are organ-associated antigens that help in the identification of the nature of a given primary tumor; such antigens include α-fetoprotein, placental alkaline phosphatase, human chorionic, prostate specific antigen, gonadotrophin, and others [25]. Additionally, tumors and tissues can assist in the morphological identification of similar lesions as well as the identification of the origin of the undifferentiated metastases of organ or tissue. Analysis of tissue aspirates lymph nodes, bone marrowetc alongside selected MAbsby employing immuno-cytological technique help in the detection of occult metastases MAbs increase the sensitivity in detecting even small quantities of invasive or metastatic cells. MAbs specific for cytokeratins can distinguish disseminated individual epithelial tumor cells in the bone marrow [25].

Selected MAbs help in the detection of occult metastases by immuno-cytological analysis of lymph nodes, bone marrow, other tissue aspirates, as well as other tissues [56]. Three monoclonal antibodies (T16, C26, and AE-1), are known to be capable of recognizing membrane and cytoskeletal antigens expressed by epithelial cells to detect tumor cells, in performing a sensitive immuno-histochemical assay. This immuno-histochemical staining of bone marrow aspirates is very useful to detect occult bone marrow metastases in patients with apparently localized prostate cancer and has also led to an improved ability to detect occult breast cancer cells not only in bone marrow aspirates but also in peripheral blood [20].

Treatment

Another paramount application of hybridoma-derived monoclonal antibodies is treatment. For instance antibody therapeutics can potentially treat diseases ranging from autoimmune disorders to cancer and viral or bacterial infections. Recent reports indicate that numerically monoclonal antibodiesin development are far much greatersurpassing any other kind of therapeutics[57]. The materialization of antibodies as an attractive therapy is due to the evolution of MAbs technology that has been revolutionalizedo ver the past 25 years which has improved 100 percent reliance on mouse protein through chimeric and humanised proteins to fully fledged human antibodies [58]. Though not necessarily treatment, it still aids the treatment process in the use of monoclonal antibodies to cytokeratin in the examination of the sentinel axillary lymph node for metastatic breast cancer which increases nodal positivity by up to 10%[10]. Consequently,MAbscan also aidin speeding up the start of recommended treatment [59].

Advantages and Limitations of Using MAbs

Advantages

There are a number of significant potentials for monoclonal antibody as whole though this review tackles specifically the advancement of hybridoma and hybridoma-related technologies, the following table gives a general incite of some notable monoclonal antibody technique's benefits of which hybridoma and hybridoma-derivatives earn recognition.

Monoclonal Antibody Technique	Advantage
MAbs specific for cytokeratins	Enables the distinguishing ofthe epithelial tumor cells that are disseminated individually in the bone marrow
Immuno-histochemical	Ideal for detecting breast cancer cells even when in small quantities Simple, quite specific, and sensitive in detection of breast cancer cells' quantification
Immuno-cytochemical	Increases diagnostic accuracy in easily recognizing malignant disease complications by effusion, neoplastic cells which are not seen easily Patients are saved from painful, uncomfortable and expensive procedures to be performed on them. Catalyze in speeding up the commencement of pertinent treatment Recognition of about two antigens residing in the same smear - double staining with light chain antibodies as well as inclusion of T and B cell markers can indicate the neoplastic origin of a lymphoma
Techniques of MAbs in Functional and Molecular Biology	Used to show thoroughly expression study of antigens in different cell types and tissues, and to isolate and further analyse sub-populations of cells. Tools for purifying target antigens and for cDNA cloning in order to genetically typify the target antigen
Hybridoma	Allow the direct sampling of the immune repertoire via single B cell analysis. Allow a more thorough interrogation of the B cell population, which improves the likelihood of finding rare antibodies with highly desirable properties, and production of large and various panels of antibody lead molecules Help in the detection of small quantities of metastatic or invasive cells by normal histopathological staining with eosin and haematoxylin to make it not to be frequently sensitive The product of a single hybridoma has the capability to specifically react with the same epitope on antigens. With hybridoma it is possible to select for specific epitope specificities and generate antibodies against a wider range of antigenic determinants.

Table 2: Notable monoclonal antibody technique's benefits of which hybridoma and hybridoma-derivatives earn recognition [20,59-65].

Limitations

Even though there are numerous advantages of monoclonal antibody, there are also some drawbacks that need to be taken into consideration when tackling about MbAs.The following table summarizes the notable disadvantages of some monoclonal techniques hybridoma inclusive:

Monoclonal Antibody Technique	Limitations
Naïve Antibody Librariessystems	Requires random combination of antibody variable region genes for its constructionand this results into reduced specific diversity Require *in vitro* resemblance maturation to produce molecules with an acceptable potency profile and this results into genesis of liabilities into the molecule that affects stability and pharmacokinetics Require a reliance on phage to display antibodies which is also involved in biasing the repertoire toward those molecules capable of being expressed by E. coli and displayed on a phage particle as an antibody fragment, such as a single-chain variable region fragment (scFv)
Immuno-cytochemical	Have in their content only those tumor that are not specific and tumor-associated MAbs which are incorporatedand in the end result into unnecessary cross-reaction with normal cells to its disadvantage
(CHO)Chinese Hamster Ovary	Require long development time from gene to production cell line Inability to adequately control N-glycosylation. In case of humans lack the pathway for the synthesis of N-glycolylneuraminic acid as an example, EPO produced from CHO cells contains both N-acetylneuraminic acid and N- glycolylneuraminic acid Glycoconjugates that contain N-glycolylneuraminic acid might be subject to a go-ahead by anti-Nglycolylneuraminic acid antibodies present in human serum
Hybridoma	Generally the average resemblance of monoclonal antibodies is on the lower side than that of polyclonal antibodies. When it'sagainst conformational epitopes on native proteins there is likelihood to lose reactivity with antigens that have been minimally disturbed.. Time and effort commitment leading into cumbersomeness Difficult to control the epitopes to which antibodies are produced Antigens such as membrane proteins and nucleic acids are so sensitive Hybridoma derived antibodies require much time to be formed for instance 4 to 6 months to create hence time consuming

Table 3: Notable disadvantages of some monoclonal techniques hybridoma [20,66-70].

Conclusion

Looking at the development of modern advanced techniques to genetically modified organisms, we can be pleased that this is bringing about derivation of important experimental tools such as mice in which the mouse immunoglobulin genes are substituted by genes of human origin. Thus, the original hybridoma technique has received a new lease of life, because using such mice it is possible to generate completely human MAbs which are useful in the major applications of hybridoma-derived MAbs. MAbs produced by hybridoma cells through hybridoma technology open a remarkable technique to

prevent, diagnose, and treat several serious human diseases including, chronic inflammatory diseases, cancer, infectious, and cardiovascular diseases. Since MAbs are targeted to the molecule of interest, treatment of diseases with MAbs is much more efficient than drugs because drugs have a great tendency to indiscriminately attack nonspecifically to the host's own cells causing adverse side effects.

Nevertheless, hybridoma technology is rarely used in industries, as the fusion of B lymphocyte and myeloma cell partner is inefficient (5×10^{-6} efficiency with conventional PEG fusion) 8 and time consuming (about 7 months). Consequently, it is essential to seek efficient and rapid approaches to generate functional antibodies derived from human immune system. A number of advanced alternative methods to hybridoma technology have been produced over the last three decades and each of them achieves certain success. However, all of them have not been able to harvest perfect antibodies which are produced naturally by human immune system. Although, the importance of MAbs in medical and diagnostic laboratories has been increasing significantly finding even more reliable and easily manipulated approaches directed to generate functional human MAbs is still vital.

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