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Review

Hybridoma technology for the production of monoclonal antibodies

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ABSTRACT

Given the size and scope of the global pharmaceutical market, monoclonal antibodies (mAbs) have a promising future in a broad range of areas such as autoimmune diseases, infectious diseases, and cancer therapies. A decisive medical treatment is achievable since mAbs only identify their target antigens and not additional irrelevant proteins. As global demand grows, hybridoma technology, which enables the production of specific mAbs for target antigens, is gaining even more importance. Since the invention of the cell fusion technique known as 'hybridoma technology' to continuously produce targeted mAbs *in vitro*, their generation has become more widespread. The crucial point is the use of cancer cells. Antibody-producing B lymphocytes can become immortal through somatic fusion with myeloma cells, with the resulting hybridoma cells retaining not only the innate functions of antibody-producing B lymphocytes but also the malignant capacity for infinite proliferation *in vitro*. This review includes general information regarding hybridoma technology and hybridoma-based mAb generation.

Keywords: Hybridoma, immunoglobulin monoclonal antibodies, polyclonal antibodies.

Antibodies are glycoproteins produced by B cells, also known as immunoglobulins. They are an essential component of the humoral immune system that protects against pathogens (such as viruses, bacteria, and parasites). They exist in the organism in two forms: soluble in blood and plasma and bound to B lymphocytes.[1]

Antibodies consist of two structural components: the heavy and light chains. The light chain has one variable and one constant region, whereas each heavy chain has one variable and three constant regions. The variable region of antibodies is responsible for the recognition of pathogens and antigens. Antigens and antibodies have a similar relationship to lock and key. Each antibody has a specific paratope (i.e. lock) that can bind to a particular antigen (i.e. key). Each B lymphocyte produces only one type of antibody in response to a particular antigen. There are five different types of heavy chains according to the structure of antigen-binding crystallizable fragment (Fc). Accordingly, on the basis of the different Fc regions, antibodies are grouped under five different isotypes: immunoglobulin (Ig) M, IgG, IgA, IgD, and IgE. Of all isotypes, IgG is the smallest and most common isotype with the highest therapeutic potential. Immunoglobulin Gs that can pass into extravascular spaces constitute 70-80% of total antibodies and have a longer half-life than other isotypes.[2]

Antibodies were developed about 40 years ago and have been used in biomedical research for many years. They are employed in a wide range of applications, including flow cytometry, magnetic cell categorization, immunological assays, and therapeutic methods, due to their high specificity and selective binding capabilities.[3] Depending on their production method, antibodies are classified into two main subgroups: monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs).[4,5] Both subtypes have advantages and disadvantages according to the intended purpose.

Polyclonal antibodies refer to all immunoglobulins secreted by different B

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lymphocytes that react with more than one epitope of a specific antigen. They are produced by injecting an immunogen into an animal using a prime-boost immunization strategy to generate high-titer antibodies against the particular antigen. After immunization, the serum of the immunized animal is collected and the pAbs are used for research or therapeutic purposes either directly or after purification.[6] The polyclonal structure and biophysical diversity of pAbs are their most significant advantages. The polyclonal structure allows for the targeting of multiple antigenic constructs, while the biophysical diversity ensures stability in the face of environmental changes.[7,8] Aside from the aforementioned benefits, there are drawbacks such as limited application, batch-to-batch variability, the risk of disease transmission through blood, and only a small antibody fraction in the antibody pool that can exhibit the desired effect. Particularly in cases of low specificity, relatively high doses may be needed to achieve the expected clinical benefit.[9,10] Another drawback is that polyclonal serum cannot be used in the treatment of chronic diseases.[11] Due to the drawbacks of pAbs, the need for mAbs has become even more apparent.[12]

Monoclonal antibodies are mono-specific and produced by identical (clonal) B cells with high affinity and specificity for a single epitope of an antigen. Hybridoma technology was first used in 1975 to produce mAbs, allowing for continuous and unrestricted production of mAbs with little or acceptable variation from batch-to-batch. Due to the aforementioned properties, mAbs have become a valuable tool in the fields of biochemistry, molecular biology, and medicine.[13]

Antibody-based biologic agents have become one of the best-selling product groups today. In recent years, developments in mAb production technologies have facilitated the identification of different antigens for diagnostic and therapeutic purposes.[14,15] Over the years, different production technologies have been developed for the production of mAbs, such as hybridoma technology, single B cell cloning, and culture methods. Unlike hybridoma technology, other technologies mostly rely on the recombinant production of mAbs. Each of these technologies has distinct advantages, disadvantages, and applications.[16-18] However, hybridoma technology is the most primitive, fundamental, and successful mAb production methodology.[14] This technology is very robust and useful in discovering thousands of antibodies for different applications.[19] The most fundamental and practical benefit of hybridoma technology is that once hybridoma clones are established, mAb production can be carried out in a simple and efficient manner.[15,17] More than 90% of antibodies currently approved by the United States Food and Drug Administration (FDA) are produced by hybridoma technology.[14,20,21]

HYBRIDOMA TECHNOLOGY

Kohler and Milstein[22] discovered hybridoma technology in 1975, for which they were awarded the Nobel Prize in physiology and medicine in 1984. Hybridoma cells are formed by fusing short-lived, antibody-producing B cells with immortal myeloma cells. Each hybridoma cell produces a large amount of mAb specific to a single epitope. Specific hybridoma cell clones can be cryopreserved for extended periods of time for reuse for continuous mAb production.[14]

BALB/c mice are generally preferred for spleen cells needed for mAb production.[23] Initially, hybridoma technology was limited to mouse antigens. However, in parallel with the developments in this field, it has now become possible to develop mAbs against a wide range of antigens from various species including rabbit,[24] human,[25,26] chicken,[27] goat, sheep,[28] cow,[29] mouse,[30] guinea pig, and rat.[31] In the early years of its discovery, hybridoma technology received little attention since its success solely depended on the availability of a suitable fusion partner from mice. However, in the following years, researchers were able to use this technology to create human and rabbit hybridomas. The instability of hybridoma clones produced with heterologous species is another limitation encountered in studies of mAb production from other species.[15] The reason for this instability seen in hybridoma clones is chromosomal instability caused by the fusion of two cells from different species.

Hybridomas are classified into two types: homo-hybridomas and hetero-hybridomas. Both IgG-secreting B cells and their fusion partners are of the same species in homo-hybridomas. Antibody-secreting B cells and their fusion

partners are of two types in hetero-hybridomas. Homo-hybridomas are more genetically stable than hetero-hybridomas and secrete more stable IgG. Since hetero-hybridomas gradually lose their chromosomal recombinants due to genetic instability during clonal selection, this raises the stability issue.

THE MOST COMMON SPECIES USED IN THE PRODUCTION OF HYBRIDOMA

Mouse

Mouse pAbs and mAbs captured the largest portion of the market in 2019 due to their specificity and ease of manufacturing on a large scale. Structural similarities between human and mouse antibodies have been the main reason for this situation. The ease of development and simplicity of mouse hybridoma technology has made it the most preferred in research and therapeutic studies.[32]

The production of mouse hybridomas is a multi-step process that takes advantage of the host animal's ability to produce specific, high-affinity, and functional mAbs. The development and optimization of the specific immunogenic antigen is the focus of this process. Following optimization, the mouse is immunized for several weeks with adjuvant and antigen. Sera from immunized mice are tested for antigen reactivity and specificity. Therefore, animals with high levels of neutralizing antibodies are chosen for splenocyte isolation.[30] Isolated spleen cells are fused with myeloma cells using fusogenic agents such as viruses, chemicals, and electric current/pulses. Commonly used myeloma cells are X63-Ag 8.6539[33] and Sp2/0-Ag 1410 from the BALB/c mouse.[34] The fused cells are then selected in a hypoxanthine-aminopterin-thymidine (HAT) medium. Myeloma cells are sensitive to the HAT environment as they lack the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene required for de novo nucleotide synthesis. Non-fused B cells die due to their short lifespan. Only hybridoma (B cellxmyeloma) cells can survive in this process since they receive the functional HGPRT gene from B cells. The obtained hybridoma cells have the ability of B cells to secrete antibodies and the ability to proliferate continuously (immortality) of myeloma cells. Screening is done using the dilution method or semisolid selective medium to select only those hybridoma cells that produce antibodies of appropriate specificity.

Antibody production with mouse hybridoma technology is highly efficient. For this reason, it is widely used in the production of thousands of antibodies required for different purposes. It remains the first preferred methodology by academic and industrial working groups, especially when it comes to obtaining antibodies for analytical purposes.[35]

The first therapeutic antibody, approved by the FDA in 1985 and developed with murine hybridoma technology, was used to reduce graft rejection in tissue transplant patients.[15] The first mAb used in organ transplantation is OKT3. There has been an extensive experience in its use for both the prevention and treatment of organ transplant rejection over the last decade. The mechanism of action of OKT3 can be explained as blocking T cell function by modulating CD3 and T cell receptors and acting as an immunosuppressant.[36] Since its discovery, a number of improvements have been made, from the preparation of the chimeric version to the humanized version, in order to further reduce the negative effects of OKT3 and increase immunological efficiency.[37] Monoclonal antibodies obtained from mice have some limitations in their therapeutic use as they may cause immunogenic reactions in the target host. To overcome such limitations, studies have been conducted on the production of chimeric or humanized mAbs in which immunogenic content of mouse origin has been extracted.[38]

Rabbit

Since the discovery of mouse mAbs, rabbit hybridoma has been used frequently in recent years as an essential tool in research, diagnosis, and therapy.[3] While the rabbit immune system produces antibodies with high affinity, it has been identified as a useful tool for the recognition of many molecules, including phospho-peptides, carbohydrates, and immunogens that are not immunogenic in the mouse.[21] Compared to mice, antibodies produced in rabbits have about 10 to 100 times greater affinity. Compared to humans and mice, gene conversion and somatic hypermutation phenomena in rabbits cause more mutations in the antibody repertoire and a more

diverse and complex immune response to the target antigen.[39,40] Rabbit IgGs are simpler compared to mouse and human antibodies. Rabbit IgG has only one subclass (Cγ gene) and the majority (90-95%) of light chains are derived from the Cκ1 isotype. After the development of mouse hybridoma technology in the 1970s, several attempts have been made to produce rabbit mAbs due to the positive properties of rabbit antibodies. However, these studies were significantly limited by the lack of rabbit myeloma cell lines. In order to solve this problem, generating myeloma-like cell lines from rabbit B cells by viral transformation has been found to be difficult and highly inefficient.[41] For this reason, studies on the generation of rabbit-mouse hetero-hybridomas have intensified. Unfortunately, all first-generated hetero-hybridomas resulted in poor fusion efficiency, genetic instability, and nonfunctional heavy and light chain pairings. In 1988, Raybould and Takahashi[42] performed the fusion of rabbit spleen B cells with the mouse myeloma cell line SP2/0-Ag14 using polyethylene glycol and produced the first stable rabbit-mouse hetero-hybridoma. They observed stable rabbit IgG expression for several months from the generated hetero-hybridoma. However, due to being a hetero-hybridoma, other investigators have observed genetic instability and consequent reduction in mAb secretion.[42]

Researchers from the University of California developed an enhanced rabbit hybridoma fusion partner by repeated subcloning. After the subcloning process of these hybridomas, a new cell line, named 240E-W, with high fusion efficiency and stability was produced.[43] The 240E-W cell line was then modified to a superior version designated as 240E-W2, which was patented.[16]

Human

Human hybridoma technology is a natural and effective approach that allows the production of human antibodies for diagnostic and therapeutic purposes without the need for additional modifications.[44] It is believed to be the most promising and suitable technological platform for the isolation of therapeutic mAbs. However, the fact that most of the current fusion partners are rodent or hetero-hybridomas limits the success of human hybridoma technology. Several hetero-hybridomas have been successfully used to generate human-derived mAbs for diseases such as human immunodeficiency virus,[26,45] chikungunya virus,[46,47] and dengue virus.[48] However, it was observed that such hetero-hybridomas lost their ability to secrete antibodies due to instability in their genetic structures, and the produced mAbs were insufficient in terms of their pharmacokinetic properties such as distribution, metabolism, and excretion compared to human antibodies.[44] The first problem experienced in the production of human hybridomas during the use of this technology is the low fusion efficiency of human B cells with myeloma cells (0.001%), and the second problem is the low level (0.01%) of antigen-specific B cells circulating in the peripheral blood.[49] In hybridoma production, the fusion of two cells is carried out with three different methods: polyethylene glycol (PEG), Epstein-Barr virus, and electric current. The PEG-mediated fusion is the most preferred conventional method due to its simplicity and being the most suitable fusing agent for hybridoma production. Polyethylene glycol leads to dehydration of the lipid groups, thereby disrupting the symmetry of the membrane bilayer. This causes the plasma membranes of two adjacent mammalian cells to fuse, thus forming a single cell with two or more nuclei. A major disadvantage of PEG-mediated fusion is that it can mediate nonspecific fusion between different cell types and form hetero-hybridomas.[50] It has been used successfully for fusion in viral agents. For this purpose, the Sendai virus and vesicular stomatitis virus are the most commonly used viruses. The most efficient and innovative method in cell fusion studies is fusion with electrical pulses. This method is based on the principle of fusion of cells in the presence of high-intensity electric current (electrofusion), which causes temporary membrane permeability. It has higher fusion efficiency than chemical or virus-mediated fusion methods. However, the efficiency of the electrofusion method decreases, especially when the dimensions of the two fusion partner cells are different.[51,52] In this context, it has been determined that the human-mouse myeloma analog 2.5 cell line has the maximum fusion efficiency in the electrofusion method compared to other myeloma cells.[50,53] The most important advantage of human hybridoma technology is that the antibodies produced by this method are more suitable for therapeutic applications than their

counterparts from other species since they are of human origin.

Chicken

Due to the evolutionary difference between mammals (eg, humans, mice) and chickens, the chicken immune system recognizes a large number of epitopes in mammalian proteins as foreign, thereby eliciting a stronger antibody response.[54] As the phylogenetic difference between the antigen and the vaccinated host increases, the response of the immune system increases at the same rate.

Immunoglobulin Y (IgY), the principal isotype in chicken antibodies, has similar structural and functional homology to mammalian IgG and IgE isotypes. However, considering the constant regions of the antibodies, it was seen that while IgY has four constant regions, IgG has three constant regions.[55-57] It has been determined that IgY in chicken serum is transferred to the embryo via egg yolk.[58] Immunoglobulin Y antibodies have previously been used against bacterial and viral infections.[59,60] Humanization of these antibodies could have great potential to serve biopharmaceutical purposes.[61,62] The development of transgenic chicken with human immunoglobulin loci has accelerated the therapeutic use of transgenic chicken mAbs in humans.[63,64]

ADVANCES AND CHALLENGES IN HYBRIDOMA TECHNOLOGY

Antibodies produced with hybridoma technology have the advantage of being used directly and can be stored frozen for future use indefinitely. In addition, myeloma cells, which are fusion partners, have a transcriptional ability that can be remodeled to continuously secrete large amounts of antibodies.[65] The development of stable cell lines has advanced the antibody production system by eliminating the drawbacks of consistent antibody production resulting from unstable hybridomas. The Chinese hamster ovary cell line is one of the most preferred cell lines for large-scale production of mAbs. In recent years, the reprogramming of immunogenomics with clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) has enabled the rapid generation of cell lines capable of producing the desired antibodies.[66]

The most important problem limiting the use and applicability of hybridoma technology to other species is the lack of suitable fusion partners. To overcome this problem, the transgenic mouse model H-2Kb-tsA58 has been developed.[67] Another major challenge with hybridoma production is the supply of purified antigens required for a specific immune response. In particular, the expression and purification of the recombinant protein are both time-consuming and expensive. In addition, adjuvants used to enhance the immune response may cause undesirable immune reactions in vaccinated animals as they may alter the natural conformation of proteins.[45] Adequate immunization of animals is also a possible challenge. Failure to obtain sufficient immunity directly negatively affects the success of the hybridoma cell to be obtained in the next step. At this point, factors such as the route of administration of the antigen, the dose of the antigen, the adjuvant used, the number of booster doses, and the vaccination protocol may directly or indirectly affect the effectiveness of the immunization step.

MONOCLONAL ANTIBODIES AS A BIOTHERAPEUTIC AGENT

It is clear that the use of mAbs against carcinogenic agents has come a long way, and while they are now a standard component of cancer therapy, more research is needed.[68] Monoclonal antibodies have always been preferred over chemical compound-based therapies as their high specificity and affinity for the antigen. However, they show minimal side effects due to their unique pharmacotoxicity and pharmacokinetic properties. Being antigen-specific, they stand out as an important option in terms of medical treatment and molecular drug development.[69]

The unsuccessful results of the first clinical trials of cancer treatment, which was started under primitive conditions about 20 years ago, led some experts to evaluate antibody-based cancer therapy as an unsuccessful hypothesis.[70] Although the mAbs used in these studies were of mouse origin, some promising results were obtained, but the problems experienced with the use of those in humans caused the studies to be limited.[71] An overreaction in the human immune response to therapeutic mAbs of mouse origin can result in

their rapid clearance from the system and severe immune system disruption. Despite all these limitations, studies on the use of mAb in cancer treatment continue. In this context, studies are conducted on the use of IgG for various purposes such as directly targeting cancer, changing the host immune response against cancer, providing cytotoxic substances to cancer, and resuming the cellular immune response against cancer.[68]

Currently, the effectiveness of antibodies in the treatment of cancer and other deadly diseases has become increasingly recognized. Most of the antibodies used for this purpose are specific for the specific antigens expressed by the disease agent.[72] The success of therapeutic mAbs has been limited when viruses have been selected as targets. However, neutralizing antibodies play an important role in antiviral immunity and come to the fore in the fight against viral diseases.[73,74] It has been shown that the mortality rate is reduced by up to 95%, especially in treatment regimens where mAbs are administered in the early period.[75]

In the absence of vaccines against various deadly viral diseases, preventing loss of life and controlling diseases requires the development of therapeutic antibodies.[76] During an epidemic, it is always much quicker and easier to produce pAbs or mAbs to protect the population at risk than it is to produce a vaccine. Although vaccines are one of the most economical and effective ways to control infections, the need for adequate immune response and time to develop protective immunity is always an obstacle.[48,49]

With the development of high-throughput mAb production technologies, hybridoma technology is the most preferred method compared to other methodologies.[77] Advances in methods of recombinant DNA technology, such as chimerization and humanization, have greatly increased the potential of hybridoma technology.[14] The monoclonal antibody market as a diagnostic and therapeutic reagent has grown tremendously over the past five years. Commercial development of therapeutic mAbs began in the early 1980s, and in 1986 the first therapeutic mAb was approved by the FDA for the prevention of kidney transplant rejection. With the widespread use of mAbs over the years, the global sales revenue of mAb products was approximately $115.2 billion in 2018. The global mAb therapeutic market is expected to reach market revenue of approximately US$218.97 billion by the end of 2023, with a compound annual growth rate of 12.80%.[78]

In conclusion, the difference in results obtained in tailored treatments has recently increased the importance of the concept of personalized treatment. This innovative treatment approach has resulted in an increase in the therapeutic use of mAbs. Monoclonal antibodies are currently one of the pharmaceutical product categories with the largest market share. More mAbs are expected to become available in the coming years as interest and research in mAbs grow. Monoclonal antibodies are currently produced primarily in mice or rats and have some limitations. These constraints, however, can be reduced thanks to advances in genetic engineering. Various techniques are being developed in particular to make mAbs produced in the laboratory as humanoid as possible.

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