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Review

BIOSAFETY ASPECTS OF HYBRIDOMA TECHNOLOGY: NATURE OF RISKS AND APPROACHES TO THEIR MANAGEMENT

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This study investigates the biosafety aspects of hybridoma technology, focusing on the identification and management of associated risks. Monoclonal antibodies, essential tools in immunology, biotechnology, and medicine, are primarily produced through hybridoma technology. This process involves fusing B lymphocytes from immunized animals with myeloma cells to create hybridomas, which are then cultured to produce specific antibodies. The research highlights significant contamination risks, particularly from rodent-borne viruses and other pathogens, during both *in vivo* and *in vitro* cultivation. It systematically analyzes existing strategies for identifying and mitigating these risks at various stages of monoclonal antibody production, including hybridoma identification, cell fusion, and antibody purification. The study underscores the importance of stringent biosafety protocols and optimized purification methodologies to ensure the production of high-quality, contaminant-free monoclonal antibodies. Additionally, it emphasizes the necessity of comprehensive risk assessments and the implementation of advanced contamination control systems in laboratories. The conclusions drawn from this study provide valuable insights into enhancing the safety and efficacy of monoclonal antibody production. By addressing these biosafety concerns, the research supports the widespread application of monoclonal antibodies in scientific and medical fields, ensuring their reliability and effectiveness in various diagnostic and therapeutic contexts.

Keywords: hybridoma technology; monoclonal antibodies; biosafety; contamination risks.

Introduction

Monoclonal antibodies (mAbs) constitute indispensable immunological instruments extensively utilized across a broad spectrum of scientific disciplines, including immunology, biotechnology, biochemistry, and applied biology. Contemporary investigations into mAbs, conducted in research laboratories worldwide, continue to elucidate novel prospects for their implementation in diverse scientific and biomedical applications [1–3]. Given that large-scale monoclonal antibody production predominantly relies on ascitic fluid preparations derived from murine or rat models, a critical concern necessitating meticulous evaluation pertains to the potential presence of rodent-borne viruses. These pathogens, which are capable of infecting laboratory animal colonies, pose a substantial risk of hybridoma contamination, thereby compromising the integrity and safety of antibody preparations. Additionally, the risk of viral and bacterial contamination remains pertinent during *in vitro* cultivation, particularly under conditions where rigorous sterility protocols are not strictly maintained [4, 5]. Currently, hundreds of mAbs are either commercially available or at various stages of preclinical and clinical development. To ensure the production of high-quality biopreparations, strict adherence to standardized manufacturing protocols and the implementation of optimized purification methodologies are imperative. The purification process must not only enhance the specificity and efficacy of the final product but also ensure the complete elimination of potential contaminants, thereby guaranteeing its biosafety for human application [6, 7]. Given the growing biomedical significance of monoclonal antibody-based therapeutics, a comprehensive assessment of existing methodological approaches employed in hybridoma technology, alongside the identification and mitigation of contamination risks at different stages of mAbs production, remains a topic of considerable scientific relevance. Such risks emerge at multiple procedural stages, including hybridoma identification, *in vitro* cultivation, antibody purification, and experimental procedures involving laboratory animals. Consequently, the primary objective of this study was to systematize and analyze existing strategies for identifying and eliminating contamination risks inherent to hybridoma technology, with a particular focus on the potential hazards associated with viral infections and exposure to toxic substances.

Hybridoma technology for monoclonal antibody production: critical biotechnological aspects

One of the most widely employed methodologies for the generation of mAbs is hybridoma technology [8]. This process encompasses both *in vivo* and *in vitro* techniques. In the framework of this technology, B lymphocytes, which are responsible for antibody production, are harvested from immunized mice (or alternative animal models) and subsequently fused with immortal myeloma cell lines, thereby generating hybrid cells. These hybridomas are then cultured under controlled laboratory conditions, facilitating the production of mAbs targeting a specific antigen [9–11].

Hybridoma technology serves as a fundamental approach for the production of mAbs with specificity towards distinct antigens [12–14]. These generated cell lines exhibit the potential for cryopreservation over extended periods, ensuring their long-term availability. The range of antigens includes, but is not limited to, hormones, enzymes, as well as internal and external viral, bacterial, and eukaryotic cell structures [14].

Monoclonal antibodies produced through hybridoma technology find widespread application across both medical and scientific research domains. They are pivotal in the diagnosis and therapeutic management of numerous diseases, including cancer, infectious pathologies, and autoimmune disorders, while also serving as invaluable tools for the study of biological processes at the cellular and organismal levels. Moreover, they facilitate the detection of antigens within biological samples, such as blood or tissue biopsies, thereby contributing to early disease identification and enhancing therapeutic efficacy [8, 13].

Hybridoma technology for the production of mAbs encompasses a series of meticulously coordinated stages (the Figure): animal immunization, obtaining antibody-producing cells from the spleen or lymph nodes, myeloma cell cultivation, B-cell and myeloma hybridization, hybridomas growth, selection of active clones, hybridomas cloning, screening (characterization) of hybridomas, cultivation of hybridomas, purification and separation of mAbs. A more detailed examination of each stage will be presented in the following sections.

The biosafety measures implemented at all stages of hybridoma technology align with the laboratory biosafety standards outlined in the WHO Laboratory Biosafety Manual, particularly in relation to the following key aspects: stringent control of viral and bacterial contamination; safe handling and utilization of laboratory animals; rigorous purification and sterilization of culture media and biological materials; and adherence to biosafety level (BSL) classifications, specifically BSL-2 and BSL-3 containment requirements.

The immunization phase entails the administration of selected antigens into laboratory animals (typically rabbits or mice) via a series of injections conducted over an extended period, usually spanning several weeks. The specific regimen and duration of immunization may vary based on the nature of the antigen and other influencing factors. It is generally advantageous to immunize animals with purified antigens, as this enhances the efficiency of the hybrid screening process. This phase facilitates the differentiation of B cells into memory B cells and plasma cells. The animal is humanely euthanized once a sufficient concentration of antibodies has been generated in its blood [14].

Vigilant monitoring of the animals' health and the integrity of the antigens is essential during immunization. The presence of inadvertent infections or biological contaminants in the antigens can lead to contamination of the B cells, thus compromising their utility for subsequent procedures. The isolation of B lymphocytes is performed by excising the spleen of the immunized animal under stringent aseptic conditions. This organ is then processed through a density gradient centrifugation to separate the B lymphocytes. The presence of antibodies in the serum is quantified using analytical techniques such as flow cytometry or enzyme-linked immunosorbent assay (ELISA). Following this, the activated B lymphocytes are fused with myeloma cells to generate hybridomas [14].

Obtaining myeloma cell lines involves safety aspects of acquisition and storage.

The myeloma cell lines utilized as parental cells in the creation of hybridomas must be derived from the same strain of the immunized mouse (for instance, myeloma cells for BALB/c mice should originate from the BALB/c strain) and must not secrete endogenous immunoglobulin chains. Furthermore, these parental myeloma cells should be devoid of mycoplasma contamination [16], exhibit efficient fusion capacity, and facilitate the generation of stable hybridomas that consistently secrete the desired mAbs. Notably, SP2/0 and X63Ag8.653 are widely recognized myeloma parental cell lines that fulfill all these essential criteria.

In the culture of myeloma cells, hybridomas must proliferate continuously and selectively, effectively inhibiting the growth of the parental myeloma cells. The chosen myeloma cell lines should

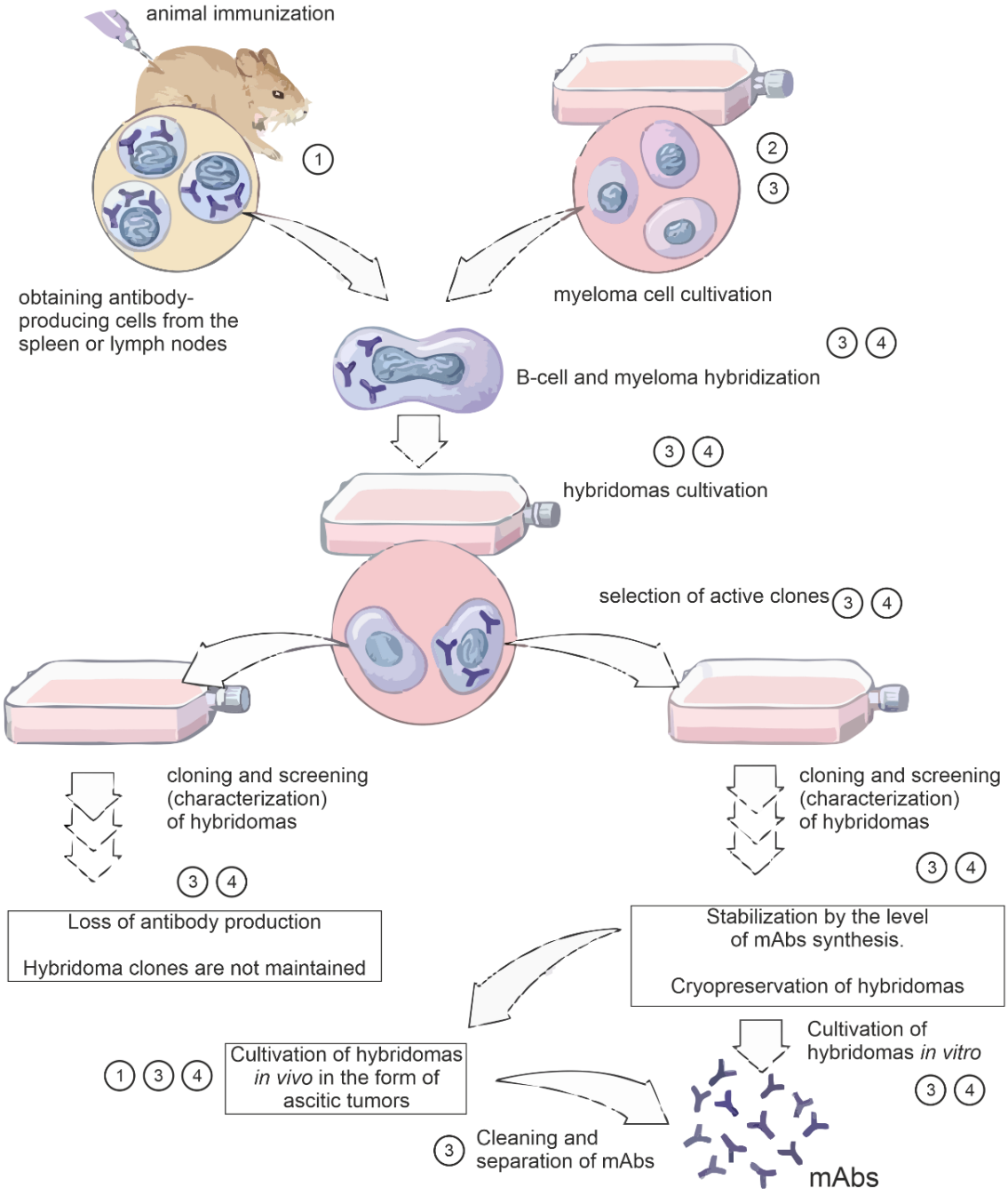

Figure: Hybridoma technology stagers and biosafety risk factors: 1 – infected animals; 2 – cell cultures microbiological contamination (bacteria, fungi, viruses); 3 – chemical and biological contamination of cultural media and reagents; 4 – hybridomas microbiological contamination (bacteria, fungi, viruses) (adapted from [15] with permission from the copyrightholder)

exhibit a deficiency in nucleotide salvage, implying a loss of the ability to synthesize nucleotides through the salvage pathway. These cells are maintained in the presence of 8-azaguanine, which prevents the synthesis of the HGPRT enzyme, a crucial component for nucleotide salvage. Parental myeloma cells are cultured for a minimum of one week prior to the fusion process. The cells are seeded at a density of approximately 5×10^4 cells/mL and subcultured every two days. For the fusion process, cells in the early to mid-logarithmic phase of growth are selected [14].

In recent years, the detection of oncovirus-like particles in myeloma cells has prompted considerable scientific debate. The discovery of Type C oncovirus particles within hybridoma cells derived from BALB/c myeloma cell lines suggests that ascitic fluids obtained from *in vivo* cultures of these cells, or supernatants harvested from *in vitro* hybridoma cell cultures, may be potentially contaminated with retroviruses. Xenotropic retroviruses, in particular, are of notable concern due to their exclusive ability to infect foreign hosts, thereby posing a risk when working with human tumor xenografts in murine models [17, 18].

Myeloma cell lines that are not actively engaged in hybridoma formation may be preserved through cryopreservation in an appropriate medium, under carefully controlled conditions. These cells should be stored at low temperatures, typically at –80 °C or lower, to maintain their viability over extended periods. Prior to freezing, myeloma cells are commonly treated with a cryoprotectant solution to mitigate the risk of ice crystal formation, which can induce cellular damage. It is essential to maintain a stable pH within the range of 7.2–7.4, closely approximating the neutral pH of human blood, as this is critical for the preservation and longevity of the cells. However, it is important to acknowledge that, under specific circumstances such as when stored in alternative buffer solutions or during certain experimental protocols, adjustments to the pH of the storage medium may be necessary. In such instances, it is imperative to ensure that the appropriate pH is maintained during the freezing process to prevent substantial deviations that could negatively impact the viability and integrity of the myeloma cells. Moreover, these cells should be stored in specialized containers or cryovials that are designed to prevent contamination and ensure the secure and reliable long-term storage of the samples.

Regular surveillance of the storage conditions of myeloma cells, coupled with systematic evaluations of their biological activity, is crucial to maintaining their integrity. Another critical aspect of their preservation is the prevention of biological contamination, which plays a pivotal role in ensuring their long-term viability and suitability for subsequent applications. To mitigate the risk of contamination, a variety of methods and protocols can be employed. Sterilization, which refers to the complete eradication of all microorganisms – encompassing bacteria, viruses, fungi, and other pathogens – is achieved through various techniques, such as autoclaving, ultraviolet irradiation, and gas sterilization, among others. In contrast, disinfection involves the reduction or elimination of microorganisms on surfaces of equipment, utensils, and other materials in direct contact with myeloma cells, utilizing chemical agents like alcohol, hydrogen peroxide, chloramines, and formalin. Additionally, it is essential to isolate and store myeloma cells in dedicated containers, ensuring that they remain segregated from other materials or samples that might pose a contamination risk. Regular monitoring for the presence of biological contaminants through testing is vital for early detection of potential issues, facilitating rigorous control over the quality and purity of the cell cultures [17].

The overarching objective in safeguarding myeloma cells from biological contamination is to maintain their optimal quality and purity, thereby enhancing their utility in both scientific and medical applications.

The cell fusion process, essential for the generation of hybridomas, involves several preparatory steps prior to fusion. Myeloma cell lines are first cultured in a medium supplemented with thymidine and hypoxanthine, collectively known as HAT medium, to create a selective environment. During fusion, activated B-lymphocytes merge with HAT-sensitive myeloma cells, typically achieved by centrifuging the myeloma cells together with freshly activated B-cells in a medium that promotes fusion. Polyethylene glycol (PEG) is commonly employed in this procedure, as it facilitates the fusion of the plasma membranes of the myeloma cells and the antibody-producing B-lymphocytes, resulting in the formation of heterokaryons – cells containing more than one nucleus. An alternative, yet more efficient, method of fusion is electrofusion, wherein cells are subjected to high-voltage or low-voltage electric pulses. This approach is generally considered more effective than the PEG-mediated fusion technique [14].

Following fusion, the next stages encompass the selection, screening, cloning, and propagation of hybridoma cells. While fusion in a PEG-containing medium yields hybridomas, the process is not entirely efficient, with only about 1-2% of the total cells resulting in hybridoma formation. Furthermore, only approximately 1 in 100 of these fused cells will be viable hybridomas. The remaining unfused cells are eliminated through selective methods. To facilitate the identification of hybridomas, the fused cell mixture is incubated and subsequently cultured for 10–14 days in HAT medium. The aminopterin component of this medium inhibits nucleotide synthesis via the de novo pathway,

while hypoxanthine and deoxythymidine enable the survival of cells with functional HGPRT genes. As malignant tumor cells that fail to fuse lack the HGPRT gene, they perish within days. Thus, the surviving cells in the medium are hybridomas that possess functional HGPRT genes derived from the B-lymphocytes, enabling them to thrive and proliferate in the selective HAT medium, ultimately facilitating their identification as viable hybridoma cells [14].

Subsequent to the HAT selection, hybridoma cells are transferred to culture plates, with each well containing a single hybridoma cell, a process achieved through the limiting dilution method. The genetic contribution from the B-cell line within these hybridoma cells enables the production of a specific antibody targeting a particular epitope, commonly referred to mAbs. It is possible that other wells may harbor hybridomas generating antibodies that are specific to different epitopes of the same antigen. Following the division and isolation of various hybridoma populations, screening is conducted to identify those producing antibodies specific to the desired epitopes of the target antigen [17].

The hybridomas that yield the desired antibodies are subsequently selected and transferred to larger culture vessels or flasks. These hybrid cultures are clonally expanded multiple times to generate a homogeneous population of clones, which are then utilized for the production of mAbs. Additionally, the selected hybridomas can be cryopreserved in liquid nitrogen, stored in ampoules, for later use in future experiments. Cryopreserved hybridomas are thawed and recultured only when the need for fresh mAbs arises. Regular assessments of the quality of the produced antibodies are highly recommended, as long-term storage may lead to a decline in the viability and functionality of the hybridoma cells over time [14, 17].

Hybridoma cell cultivation may be performed through both *in vivo* and *in vitro* methods, with the cells being maintained within an appropriate culture medium.

The *in vivo* approach involves utilizing mice for the production of mAbs. A range of approximately 10^5 to 10^{10} viable hybridoma cells is intraperitoneally injected into the animals. Following a period of several weeks, ascitic fluid is harvested from anesthetized mice. However, the ascitic fluid typically contains contamination from mouse-derived immunoglobulins, necessitating subsequent purification procedures to isolate the mAbs. In cases where antibody purity is of paramount importance, the *in vivo* method may be less advantageous due to this contamination.

The injection of a significant number of hybridoma cells is linked to an increased incidence of morbidity in the animals. Therefore, careful daily monitoring of the animals is required, alongside regular assessments of their body weight. A weight gain exceeding 20% of the animals' initial body weight should be prevented. Special attention must be directed towards detecting signs of pain or distress, such as a hunched posture, rough fur, and impaired mobility. Additionally, any respiratory distress or indications of shock, such as pale eyes and ears, along with lethargy, must be vigilantly observed. Should any of these symptoms manifest, the animals should be euthanized prior to the fluid collection procedure to mitigate suffering [19].

During the application of the *in vivo* method, there exists the potential for contamination of ascitic fluid preparations, which contain mAbs, by endogenous rodent viruses. It is particularly likely that hepatotropic viruses, which are shed into the peritoneal cavity during either the acute or chronic phases of infection, may contaminate the ascitic fluids. Furthermore, any infection that induces viremia has the potential to introduce viral contamination into the ascitic fluid through blood leakage during the collection process. To mitigate the risk of ascitic fluid contamination, it is essential to conduct comprehensive screening of laboratory animals prior to initiating cultivation. Any animals exhibiting signs of illness should be isolated in separate facilities until they have fully recovered [4, 20].

Alternatively, the *in vitro* method is employed for hybridoma cell cultivation under controlled laboratory conditions. This approach involves growing hybridoma cells in a culture medium, followed by the isolation of mAbs from the medium. The *in vitro* method is generally preferred for hybridoma cell cultivation due to its reduced likelihood of contamination, resulting in the production of highly pure antibodies.

Cell culture processes, however, are susceptible to contamination by extraneous agents, primarily bacterial and viral pathogens. Viruses, in particular, present a significant concern as they are often more difficult to detect compared to other microbial contaminants and may potentially propagate human pathogens when mammalian cell cultures are involved. To minimize contamination risks, it is recommended to adhere to the following guidelines: select appropriate starting materials and raw materials with a minimal risk of adventitious viral contamination; test cell banks and production materials for the presence of known viruses; and incorporate strategies for the removal and inactivation of poten-

tial adventitious and endogenous viral contaminants during the product purification stages [14, 21].

Issues in cell culture identification: methods and means

The identification of hybridoma cells presents a series of challenges that must be meticulously addressed to ensure accurate results. One of the most prevalent issues arises from the morphological and phenotypic similarities between hybridoma cells and their parental counterparts, which complicates the process of distinguishing between them, particularly when researchers are not fully acquainted with the distinct characteristics of the progenitor cell lines.

Furthermore, hybridoma cells are often characterized by genomic instability, which may manifest as chromosomal abnormalities, such as the presence of supernumerary chromosomes or aneuploidy, wherein cells exhibit an abnormal chromosomal count. Such genetic instability can further hinder accurate identification.

Another complicating factor in the identification process is the potential for contamination. Exogenous genetic material from unintended sources may inadvertently integrate into the hybridoma cell, leading to erroneous identification. To minimize the risk of contamination, it is imperative that strict adherence to established protocols for sample collection, handling, and processing is maintained [8, 17]. Additionally, quality control assays, including those specifically designed to detect unique genetic markers, are essential tools for identifying the presence of contamination.

Accurate identification of hybridoma cells thus requires the employment of diverse and complementary techniques. For instance, molecular genetic approaches such as polymerase chain reaction or fluorescence *in situ* hybridization can be utilized to assess the genetic composition of the cells, enabling precise identification. Furthermore, cell culture methodologies, such as morphological analysis and cell cycle assessment, are integral in examining the characteristic features of the cells. A critical aspect of the hybridoma identification process is the selection of viable cells, which can be efficiently achieved through the application of various selection techniques tailored to the specific needs of the study.

Light scattering and red fluorescence criteria are employed to facilitate the precise identification of viable hybridoma cells. The signals derived from two fluorescence detectors within a fluorescence-activated cell sorting (FACS) system are processed through a two-color compensation network, enabling the separation of independent signals corresponding to cell fluorescence (red) and microsphere fluorescence (green). Following this, live cells, distinguished by light scattering and autofluorescence characteristics, can be sorted based on the number of bound microspheres by utilizing the green fluorescence channel in the FACS apparatus [22].

The membrane-type immunoglobulin-directed hybridoma screening (MIHS) method represents a fluorescently labeled approach for the detection of hybridomas. In this method, a screening peptide selectively binds to membrane-bound immunoglobulins expressed by antibody-secreting hybridomas. Fluorescently tagged hybrids, which are anticipated to produce antibodies specific to particular antigens, are subsequently isolated and expanded through flow cytometry techniques [23].

The most frequently employed methodology for determining the concentration and viability of hybridoma cells is exclusionary microscopy utilizing trypan blue. This technique capitalizes on the ability of cells with intact membranes (designated as viable cells) to exclude the dye, whereas cells that absorb trypan blue are classified as non-viable. Additionally, the incorporation of fluorescent dyes such as propidium iodide, in conjunction with annexin V or caspase-5, in multiparameter flow cytometry allows for the quantitative evaluation of live and dead cells, as well as the identification of apoptotic populations. This approach can be effectively conducted using relatively straightforward 96-well plate-based systems along with specially developed assay kits for comprehensive analysis [17, 24, 25].

Western blotting represents a widely utilized technique for the identification of hybridoma candidates, clones, subclones, and mAbs that specifically bind to one or multiple proteins of a defined molecular weight. Proteins are initially separated via SDS-polyacrylamide gel electrophoresis, wherein they migrate according to their molecular mass. Subsequently, the proteins are transferred onto a membrane composed of nitrocellulose or polyvinylidene difluoride. The membrane, now bound to the proteins, is first incubated with either a known or investigational antibody reagent and subsequently exposed to a secondary antibody conjugated to an enzyme or substrate for colorimetric detection, or alternatively labeled with a radioactive isotope for further analysis [26].

Specific problems of monoclonal antibody purification

The advent of hybridoma technology for the production of mAbs has not only facilitated significant advancements in immunology but has also proven to be indispensable across nearly all domains of biomedical research. In particular, this innovation has greatly impacted *in vivo* diagnostic techniques and various therapeutic interventions, including anti-tumor therapies, immunomodulation, and passive immunization. A substantial number of mAbs are now available for clinical use or in the process of undergoing rigorous testing. However, akin to other breakthroughs in contemporary biotechnology, the widespread application of mAbs has prompted new concerns regarding their safety profile. The potential for adverse effects and complications necessitates thorough preclinical and clinical toxicological evaluations, along with comprehensive risk assessments. Such assessments are also mandated by regulatory bodies in order to grant approval for their incorporation into medical practice. The primary sources of potential risks associated with monoclonal antibody administration include contaminants arising from preparation or manufacturing processes, intrinsic properties of the immunoglobulin molecules, and the host's immune response to the introduced protein [17, 27].

Potential contaminants in mAbs include viruses, DNA components, biologically active substances such as growth factors, and various other immunogenic entities. Of these, the most pressing concerns are contamination with pathogenic viruses and residual DNA. There have been documented cases of viral contamination in biological preparations intended for human administration, such as the presence of avian leukosis virus (ALV) and hepatitis B virus (HBV) in the yellow fever vaccine, as well as Simian virus 40 (SV40) in the polio vaccine. These instances underscore the importance of demonstrating the efficiency of the purification process in removing or deactivating a broad spectrum of viral strains.

The purification protocol must consistently produce a final product suitable for human therapeutic use, ensuring the effective removal of contaminants, including DNA, adventitious and endogenous viruses, endotoxins, aggregates, and other impurities, while maintaining an acceptable yield. Furthermore, it is imperative to address and eliminate contaminants that may arise during the purification process itself, such as residual Protein A, substances leached from chromatography resins and filters, process-related buffers, and agents (e.g., detergents) employed for viral reduction [17].

The initial phase of antibody isolation entails the collection of the harvested cell culture supernatant, achieved by the removal of cells and debris, resulting in a clarified and filtered solution appropriate for subsequent chromatography. Typically, this is achieved through techniques such as centrifugation, depth filtration, and sterile filtration, although alternative methods may be employed depending on the scale and specific capabilities of the production facility [6–9, 27, 28].

The purification techniques employed at this stage are among the most efficient. Centrifugation, in combination with depth filtration, has been a longstanding approach for the preliminary recovery of mAbs, demonstrating suitability for both pilot-scale and industrial-scale production. Continuous disk centrifuges effectively remove cells and large cellular debris; however, the process may induce cell damage, particularly when the feed material consists of low-viability cell culture fluid. Additionally, centrifugation is limited in its capacity to remove submicron-sized particles.

Depth filtration, a standard method for clarifying cell culture broths, serves to augment membrane filter performance or protect chromatography and viral filters. These filters, typically composed of cellulose, diatomaceous earth, and ion-exchange resins, operate through a combination of size exclusion and adsorptive binding mechanisms to facilitate separation. They are frequently employed post-centrifugation due to the limitations of centrifugation in effectively removing smaller particles [6].

Membrane and filtration technologies are integral to the isolation and purification of mAbs and other recombinant DNA-derived products, spanning the entire process from initial broth clarification to the final sterile filtration of purified bulk solutions [27, 28].

Flocculation/Sedimentation: The accumulation of cell debris and colloidal material in cell culture fluids may overwhelm the clarification process, potentially leading to an increased demand for surface area in depth filters that exceeds the processing capacity of available filtration equipment. To mitigate this challenge, recent investigations have focused on pre-treatment strategies based on sedimentation and flocculation.

Flocculation in suspension cultures, as well as selective flocculation of cell contaminants from soluble proteins, is commonly achieved through the use of acidic or cationic polyelectrolytes. These polyelectrolytes function by adsorbing onto particu-

late matter, thereby creating oppositely charged surface domains. As a result, particles can form aggregates through electrostatic attraction between complementary charged regions on the surfaces of interacting particles. The predominant mechanism for polymer adsorption onto cells and cellular debris is electrostatic interaction. Positively charged polyelectrolytes are typically more effective in facilitating cell flocculation, whereas neutral or negatively charged polymers often exhibit limited efficacy in this regard [9, 28].

In the biopharmaceutical industry, chromatography remains a pivotal and widely employed technique for the separation and purification of biomolecules, owing to its outstanding resolution capabilities. This method leverages the inherent physical and chemical differences between biomolecules to achieve effective separation. For the purification of mAbs, protein A-based chromatography is a cornerstone technique, offering high purity and recovery in a single-step process. To further refine the product, one or two additional chromatography steps, such as cation exchange and anion exchange chromatography, are typically utilized as polishing stages. These steps enhance the removal of residual impurities, including viruses, host cell proteins, DNA, aggregates, and unwanted product variants. Additionally, to ensure adequate viral clearance, low-pH hold chromatography is commonly incorporated following protein A purification and viral filtration stages.

Affinity chromatography, the most selective form of chromatography employed in biotechnology, isolates proteins based on reversible interactions with a specific ligand covalently attached to the chromatographic matrix. This approach serves as an excellent capture phase in the broader purification process.

The robust interaction between protein A and the Fc region of immunoglobulin G (IgG) antibodies forms the basis for the efficient purification of IgG, its fragments, and subclasses. In the context of protein A chromatography, the standard procedure involves the passage of clarified cell culture supernatant through a column under mildly acidic to neutral pH conditions (pH 6–8). During this process, antibodies specifically bind to the column, while unwanted contaminants such as host cell proteins, cell culture media components, and potential viral particles are allowed to pass through. An intermediate wash step is typically employed to remove nonspecific impurities bound to the matrix, followed by elution of the purified product at an acidic pH range (pH 2.5–4.0) [8, 27].

Ion exchange chromatography is frequently incorporated in monoclonal antibody purification protocols, often as a critical step in the process. This technique offers substantial selectivity in separation while utilizing relatively cost-effective resins, making it suitable for both early and late stages of purification. For antibodies with a basic isoelectric point (pI), cation exchange chromatography may be utilized as a capture step, although it is more commonly employed as a polishing stage following protein A affinity chromatography. Ion exchange chromatography is particularly effective in reducing high molecular weight aggregates, charge variants, residual DNA, host cell proteins, leached protein A, and viral contaminants [6].

Anion exchange chromatography, which involves the use of resins containing immobilized positively charged groups, can be either weakly basic (e.g., diethylaminoethyl cellulose) or strongly basic (e.g., quaternary aminoethyl, trimethylammonium ethyl, or quaternary aminoethyl). This methodology is a highly effective tool for the removal of impurities associated with the manufacturing process, such as host cell proteins, DNA, endotoxins, and leached protein A. Additionally, it is efficient in targeting product-related impurities, including dimers and aggregates, endogenous retroviruses, and adventitious viruses such as parvovirus and pseudorabies virus. Depending on the specific pH characteristics of both the antibody and the impurities, anion exchange chromatography may be applied in either flow-through or bind-and-elute modes [6].

Culture media biosafety

Culture media are integral to the successful production of mAbs, serving as essential components for supporting the growth and maintenance of hybridoma cells. The formulation of media for batch processes involves the development of both batch media and feed concentrates. A variety of systematic approaches can be employed to optimize media formulations, including single-component titration, spent media analysis, and mixing techniques. From a biosecurity perspective, to mitigate potential risks associated with transmissible spongiform encephalopathy and other contaminants, the use of bovine serum and animal-derived raw materials should be minimized wherever feasible. After two decades of continuous refinement in media composition and host cell adaptation, fully defined chemical media have been established, incorporating amino acids, vitamins, microelements, inorganic salts, lipids, and insulin or insulin-like

growth factors, and have proven effective in large-scale monoclonal antibody production. However, it is noteworthy that not all antibody-producing cell lines can achieve substantial yields when utilizing chemically defined media alone. Consequently, a common strategy to enhance cell density, viability, and overall productivity is the supplementation of chemically defined media with animal component-free hydrolysates [16, 27, 28].

The principal nutrient media used in hybridoma generation include RPMI-1640 and Dulbecco's Modified Eagle Medium (DMEM), which are employed either individually or in combination after reconstitution in universal containers and equilibration with atmospheric air. The key distinction between the two media lies in the presence of specific components; RPMI-1640 lacks pyruvate, whereas DMEM does not contain asparagine [29–32].

The biosecurity of culture media is of paramount importance in hybridoma technology for monoclonal antibody production, as improper handling and contamination can compromise both environmental safety and the integrity of monoclonal antibody preparations. To safeguard against such risks, adherence to stringent biosecurity principles is essential throughout the cultivation process.

The maintenance of stringent sterility protocols is essential to ensure the integrity of nutrient media in hybridoma cell cultivation. It is paramount to prevent microbial contamination, as microorganisms may adversely affect cellular growth and pose potential health risks to humans.

Typically, the individual components of the media are prepared as stock solutions in autoclaved containers, and sterilization is achieved via autoclaving or sterile filtration. Following this, the stock solutions are stored at 4 °C, requiring aseptic handling to preserve their sterility. In the event of microbial contamination, such as fungal or bacterial growth, the contaminated stock solution must undergo re-autoclaving, and its contents should be discarded [33, 34].

The conventional method for sterilizing liquid nutrient media involves autoclaving at 121 °C for 15 minutes, followed by a cooling phase. It is critical to examine the appearance of the media post-sterilization, as any changes in color or the formation of precipitates indicate that the medium is no longer suitable for use. Various factors can contribute to such changes, necessitating a comprehensive review of the preparation process. Heat-sensitive components, such as vitamins, are typically introduced post-autoclaving, using a filtration method [35, 36].

Microwave sterilization presents an alternative technique for the sterilization of media. In this approach, the achieved temperature remains below 84 °C, which permits the addition of certain unstable components before microwave treatment. However, vitamins should only be incorporated after the microwave sterilization process is complete. This method is rapid, typically requiring no more than 10 minutes, and circumvents metal contamination that may occur during autoclaving. Nevertheless, microwave sterilization is best suited for small volumes of media (≤1–1.5 liters). Additionally, such sterilization procedures should be performed after nutrient media usage to eradicate any residual microorganisms that may persist in the environment.

The application of antiseptics is an integral aspect of the cell cultivation process within nutrient media. Instruments and surfaces must be treated with antiseptics to prevent the inadvertent introduction of contaminants into the system [36].

Biosafety cabinets offer an effective means of controlling the risk of contamination during the handling of nutrient media. In addition to this, the proper storage and transportation of nutrient media are imperative to prevent contamination and degradation. Storage should be maintained under optimal temperature conditions (2–8 °C) and appropriate humidity levels to ensure both sterility and quality. Furthermore, it is essential to adhere to the expiration dates specified for each nutrient medium, as their efficacy cannot be guaranteed beyond this period [28].

Biosafety aspects of the use of animals in hybridoma technology

The process of hybridoma generation exploits the inherent capacity of the host organism to produce functional, highly specific, and high-affinity mAbs. Presently, numerous mAbs have been developed through this technology and are extensively utilized for diagnostic, preventive, and therapeutic purposes across a spectrum of diseases. Initially, the scope of hybridoma technology was predominantly confined to the generation of antibodies specific to mouse antigens; however, with significant advancements in the field, the technology has expanded to include other species, such as rabbits, chickens, goats, sheep, cows, guinea pigs, and rats, as sources for monoclonal antibody production. The selection of an appropriate animal species for monoclonal antibody generation is influenced by a variety of critical factors. These include the pres-

ence of homologous proteins within the immunized species, the availability of a compatible fusion partner, the quantity of antigen or protein available for immunization, the time frame required to elicit a robust antibody response, and the specific applications for which the resultant mAbs are intended. Mice remain the most commonly utilized species for monoclonal antibody production, followed by rabbits. Additionally, chickens are considered a viable alternative, given their clear phylogenetic connection between the antigen source and antibody producer. However, a significant challenge in generating mAbs across different species arises from the inherent instability of hybridoma clones, particularly those resulting from the fusion of cells originating from heterologous species. This instability often manifests as chromosomal aberrations in the hybridoma clones, complicating their use in subsequent applications. To address this issue, various strategies have been developed in recent years aimed at enhancing the efficiency and stability of hybridoma fusion, thereby improving the overall success rate of monoclonal antibody production [38, 39].

Animal-related research inherently involves various risks that can be broadly categorized into chemical, biological, physical, and radiological hazards. Many experimental protocols frequently encompass one or more of these categories, necessitating a comprehensive risk assessment to evaluate the distinct risk profiles, the simultaneous presence of multiple hazards, and the most effective strategies for protecting both personnel and animals involved in the research process.

The chemical substances employed in research possess potential hazards for both human researchers and animals, necessitating rigorous control over the quantitative and qualitative composition of all substances. Agents used for cleaning and disinfecting environments, such as floors, walls, and animal enclosures, may pose significant risks to workers and necessitate enhanced precautionary measures, including the use of personal protective equipment. Chemical hazards also extend to the application of anesthetic gases and euthanasia agents, many of which are regulated as controlled substances [40].

A particularly concerning risk in certain animal research models is the exposure to allergens and zoonotic diseases, which can affect researchers. Engineering controls represent the most effective means of mitigating the risks associated with allergens and other biological hazards encountered in animal research. The technical control measures implemented within animal facilities often include chemical fume hoods, biological safety cabinets, downdraft tables, changing rooms, and animal transfer stations. Additionally, the use of rooms equipped with high-efficiency particulate air (HEPA) filters and ventilated animal racks is essential for ensuring personnel safety. Zoonoses, which are particularly prevalent in large animal models such as sheep, cattle, and primates, pose a significant threat, as healthy animals may harbor endogenous infections. As such, bites or scratches from animals in non-infectious disease models can potentially transmit pathogens. Researchers working with animals that may carry infectious agents are required to undergo extensive training, which includes education on recognizing signs and symptoms indicative of potential infections [4, 28, 40].

Following the completion of research, the disposal of animal carcasses used in monoclonal antibody production is subject to stringent safety protocols and environmental protection regulations. The methods of disposal are varied and depend on the specific safety requirements of the research. Common practices include cremation or incineration at specialized waste disposal facilities. These procedures are carried out with the utmost adherence to safety standards to prevent the spread of diseases or environmental contamination.

Improper disposal can lead to hazardous chemical reactions and the release of toxic substances, which may contaminate air, water, and soil, thereby presenting risks to both human populations and other animals in the vicinity. Furthermore, animal carcasses may harbor pathogenic microorganisms or other biological agents that could serve as sources of disease transmission and pose ecological threats.

Safety of monoclonal antibody-based products

The primary technologies employed for the generation of mAbs include hybridoma and recombinant technologies. mAbs derived via hybridoma technology serve as invaluable tools in diagnostics, biomedical research, and select therapeutic applications. However, their direct clinical use is significantly constrained by immunological, technological, and regulatory limitations. The key factors preventing the therapeutic application of hybridoma-derived antibodies include immunogenicity, compatibility issues, suboptimal physicochemical properties, and ethical concerns. Consequently, recombinant mAbs, which overcome these inherent limitations, are preferentially utilized for therapeutic purposes.

The most critical contaminants in mAbs-based products for therapeutic application are endotoxins, viruses and mycoplasma, ranked from highest to lowest severity, which pose substantial risks to product safety, efficacy, and regulatory compliance. The most critical contaminants in monoclonal antibody-based products for non-therapeutic application are viruses and mycoplasma.

The issue of contamination in therapeutic mAbs extends beyond the scope of this study, as the present work is primarily dedicated to the biosafety aspects of hybridoma technology. Regarding mAbs produced via hybridoma technology, the most probable sources of contamination include the cultivation stage of antibody-producing cell lines and the final formulation/packaging stage of the finished product.

To mitigate bacterial contamination, including mycoplasma, hybridoma technology employs antibiotic supplementation and strictly aseptic conditions during cell culture and processing. The presence of endotoxins does not exert a significant impact on the final monoclonal antibody preparations obtained through hybridoma technology. However, an exception is mAbs specifically targeting endotoxins, where specialized approaches and advanced analytical biotechnologies are required to ensure purity and efficacy.

Our experimental findings indicate that proteinaceous contamination in monoclonal antibody preparations can be effectively eliminated through gel filtration, utilizing sorbents with distinct physicochemical properties. This methodological approach has demonstrated significant improvements in the performance parameters and analytical sensitivity of ELISA test kit developed on the basis of mAbs.

Conclusions

This study provides a comprehensive analysis of biosafety aspects associated with hybridoma technology, emphasizing contamination risks and mitigation strategies in monoclonal antibody production. The findings underscore the necessity of strict biosafety measures at all stages of hybridoma technology to ensure the production of high-quality, contamination-free mAbs suitable for biomedical applications.

The research highlights that viral and bacterial contamination, particularly from rodent-borne pathogens, remains a significant challenge in both in vivo and in vitro hybridoma cultivation. The study identifies key contamination sources, including cell lines, culture media, and laboratory animals, necessitating rigorous screening, purification, and quality control measures. To minimize contamination risks, it is essential to employ validated biosafety protocols, such as polymerase chain reaction testing, nanofiltration, and viral inactivation techniques.

The study also demonstrates that hybridoma-derived monoclonal antibodies require extensive purification to eliminate adventitious agents, host cell proteins, and nucleic acid residues. The implementation of affinity chromatography, ion-exchange chromatography, and low-pH virus inactivation has proven effective in enhancing product purity and biosafety. Furthermore, the integration of chemically defined culture media significantly reduces the risk of contamination compared to traditional serum-based media, aligning with global biosafety recommendations.

From an ethical and regulatory perspective, the research underscores the importance of adherence to WHO biosafety standards, particularly in animal handling, cell line validation, and laboratory containment practices (BSL-2 and BSL-3). The study recommends enhanced monitoring of laboratory animals, implementation of engineering controls such as HEPA filtration, and strict waste disposal protocols to mitigate zoonotic and environmental risks.

Interests disclosure

The authors report that there is no conflict of interest.

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БІОБЕЗПЕКОВІ АСПЕКТИ ГІБРИДОМНОЇ ТЕХНОЛОГІЇ: ПРИРОДА РИЗИКІВ, МЕТОДИ ТА ПІДХОДИ ДО УПРАВЛІННЯ НИМИ

Дослідження висвітлює аспекти біобезпеки гібридомної технології, зосереджуючись на ідентифікації та управлінні пов'язаними ризиками. Моноклональні антитіла, необхідні інструменти в імунології, біотехнології та медицині, виробляються за допомогою гібридомної технології. Цей процес передбачає злиття В-лімфоцитів імунізованих тварин із клітинами мієломи для створення гібридом, які потім культивують для отримання специфічних антитіл. Дослідження підкреслює значні ризики зараження, особливо вірусами, які переносяться гризунами, та іншими патогенами, під час культивування як *in vivo*, так і *in vitro*. Систематично проаналізовано існуючі стратегії виявлення та пом'якшення цих ризиків на різних етапах виробництва моноклональних антитіл, включаючи ідентифікацію гібридом, злиття клітин і очищення антитіл. Дослідження підкреслює важливість суворих протоколів біобезпеки та оптимізованих методологій очищення для забезпечення виробництва високоякісних моноклональних антитіл без забруднень. Крім того, необхідними є комплексна оцінка ризиків і впровадження передових систем контролю забруднення в лабораторіях. Висновки, зроблені в результаті дослідження, дають цінну інформацію про підвищення безпеки та ефективності виробництва моноклональних антитіл. Вивчаючи ці проблеми біобезпеки, дослідження підтримує широке застосування моноклональних антитіл у науковій та медичній сферах, забезпечуючи їх надійність і ефективність у різних діагностичних і терапевтичних контекстах.

Ключові слова: гібридомна технологія; моноклональні антитіла; біобезпека; ризики зараження.