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Review Article

Research and progress in the preparation of monoclonal Rh blood group antibodies

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ABSTRACT

The Rh blood group system is considered significant within human blood group classification, alongside the ABO system known for complexity. Studies have shown that the Rh group protein antigen is encoded by the *RHD* and *RHCE* genes, which are responsible for the synthesis and expression of more than 50 Rh antigens. Nevertheless, the five most clinically significant antigens are RHD, RHC, RHc, RHE and RHe. Currently, various Epstein-Barr virus (EBV) immortalized human-mouse or human-human hybridomas with multiple Rh antigens have been established internationally. These hybridomas exhibit notable stability and specificity, making it commonly utilized in clinical applications such as blood transfusion, blood group identification, and management of pregnancy-related conditions. This paper provides a comprehensive review of the research and development related to the preparation of Rh blood group antibodies domestically and internationally. The findings will contribute to the research foundation of Rh blood group antibodies and production of cell banks.

Keywords: Rh blood group system, EBV immortalization, hybridoma, monoclonal antibody, clinical diagnosis

INTRODUCTION

Based on the nomenclature principles established by the International Society of Blood Transfusion (ISBT) for red blood cell (RBC) surface antigens, a total of 366 RBC blood group antigens have been identified and confirmed to date[1], categorized into 47 blood group systems (containing blood type collections). Among these, the Rh blood group system is considered a highly intricate system, with the encoding genes locating on chromosome 1. Rh antigens are multimeric protein complexes, with the *RHD* and *RHCE* genes being the central components[2]. However, the recombination and mutation of genes may result in the generation of various antigens, among which D, C, c, E, and e antigens are frequently observed[3]. Notably, the RHD antigen exhibits the strongest immunogenicity, and its corresponding anti-D antibody is the primary cause of neonatal hemolytic disease. In contrast, the immunogenicity of C, c, E, and e antigens is relatively weaker. Antibodies against these antigens may induce the occurrence of neonatal hemolytic disease and pose challenges in clinical transfusion. Given these clinical implications, it is of great clinical significance to focus on developing antibodies corresponding to antigens within the Rh blood group system.

The preparation of Rh blood group antibodies can expand the options for clinical RBC detection. Clinical blood group detection reagents, particularly those for the Rh blood group system, are predominantly supplied by manufacturers in European

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and American countries. These imported reagents are not only costly but also frequently face supply chain limitations. Currently, there are no maturely developed Rh antibody reagents being utilized in China. Research indicates that individuals of foreign descent with Rh-negative blood account for approximately 15%[4–5], compared to only 3%–4% of individuals with Rh-negative blood in the Chinese population. As a result of advancements in bioethics and legal provisions, Rh monoclonal antibodies have been prepared using improved hybridoma technology. Initially, Rh blood group antibodies were sourced from B cells of donors with high titers of Rh antibodies *via* Epstein-Barr virus (EBV) transformation and amplification, but this method yielded limited quantities of low-quality antibodies[6–8]. The researchers enhanced the methodology by fusing EBV-transformed B cells with either mouse myeloma or human lymphoblasts. Hybridoma cells secreting Rh antibodies were formed; however, the stability of antibody secretion by hybridoma cell lines was found to be suboptimal[9–14].

In recent years, Chinese academics have conducted extensive research on antibodies in the Rh blood group system. Following standard EBV transformation and fusion procedures, the researchers removed T cells to obtain monoclonal anti-Rh hybridoma cell lines. However, these hybridoma cell lines have not yet been implemented for large-scale antibody production[15–17]. Presently, most EBV-transformed B cells are derived from human peripheral blood. Wilson *et al*. discovered the presence of B cells capable of producing Rh blood group antibodies in the umbilical cord blood of newborns[18–21]. Alternative approaches include the generation of Rh blood group monoclonal antibodies through animal immunization[22–25]. International researchers have also utilized genetic engineering modification techniques in the production of monoclonal antibodies, including phage display technology and single-cell BCR sequencing technology[26–30].

CLINICAL APPLICATION OF RH BLOOD ANTIBODIES

One primary role of the human immune system is the production of antibodies by B cells. Nowadays, the predominant antibodies employed are either IgM class or IgG/IgM mixtures[31]. This phenomenon occurs due to the pentameric structure of IgM antibodies, which enables direct visible agglutination with RBCs. In contrast, the antibodies frequently utilized in clinical settings to prevent the alloimmune response of RhD are typically IgG, a safer option.

The production of antibodies in the Rh blood group system primarily occurs during pregnancy and blood transfusion. Transfusing blood with an incompatible Rh phenotype may trigger adverse transfusion reactions such as delayed hemolytic reactions and irregular antibody production[32–33]. So far, the Rh blood type phenotype library has been established clinically, facilitating expedited clinical treatment procedures. Meanwhile, it decreases the incidence of hemolytic blood transfusion reactions and neonatal Rh hemolytic disease, while also enhancing the safety of blood transfusion[34–35]. Subsequently, developing a rare Rh blood type phenotypic bank can enhance transfusion support for special populations.

PREPARATION METHODS FOR RH BLOOD GROUP MONOCLONAL ANTIBODIES

Hybridoma technology

Murine hybridoma

Mice are frequently utilized as immunized animals due to their traits of high reproductive rates, ease of breeding, and clear expression of immunological responses. Early studies showed that Rh-negative human RBCs (ccdee type) were employed to immunize BALB/c mice, followed by the collection of spleen cells for fusion with mouse myeloma cell SP2/0 using polyethylene glycol (PEG). The resulting murine monoclonal antibodies showed the capacity to secrete RhE antibodies[36]. However, preparing monoclonal antibodies for blood type detection through mouse immunization with human Rh blood group antigens remains challenging. The effectiveness of murine immunoglobulin preparations is limited by the induction of anti-mouse immune responses, which may be attributed to the week immune response of mice to Rh blood group antigens.

Karl Landsteiner and his colleagues utilized immunized guinea pigs and rabbits with rhesus monkey RBCs, obtaining an immune serum that demonstrated dual reactivity — agglutinating both rhesus monkey RBCs and a subset of human RBCs[37]. In subsequent research, Abraham CV and Bakerman S observed variations in the titers of Rh typing antibodies present in serum by intraperitoneal injection of Rh typing antigens into guinea pigs[23–24]. Another investigation compared antibody production in multiple species by injecting purified human γ-globulin into rabbits, goats, sheep, and horses, revealing that antibodies were generated first in

rabbits, reaching a peak concentration of 1.7 to 6.4 mg/mL[22]. Therefore, guinea pigs and rabbits have become increasingly favored for studying animal immunity due to their size and antibody production advantages. With advances in hybridoma technology[25,38], researchers have observed that spleen cells derived from rabbits exhibit a larger size and are capable of generating antibodies with high specificity and affinity. The rabbit myeloma cell line 204E-W2 shows particularly high fusion efficiency with rabbit B lymphocytes. The resulting rabbit hybridoma cells have no endogenous IgG expression while demonstrating enhanced genetic stability and IgG secretion capacity. However, there is a scarcity of literature reports concerning guinea pig fusion ligand. Subsequently, attempts can be made to fuse immunized animal B lymphocytes with mouse myeloma cell lines (SP2/0, X63-Ag8.653) or with human myeloma cell line (GM1500) and lymphoblastic cell line (WIL2-729-HF2). Nevertheless, the antibody stability remains uncertain, warranting additional investigation.

Human-mouse hybridoma

EBV can immortalize donor B cells, enhancing hybridization frequency by over tenfold[7]. Researchers from both domestic and international institutions have been committed to seeking fusion ligands that target EBV immortalized human B cells. They found that mouse myeloma cell lines (SP2/0 and X63-Ag8.653) and somatic cell B lymphoblastic hybridoma SHM-D33 could be fused with human B cells secreting human RBC Rh blood group antibodies through either PEG or electrode methods to form human-mouse hybridoma cell lines[13–14].

There is a low prevalence of Rh-negative pregnant women in China. Postpartum women who develop Rh antibodies through passive immunity are indeed uncommon. This scarcity presents significant challenges in obtaining suitable B cells for antibody preparation. Despite occasional sightings, the level of Rh antibodies is typically very low. These observations indicate that the concentration of antibody-secreting B lymphocytes in blood is also minimal. Consequently, the final harvest of B lymphocytes is unable to meet the required amount for direct fusion, making transformation with EBV challenging.

Recent research has identified distinctive phenotypic and functional characteristics in B cells derived from umbilical cord blood. The antigens CD1C, CD38, CD5, and CD23, normally expressed in adult circulating B cells, are similarly elevated in B cells from umbilical cord blood[18]. Wilson and Morgan *et al*. found that the absence of a primary immune response in newborns allows B lymphocytes in fetal umbilical cord blood to readily convert into lymphoblastic cell lines upon infection with EBV[20–21]. However, the *in vitro* transformation of fetal umbilical cord blood B cells by EBV varies significantly between donors. Meanwhile, the EBV transformation efficiency is related to the primary immune response involving NK cells of CD4, CD16, and CD56 in umbilical cord blood samples[20]. However, there is limited literature available on the fusion of umbilical cord blood B cells. To date, only Edelman L *et al*. have reported successful immunization of BALB/c mice with umbilical cord RBCs and fusion of spleen cells with the X63-Ag8.653 mouse myeloma cell line to generate a monoclonal antibody that reacts with human RBCs. This approach yielded monoclonal antibodies exhibiting superior agglutination capabilities against specific human RBC types compared to conventional antibodies[39].

Human-human hybridoma

While human-mouse hybrid fusion can produce a certain number of hybridomas, partial loss of human chromosomes occurs during this process[10]. This may lead to a gradual decline in the ability of hybridoma cells to produce specific antibodies over extended periods of cultivation[7]. The primary limitation of human-mouse hybridoma is the loss of human chromosomes, resulting in decreased immunoglobulin secretion. Nonetheless, the loss of human chromosomes does not occur randomly. In human-mouse hybridoma, the human chromosome 14 (carrying heavy immunoglobulin chain genes) and the human chromosome 22 (containing lambda chain genes) are preferentially preserved, while the human chromosome 2 (containing kappa chain genes) is commonly lost[9–10]. The majority of the stable hybridomas have lambda chains, whereas the ratio of the kappa chain to the lambda chain in the human body is 2:1. It can be stated that two-thirds of the Ig molecules possess kappa chains. The outcome entails the depletion of human chromosomes, especially chromosome 2. Therefore, intraspecific hybridization exhibits greater stability, making human-human hybridoma generally the preferred option.

Currently, most human fusion cells are derived from two parent cell lines, GM1500 and WIL2-729-HF2[11–12]. Research has demonstrated that the human lymphoblastic cell line WIL2-729-HF2 can fuse with human B cells, forming human-human hybridomas. While the technique remains technically challenging and exhibits relatively low fusion efficiency, the cell line WIL2-729-HF2 has a structure like myeloma and

the ability to generate a significant quantity of immunoglobulin. Moreover, the fused hybridoma can produce culture supernatants with high titers[11].

Phage display technology

In contrast to traditional monoclonal antibody technology, phage antibody libraries do not necessitate the immortalization of donor lymphocytes or species-specific cell fusion, promoting the isolation and production of human antibodies[40]. This innovative approach differs from traditional tissue culture methods by immortalizing immunoglobulin (Ig) genes rather than the originating cells. This method is distinguished by the establishment of a physical connection between the phenotype of the antibody (specifically the Fab domain binds to the antigen) and its genotype (the heavy and light chain DNA encoding a specific Fab molecule). Common bacteriophages include single-stranded DNA (M13) and double-stranded DNA (T4, T7, and λ), with M13 being commonly preferred for the display of exogenous peptides or proteins[41–42]. In general, the amplified antibody genes are cloned into the phage expression vector and transformed into competent *Escherichia coli* (*E. coli*) cells. Subsequently, supplementary bacteriophages are introduced to infect *E. coli*, facilitating the proliferation of bacteriophages within the bacteria cells. During this process, the phages within the bacteria assemble into specific antibodies or antibody fragment genes. The predominant expression systems utilized at present include mammalian cell lines, such as human embryonic kidney cells or Chinese hamster ovarian cells[43–44]. Based on these methods, researchers have successfully obtained monoclonal antibodies against human erythrocyte antigens D, E, Kp^b, B, and H in unimmunized D-positive and Kp^b-positive individuals[26,45]. Furthermore, by replacing the light chain while maintaining the RhD antibody heavy chain framework, RhE antibodies have been successfully generated[27].

Recently, nanobodies have been widely used in research fields such as molecular diagnosis, drug delivery, tumor immunotherapy, and cell therapy, attributed to their outstanding performance. In addition to conventional monoclonal antibodies, nanobodies retain the ability to specifically bind to antigens, and consist of a singular heavy-chain variable region. They possess small molecular weight, lower immunogenicity, and stronger tissue penetration ability[46–48]. The preparation of nanobodies typically involves immunizing alpacas to obtain antibody genes, followed by the screening of optimal antibody sequences from the alpaca antibody library. It was reported that the sequence homology between nanobodies and human heavy chain antibodies exceeded 80%, and demonstrated a high potential for humanization transformation[28–29]. The Fc region of Rh blood-related antibodies can be linked with nanobodies to create recombinant humanized antibodies. However, the antibodies used in clinical blood type testing are predominantly IgM, comprising of one J chain and ten light and heavy chains. These antibodies can potentially be modified through J chain-mediated conjugation with tandem nanobodies. At present, nanobody research predominantly focuses on tumor drug research and development, where specific nanomaterials such as gold nanoparticles, graphene oxide, and polymer nanoparticles have demonstrated significant advantages in tumor treatment[49]. Despite these advances, monomeric nanobodies still face limitations such as insufficient affinity and shortened half-life. These challenges can be effectively overcome through polymerized nanobodies, which may be achieved *via* two ways: (1) *in vivo* assembly using flexible linkers or functional modification within antibody domains[50]; (2) *in vitro* construction through enzymatic ligation and chemical cross-linking reagents[51]. However, nanobodies have not yet been involved in the field of blood type detection reagents. In the future, these technical methods can be leveraged for the production of Rh blood type antibodies and nanoconjugates that target multiple antigenic epitopes.

Single-cell BCR sequencing technology

The development of single-cell BCR technology is a recent innovation in the production of diverse monoclonal antibodies that have demonstrated efficacy in the treatment of partial tumors and autoimmune diseases. This technique is based on the principle that each B cell contains only one pair of functional heavy chains and light chains, producing a single unique antibody[30]. This method includes immunogen preparation, single B cell sorting, antibody gene amplification and cloning, and antibody expression and identification. A major advantage of single-cell BCR technology lies in its high-throughput capacity and preservation of naturally paired antibody variable regions. Recent studies have utilized flow cytometry to isolate human B cells that secrete antibodies specific to the Rh blood group of human RBCs. Subsequently, the single B cells underwent reverse transcription and specific cDNA was amplified and enriched. During screening and pairing process, the EMBL-EBI and Ig Blast databases were

used to annotate the antibody information obtained through sequencing. Meanwhile, the 3D structure of the antibody's variable region was predicted. Furthermore, this study also forecasted and optimized the 3D structure of the RhD antigen protein, followed by computational docking analysis between the antibody and RhD antigen. Eventually, the heavy and light chains of the target antibody were synthesized into an expression vector such as *E. coli*, and expressed through 293F or CHO expression system, ultimately yielding fully human RhD-specific antibodies[52–54]. These antibodies demonstrate strong agglutination with RhD-positive RBCs and the titers of strong positive reactions can reach up to 1 : 1024. Clinical validation confirmed that their RhD antigen detection accuracy matched that of clinically used antibodies[54]. Due to the heterogeneous composition of antibodies utilized in clinical practice, future work should focus on expanding antibodies targeting diverse RhD epitopes through clinical exploration.

SUMMARY AND PROSPECT

In transfusion medicine, the production of serological reagents using murine monoclonal antibodies presents biological limitations, primarily stemming from challenges in obtaining antibodies that target human blood group antigens. The difficulty lies in the fact that mice have a very weak or even no immune response to the Rh blood group antigens of human RBCs. Currently, the most widespread method for producing monoclonal antibodies against human blood types involves generating hybridomas from human B cells that secrete Rh blood group antibodies. Nevertheless, sourcing these B cells from patients who produce antibodies against the Rh blood group system poses significant challenges and requires ethical and informed consent. At present, phage display technology, polymerized nanobody technology and single-cell BCR technology have focused on the natural pairing and recombination of the variable regions of antibody heavy and light chains. Furthermore, single-cell BCR technology enables the isolation of antigen-specific B cells from human peripheral blood. This review comprehensively evaluates the strengths and limitations of current preparation methods and technologies for Rh blood group monoclonal antibodies both domestically and internationally. It is reasonable to anticipate an increase in the introduction of Rh blood group monoclonal antibodies into clinical settings in the future, which will inevitably face unprecedented opportunities and challenges.

Author contributions

Conceptualization, Gengyin Wang; Methodology, Jiaqi Gu, and Gengyin Wang; Writing – Original Draft Preparation, Jiaqi Gu; Writing – Review & Editing, Gengyin Wang.

Ethics statement

Not applicable.

Informed consent statement

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Declaration of competing interest

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