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Development and Characterization of EBV-Immortalized Hetero-hybridoma Monoclonal Antibody Targeting Hepatitis B Surface Antigen

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ABSTRACT

Two hundred fifty million people suffer from chronic liver disease due to the hepatitis B virus (HBV), leading to cirrhosis and hepatocellular cancer. Despite a preventive vaccine, coverage is still not sufficient and antiviral medications are often ineffective. Monoclonal antibodies (mAbs) are crucial for a targeted therapeutic approach, enhancing the ability to control and eliminate the virus. These antibodies can neutralize the virus, prevent its spread and assist the immune system in combating the illness, particularly for persistent HBV patients. In this study, peripheral blood mononuclear cells (PBMC) from 10 hepatitis B vaccinated healthy donors, lymphoblastoid cell lines (LCLs) were generated following transformation with the Epstein-Barr virus (EBV). Subsequently, clones secreting antibodies to hepatitis B surface antigen (anti-HBs antibodies) were screened. Following this, 9 LCL clones and three clones fused with mouse myeloma were generated, and the resulting human anti-HBs monoclonal antibody was purified from the supernatant. These purified antibodies were biotinylated, and the most suitable pairs for sandwich ELISA were identified. The efficacy of these antibody pairs was tested using clinical samples positive for the Hepatitis B virus. The test demonstrated a detection sensitivity of 100% and a specificity of 99.3%. Thus, diagnostic possibility using hetero-hybridoma mAbs was verified and further study can be carried out to affirm its therapeutic potential.

Key words: Hepatitis B virus, lymphoblastoid cell lines, peripheral blood mononuclear cells, sandwich ELISA

INTRODUCTION

Approximately 297 million people globally have been exposed to the hepatitis B virus (HBV), with 296 million carrying it chronically (WHO, 2022). Annually, around 900,000 individuals die from HBV-related complications, making it one of the most prevalent infectious diseases worldwide. Countries are categorized based on HBV endemicity into high, intermediate and low groups. Chronic hepatitis B (CHB) and chronic hepatitis C infections account for over 90% of deaths and disabilities due to viral hepatitis (Premkumar and Chawla, 2021). The World Health Organization (WHO) has set a goal to eliminate viral hepatitis as a public health threat by 2030, aiming for a 90% reduction in new CHB cases and a 65% decrease in CHB-related mortality. India, classified as having intermediate endemicity, has about 50 million affected individuals, with higher prevalence in tribal areas due to factors like intra-caste marriages and limited healthcare access, reaching up to 15.9% in some regions. Pregnant women show HBsAg positivity rates of 2.3-6.3% (Ray, 2017), and unsafe injections significantly contribute to the transmission of HBV, HCV and HIV in India (Altaf, 2018).

A considerable number of children in India are unvaccinated against HBV, indicating a large reservoir of infection. To meet WHO elimination targets, access to care must be improved. While some high-risk groups have treatment options, others do not. Despite advancements in antiviral drug development, complete eradication of HBV remains challenging. Treatments for CHB include immunomodulating agents and antiviral

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nucleos(t)ide analogs (NAs), which, although effective, carry risks of drug resistance (Duan *et al*., 2023**;** Yu *et al*., 2024). Current evidence-based guidelines assist physicians in managing CHB, but drug resistance complicates treatment due to limited anti-HBV options.

Progress in HBV biology has been made through reliable cell culture systems, enhancing understanding of HBV replication and persistence. New diagnostic markers for HBV have also been identified, and novel antiviral drugs targeting various stages of HBV replication are under development (Rybicka and Bielawski, 2020). Epstein-Barr virus (EBV) is utilized to immortalize B cells for producing monoclonal antibodies (mAbs), efficiently infecting B lymphocytes for continuous proliferation (Theobald *et al.*, 2023; Xu *et al*., 2023). This property can be leveraged to generate human antibodies against HBsAg, providing an alternative to hybridoma technology (Wang *et al*., 2020). The B95-8 and P3HR-1 cell lines are commonly used sources of EBV, with the P3HR-1 line yielding a non-transforming virus and the B95-8 line producing a transforming virus (Ding *et al*., 2017). Lymphoblastoid cell lines (LCLs) offer advantages over hybridoma cells, including ease of obtaining starting material and suitability for large-scale studies, making them a cost-effective method for producing human antibodies for diagnostic and therapeutic purposes.

MATERIALS AND METHODS

Samples were collected from donors at the Blood Bank (SRKRC), with consent obtained from participants who had been vaccinated against Hepatitis B. A volume of 5 ml of peripheral blood was drawn from 10 volunteers (Table 1).

Plasma from donor's blood sample was tested for antibodies against HBsAg using the indirect ELISA method. A 96-well plate was coated with 1 µg/ml HBsAg and incubated overnight. A positive control of HBV-infected serum and a negative control of healthy, uninfected serum were used.

The culture media used for culturing the B95-8 cell line, termed CM1, consisted of RPMI 1640 (Gibco), 0.1% Nystatin (5,000 U/ml) (Gibco), 50 µg/ml Gentamycin and 10% FBS (Gibco, New Zealand). A complete media termed CM2, comprising 20% FBS, was used for cell transfection. A freezing media termed FM, consisting of 60% RPMI, 30% FBS, and 10% Dimethyl sulfoxide (DMSO), was utilized for cryopreservation. B95-8 cells were cultured in CM2 at 37°C with 5% CO_2 and proliferated until the required amount of culture supernatant was obtained. The supernatant was collected and filtered to remove remaining B95-8 marmoset cells. The resulting EBV supernatant was stored at -20°C until use.

Table 1. Primary details of the donors involved in this study

Samples	Blood group	Sex	Age
D1	A^+	Male	52
D2	B^+	Female	45
D3	B^+	Male	27
D4	B^+	Male	33
D5	O^+	Female	26
D6	B^+	Female	31
D7	B^+	Female	27
D8	A^+	Female	21
D9	B^+	Female	22
D10	A^+	Male	21

All the donors were vaccinated against Hepatitis B surface antigen and free of any other infection.

PBMCs were separated using Ficoll and re-suspended in CM2 medium for infection. Cyclosporin A and EBV supernatant were added, and incubated for three days. The cells were then transferred to a 25 cm^2 T-flask for further expansion. The LCLs were maintained for 60 days, with culture supernatant collected to monitor antibody secretion.

To evaluate antibody production in lymphocyte cell lines (LCLs), a sandwich ELISA was performed, collecting supernatant samples at 7-day intervals over approximately 60 days. The process involved coating 96-well plates with a capture antibody (Goat anti-human Ig G) at a concentration of 1 µg/ml and incubating overnight at 4°C. Following a blocking step with 5% skim milk at 37°C for one hour, the supernatant samples were added and incubated for another hour at 37°C. The complexes were then treated with an HRP-conjugated secondary antibody (Goat anti-human IgG) for one hour, and TMB substrate was added for development of reaction.

To specifically detect Anti-HBsAg, Indirect ELISA using HBs antigen (Ad and Ay Subtype, 1 µg/ml) was conducted. The collected

supernatant at intervals was incubated, and a Goat anti-human IgG-HRP conjugate was used as the secondary antibody. Detection was performed using a TMB substrate, and the absorbance was measured at a wavelength of 450 nm in the ELISA reader.

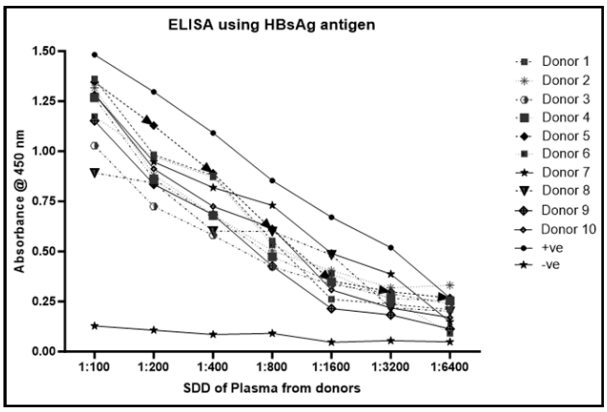
Lymphoblastoid cells fused with SP2/O-Ag14 myeloma cells were cultured in a selective medium. Hybrids producing HBs antigen-specific human antibodies were isolated and screened using ELISA. The selected clones were expanded and confirmed using dot blot, with donor serum as a positive control.

Hybridoma culture supernatants was equilibrated, loaded onto a protein column, and eluted after washing. Biotinylation of mAbs was carried out by combining biotin and antibody at a 1:10 ratio and tested for its binding efficiency.

Microtitre plates were coated with mAbs at a concentration of 2 µg/ml and incubated overnight at 4°C. After blocking, the wells were exposed to clinical sera diluted at 1:100 for 2 h, incubated with biotinylated mAbs for 1 h. The results were reported as the mean absorbance at 450 nm for each group ± standard error. Samples were positive for the assay when the optical density exceeded the mean OD+3SD value of the control sera.

RESULTS AND DISCUSSION

It was determined that all 10 donors had significant amounts of anti-HBsAg antibodies, with reactivity observed up to a plasma dilution of 1:6400 (Fig. 1). Consequently, all donor PBMCs were selected for EBV immortalization. The Trypan blue staining of the PBMC samples indicated high cell viability, ranging from 80-90% across all samples. PBMCs from five selected donors were adjusted to a concentration of 1×10^6 cells/ml for LCL preparation, resulting in the successful transformation of 10 samples, confirmed by cell elongation and morphology changes (Fig. 2). The transformation of EBV B-lymphocytes took between 7 to 14 days, and the transformed cell lines were named according to donor numbers. After three weeks, noticeable cell clumping occurred, and the LCLs were cryopreserved for future studies. The screening of LCLs for total immunoglobulin and anti-HBsAg antibody production revealed that all 10 LCLs produced total



Absorbance values at 450 nm after diluting the plasma sample ranged from 1:100 to 1:6400. All 10 donors showed presence of antibodies against HBsAg in various ranges.

Fig. 1. Determination of anti-HB_sAg antibodies by Elisa.

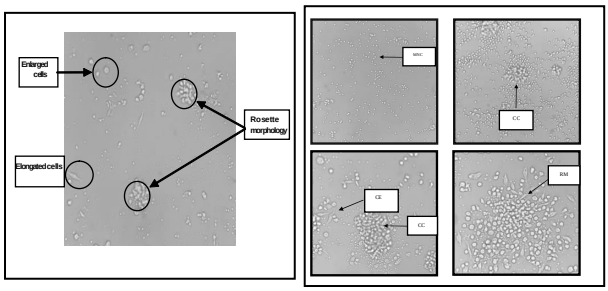
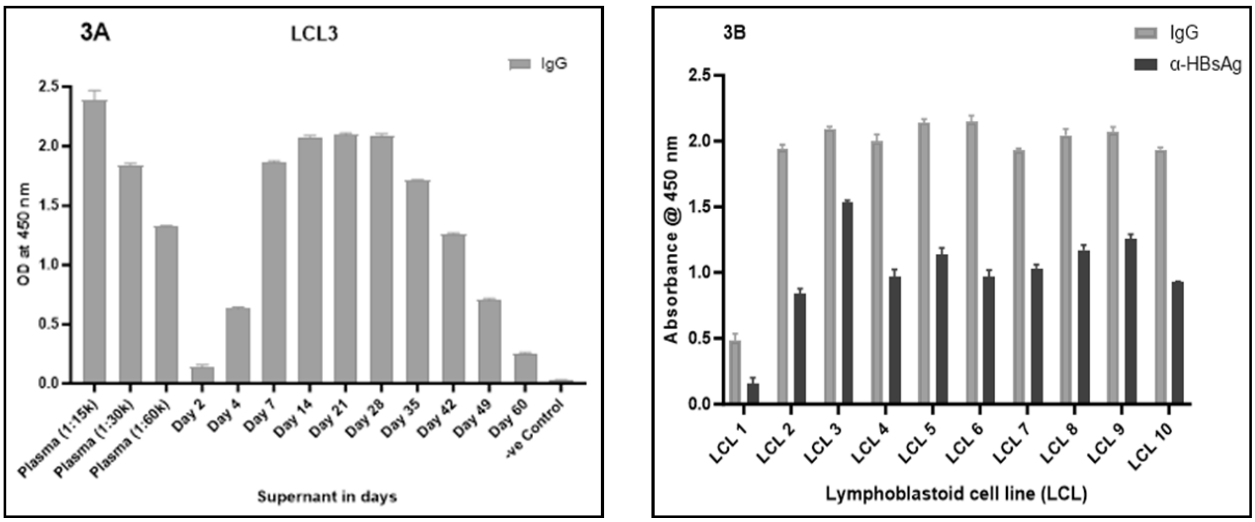


Figure outlined the EBV-induced morphological transformations in cells, captured with an inverted microscope showing cells before transformation and following infection at one, two, and three weeks, all under 40x magnification in a T25 flask. PBMC = Pre-transformation mononuclear cell, MNC = Mononuclear cell, CE = Cell elongation, CC = Clumping of cells and RM = Rosette-shaped cell grouping.

Fig. 2. Visualization of cell before and after transformation using EBV by inverted microscope.

immunoglobulins, while 9 produced anti-HBsAg antibodies, excluding LCL 1. Total IgG levels were similar across all LCLs, and a sandwich ELISA showed that total immunoglobulin production increased by day 4, peaked on day 14, remained stable until day 28, and then declined significantly by day 60. LCL 3 exhibited the highest levels of anti-HBsAg antibodies, leading to its selection for fusion (Fig. 3A, B).

Fusion was conducted with LCL 3, resulting in the selection of 21 parent clones, with six positive clones identified after further screening. Three clones (1C1, 1G8, 2C12) maintained reactivity upon expansion, and dot blot analysis confirmed that three clones produced HBs-specific antibodies (Fig. 4 A, B). The purification and biotinylation of monoclonal antibodies followed established



(3A) An ELISA test was conducted to check for the presence of anti-HBsAg antibodies and immunoglobulin G (IgG) production. A sandwich ELISA was performed using the culture supernatant from LCL 3. Different culture supernatants collected on various days were tested to assess the total IgG production. Diluted plasma (at 1:15k, 1:30k, 1:60k dilutions) served as the positive control, while Culture Media 1 (CM1) was used as the negative control. The analysis revealed a peak in IgG production during the third week. The culture supernatant of Lymphoblastoid Cell Lines (LCL)s contained both immunoglobulin and anti-HBsAg antibodies. (3B) All LCLs, except for LCL1 showed the presence of these antibodies, with LCL3 exhibiting the highest levels among all.

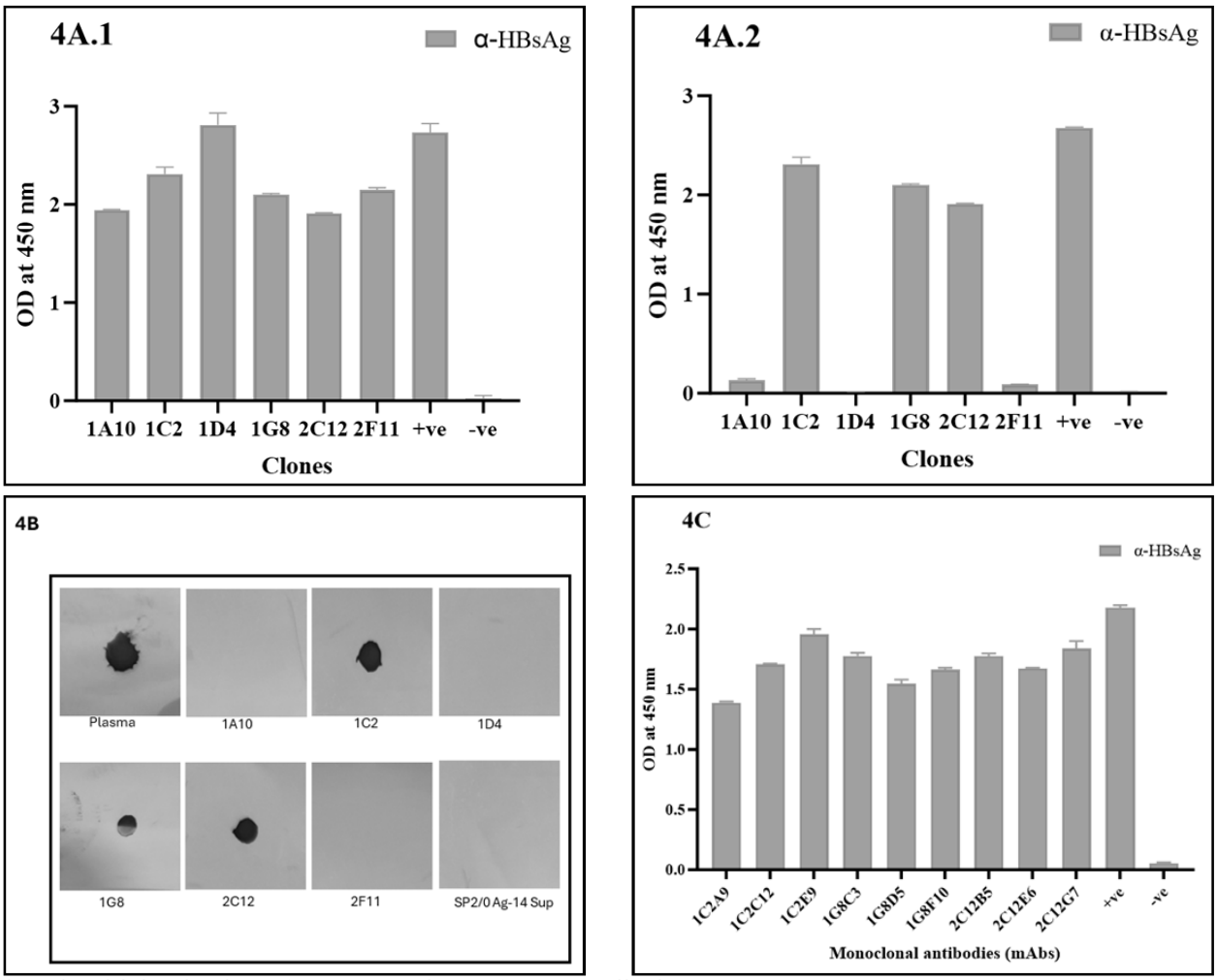
Fig. 3. ELISA for checking IgG and HBsAg production.

protocols, leading to the optimization of ELISA conditions. The pairing of 2C12G7 as the capture antibody and biotinylated 1G8C3 as the detection antibody showed higher sensitivity (Fig. 5).

Finally, a sandwich ELISA was performed to detect circulating HBsAg in clinical plasma using the established antibody combination. Plasma from patients exhibited significant reactivity ($P > 0.0001$) compared to the control samples. Data analysis involved two-way ANOVA to compare the mean OD values of each group in all the assays. HBV groups showed optical density values of (OD ± SD = 1.108 ± 0.196), while normal samples showed (OD±SD = 0.060±0.016). A total of 20 patients' sera and 60 regular donors' sera were tested using the ELISA, which demonstrated a detection sensitivity of 100% and a specificity of 99.3% (Fig. 6).

Over 30 years, methods for generating EBV-transformed B lymphocyte cell lines have been well established. There have been a few attempts to create hybridomas from LCLs as well. This study successfully demonstrated the transformation of EBV-B lymphocytes by observing the formation of rosette-shaped morphology in culture plates, as previously established in the literature. The average transformation time is as early as 7-14 days, consistent with previous studies (Lamontagne *et al*., 2021; Wang *et al*., 2023). This study aimed at generating stable Anti-HBsAg hybridomas capable of producing monoclonal antibodies with antigenic specificity. The production of hetero-hybridomas was generally unstable due to the loss of human chromosomes in the hybridomas and low fusion efficiency (Sait *et al*., 2022). However, stable generation of mouse and human hetero-hybridoma was achieved previously (Yew and Tan, 2016).

Other studies also demonstrated the production of cell lines to achieve monoclonality. To enhance the efficiency of hybridoma generation, time frame for stable LCL formation was established. The PBMCs from the donor were successfully transformed with EBV, resulting in the generation of stable LCLs. The antibody secretion period of the LCLs was studied, and it was found that under optimal conditions, these cells can consistently produce antibodies for 4-5 weeks. This information was crucial in determining the duration for the LCLs for fusion to generate hetero-hybridomas. It was established that LCLs should be utilized within 4-5 weeks for stable antibody production / rescue process or cryopreserved in liquid nitrogen. The rescue of LCLs involved

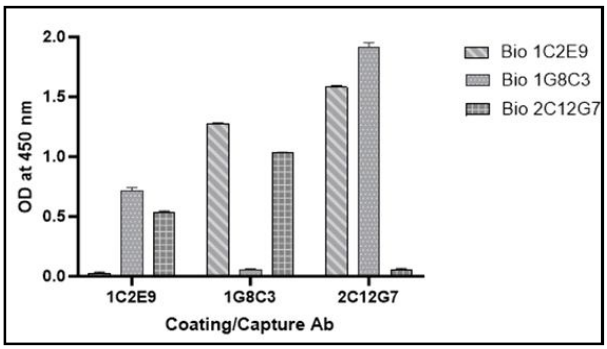


(4A.1) Initial selection of 6 parent clones from 6-well plates.
(4A.2) Selection of parent clones from 25 cm^2 flasks after multiple passaging. The graph illustrated three positive parent clones.
(4B) Western blot analysis was conducted to demonstrate the presence of specific antibodies in parent clones. 1C2, 1G8, and 2C12 tested positive.
(4C) The final screening result of the selected monoclones. +ve = Plasma, -ve = Sp2/0-Ag14 supernatant.
Fig. 4. Fusion and screening results.

their fusion with myeloma cells (Sp2/0-Ag14 cells) to produce hetero-hybridoma cells. The functionality of the hetero-hybridoma cells was verified by their ability to produce specific antibodies. Three parent clones were selected for stability and further subcloned to ensure monoclonality, thereby enhancing the consistency and efficiency of antibody production.

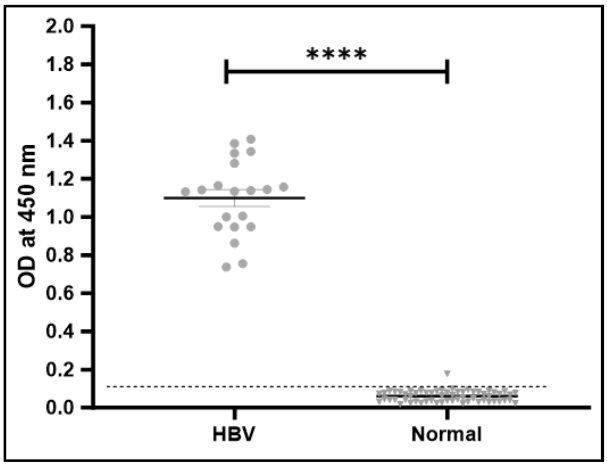
A sandwich ELISA was also developed for Hepatitis B virus diagnosis using monoclonal antibodies and antibody conjugates. The 2C12G7 monoclonal antibody, for its high specificity for the Hepatitis B virus surface antigen (HBsAg), was used to capture antibodies. Conjugates were prepared by labelling the monoclonal antibodies with biotin for the HBsAg diagnostic assay. The activity of these antibodies was tested using sandwich ELISA with clinical samples. The assay showed a sensitivity rate of 100% and a specificity rate of 99.3%, signifying the development of a highly sensitive diagnostic tool (Minic and Zivkovic, 2021).

The findings indicate that this method can generate antibodies suitable for diagnostic or potential therapeutic applications as was tried by other researchers (Valgardsdottir *et al.*, 2021). The antibodies developed using this approach offer several advantages, including being of human origin and the ability to tailor antibodies to specific targets. The study



Total six combinations of antibody pair analysis were carried out. Coating-detection combination of same mAb was avoided due to same epitope. 2C12G7-Bio 1G8C3 showed best detection values.

Fig. 5. Antibody pair analysis.



Capture antibodies were immobilized in the wells and exposed to serum samples from both infected patients and control subjects. The bound antigens were then identified using biotinylated antibodies, followed by streptavidin-peroxidase, and the results were visualized. In the graph, red dots represented the absorbance of HBV-infected sera, while black dots represented the absorbance of normal sera. The notation "****" indicated a statistically significant p-value of less than 0.0001.

Fig. 6. Capture ELISA to detect HBsAg from patients' sera.

successfully yielded clones that generated targeted antibodies. However, additional research focused on cloning and screening is needed to produce more stable monoclonal antibodies with improved scalability for production. It was found that, for diagnostic applications, the murine hybridoma remain preferable due to its efficient and reliable antibody production. Nevertheless, the EBV immortalization method held promise for generating antibodies that are valuable in the therapeutic field. It would be worthwhile to pursue further research to refine monoclonal antibodies.

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