

A BRIEF REVIEW ON HYBRIDOMA TECHNOLOGY: FUNCTIONAL PARAMETERS

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Abstract

Hybridoma technology is the method by which large quantities of identical antibodies, known as monoclonal antibodies, are produced. This is achieved by administering an antigen to a mouse, which mounts an immune response. Antibody-producing B cells are then harvested from the immunised mouse and fused with immortal myeloma cells. This fusion produces a hybrid cell line, the hybridoma, which combines the antibody-producing capacity of the B cell with the myeloma cell's ability to proliferate indefinitely in culture. A single hybridoma cell can be cloned to establish cultures of genetically identical daughter cells, each secreting the same monoclonal antibody. The myeloma fusion partner itself, by contrast, is selected specifically because it does not produce antibody, so that all antibody detected in the culture originates from the fused B cell.

Keywords: B-cells, Hybridoma, Monoclonal antibodies

1. INTRODUCTION

The generation of monoclonal antibodies was devised by César Milstein and Georges J. F. Köhler in 1975. They shared the 1984 Nobel Prize in Physiology or Medicine with Niels Kaj Jerne, who made separate contributions to immunology.¹

Procedure: Laboratory animals such as mice are given a series of injections of the test antigen over several weeks, against which the antibody is to be raised. Splenocytes are isolated from the mouse spleen, and the B cells are fused with immortalised myeloma cells by electrofusion. Alternatively, a chemical fusing agent such as polyethylene glycol (PEG) is used to fuse the B cells and myeloma cells. The myeloma cells used lack the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene, which makes them sensitive to HAT medium.² The fused cells are cultured in HAT medium (hypoxanthine-aminopterin-thymidine medium) for about 10 to 14 days. Unfused myeloma cells die under HAT selection, and unfused B cells die because they have a short lifespan in culture; only the B cell–myeloma hybrids (hybridomas) survive. The surviving culture is then diluted across 96-well plates so that each well is seeded from a single cell. Because the

antibodies in a given well are produced by a single B-cell clone, they are directed against the same epitope and are therefore monoclonal antibodies.³

Hybridomas are cells engineered to produce a desired antibody in large quantities, enabling the production of monoclonal antibodies. (1, 2) Monoclonal antibodies can be produced in specialised cells through a method now widely known as hybridoma technology.¹ Hybridoma technology was developed in 1975 by two scientists, Georges Köhler of West Germany and César Milstein of Argentina (then working in the UK), who together with Niels Jerne of Denmark (then working in Germany) were awarded the 1984 Nobel Prize in Physiology or Medicine.¹ Producing a single monoclonal antibody by hybridoma technology has been reported to cost between \$8,000 and \$12,000, and a well-resourced laboratory can typically generate only 15 to 30 new hybridoma lines per year; in settings focused on diagnostic or therapeutic-grade MAbs, additional constraints can reduce throughput further. Monoclonal antibodies are valuable for the study of parasite antigens, and it was appropriate that the WHO convened a symposium (held at the National University of Singapore, October 1981) bringing together those who had developed and refined the technology with those using it, or planning to use it, to study the organisms responsible for some of the major diseases affecting humankind. Such monoclonal antibodies have opened important new approaches to predicting, diagnosing, and treating disease. Monoclonal antibodies are used, for example, to identify subsets of B cells and T cells. This is useful both for basic research and for distinguishing different types of leukaemias and lymphomas, allowing clinicians to tailor treatment accordingly. Quantifying B-cell and helper T-cell numbers is critically important in immune disorders such as AIDS. Monoclonal antibodies are also used to track tumour antigens and, alone or conjugated to anticancer agents, to target cancer metastases. The monoclonal antibody OKT3 is used to rescue organ transplants threatened with rejection and to prevent bone-marrow transplants from triggering graft-versus-host disease.

METHODOLOGY

A hybridoma is created by injecting a specific antigen into a mouse, isolating the antigen-specific plasma cells (antibody-producing cells) from the mouse's spleen, and fusing these cells with a malignant, immortal cell called a myeloma cell. The resulting hybrid cell can be cloned to produce numerous genetically identical daughter clones, which continue to secrete the antibody. Because these antibodies originate from a single type of cell (the hybridoma cell), they are called monoclonal antibodies. The advantage of this approach is that it combines the properties of the two parent cell types: the capacity to proliferate indefinitely, and the ability to produce large quantities of pure antibody. HAT medium (hypoxanthine-aminopterin-thymidine) is used in the preparation of monoclonal antibodies. Laboratory animals (e.g., mice) are first exposed to the antigen against which an antibody is to be raised.

After splenocytes are isolated from the animal, the B cells are fused with immortalised myeloma cells – which lack the HGPRT (hypoxanthine- guanine phosphoribosyl-transferase) gene – using polyethylene glycol or Sendai virus. Fused cells are cultured in

HAT (hypoxanthine-aminopterin-thymidine) medium. In the presence of aminopterin, unfused myeloma cells die because aminopterin blocks the de novo nucleotide-synthesis pathway and these cells lack the salvage-pathway enzyme (HGPRT) needed to bypass the block. Unfused B cells also die, as they have a short lifespan in culture. Only the B cell–myeloma hybrids survive, since the functional HGPRT gene contributed by the B cell rescues the fused cell from aminopterin toxicity. These hybrid cells produce antibody (a property of B cells) and are immortal (a property of myeloma cells).² The culture is then diluted into multi-well plates such that each well contains only a single cell. The supernatant in each well can then be screened for the desired antibody; because the antibodies in a well are produced by a single B cell, they are directed against the same epitope and are known as monoclonal antibodies.³ Once a hybridoma clone is established, it will grow continuously in a culture medium such as RPMI-1640 (supplemented with antibiotics and fetal bovine serum) and secrete antibody (Nelson et al., 2000).^{3, 18}

The next step is a rapid primary screening process that identifies and selects only those hybridomas producing antibodies of the desired specificity. The hybridoma culture supernatant is incubated with an enzyme-labelled secondary conjugate and a chromogenic substrate; development of a coloured product indicates a positive hybridoma. Alternatively, immunocytochemical screening may be used (Nelson et al., 2000).^{3, 18} Multiwell plates are used initially to grow the hybridomas, and after selection, cultures are transferred to larger tissue-culture flasks. This maintains the health of the hybridomas and provides sufficient cells for cryopreservation and supernatant for further analysis. The culture supernatant can yield 1 to 60 µg/ml of monoclonal antibody, which is stored at –20°C or lower until required (Nelson et al., 2000). Using the culture supernatant or a purified immunoglobulin preparation, further characterisation of a candidate monoclonal antibody-producing hybridoma can be carried out in terms of reactivity, specificity, and cross-reactivity (Nelson et al., 2000).^{3, 18}

Advancements or Improvements in Hybridoma Technology

Considerable efforts have been made over recent decades to improve the yield of monoclonal antibodies obtained using hybridoma technology.^{4, 5} These efforts have included: (1) substitution of a chemical fusion promoter (PEG) for the Sendai virus originally used to promote fusion; (2) the use of myelomas that do not secrete their own antibodies and therefore do not interfere with production of the antibody of interest; (3) use of a continuous cell line (Sp2/0) as the fusion partner for antibody-producing B cells; and (4) use of feeder layers of extra cells to support newly formed hybridomas for optimal growth and antibody production.

Purification of Antibodies

Monoclonal antibodies generally require purification before use for a range of purposes. Prior to final purification, the culture may be subjected to cell fractionation to enrich the antibody protein. In *E. coli* expression systems, antibodies may be secreted into the periplasm, which simplifies subsequent purification. Alternatively, antibodies may be

purified from cell homogenate or cell debris obtained from the medium. Antibodies can be purified by either of the following methods: (i) ion-exchange chromatography; (ii) antigen affinity chromatography.

Serum Free Media for Bulk Culture of Hybridoma Cells

Media formulated for the bulk culture of animal cells have highlighted the importance of adding serum to basal supplement media. Serum is a highly complex and incompletely characterised mixture of components such as albumin, transferrin, lipoproteins, and various hormones/growth factors. Nevertheless, serum forms a key part of media for cultured animal cells. The use of serum, however, creates challenges in the purification of antibodies. In addition, it is a costly input for large-scale production of hybridoma cells for the industrial manufacture of monoclonal antibodies. In view of these challenges, serum-free media are increasingly used for cultured hybridoma cells.

Advantages of Serum-Free Media in Hybridoma Cell Culture and Preparation of Monoclonal Antibodies:

1. Greatly simplified purification of antibodies, owing to increased initial purity and the absence of contaminating immunoglobulin.
2. Reduced variability of culture medium.
3. Reduced risk of infectious agents.
4. Fewer factors requiring gene control/confirmation.
5. Greater control over bioreactor conditions.
6. Potential for increased antibody secretion.
7. Low or no reliance on animals.
8. Cost-effective.
9. Overall improved productivity.

Disadvantages of Serum-Free Media in Hybridoma Cell Culture and Preparation of Monoclonal Antibodies:

1. Not all serum-free media are suitable for all cell lines.
2. Cells may not grow to as high densities and may be more fragile than cells grown in serum.
3. Media may take longer to prepare.

Bypassing Hybridomas and Cloning of mAb Genes

The VH and VL genes for antibodies can be amplified by polymerase chain reaction (PCR) using 'universal primers' (general primers that anneal to conserved sequences shared by most antibody genes). By incorporating restriction sites into these primers, the amplified VH and VL genes can also be cloned for expression in mammalian cells or microorganisms. The starting material for PCR may be hybridomas or B cells, which may be homogeneous (if derived from single cells) or heterogeneous. In the latter case, a range of VH and VL genes will be amplified and will combine at random to generate up to 10^6 clones of antibody genes (from 1,000 different VH and 1,000 different VL genes). These genes are cloned into a phage vector, and their products (particularly Fab fragments) can

be screened for antigen-binding activity. From such a large number of combinations in a combinatorial library, it is very difficult to recover the original pairings of V genes (e.g., a genuine VH α -VL α pairing may be indistinguishable from an artefactual VH α -VL γ pairing). However, the complexity of the library can be reduced by using antigen-selected B lymphocytes (panning on antigen-coated surfaces can be used for screening).

Designing and Building of mAb Genes

The antigen-binding sites of antibodies have recently been studied in considerable detail. This has led to the modelling of entirely new antibodies, sometimes for use as therapeutic proteins. Computer-graphics-based modelling can be used to modify antibody genes or to design wholly synthetic genes. These genes can then be cloned and expressed in microorganisms, and the resulting antibodies tested for specificity and affinity for the target antigen.

Primary and Secondary Libraries for Antibody Genes

In this approach, a library of antibody genes can be assembled from genes obtained from a variety of different sources, including: (i) rearranged V genes obtained from animals by PCR (using universal primers); (ii) new V genes generated through gene conversion, a process used in birds; (iii) rearranged genes obtained from mRNA by reverse transcription; and (iv) design of entirely new V genes or D segments. The next step is to permit expression of the library in microorganisms and to screen the resulting antibodies for antigen-binding activity.

MabCure hybridoma technology

Custom Hybridoma / Monoclonal Antibody Production Service

Custom monoclonal antibody generation at Cell Essentials typically takes 4–6 months and is divided into 3 phases. The customer may choose to end the hybridoma-generation project before the start of the next phase. Cell Essentials will not proceed to the next phase of the project without written (email) confirmation from the customer. The customer is invoiced for each phase at its start. The amount of material required for immunisation of 5 mice is 1–1.5 mg, and an additional 0.8–1 mg is required for ELISA screening.⁹

Phase I: Immunisation of 5 BALB/c mice, including pre-injection tail bleeds, antigen/adjuvant injections, all antigen/adjuvant boosts, tail bleeds, and ELISA to identify the mouse with the highest titre for use in cell fusion. Duration: ~2 months. Cost: \$1,800 (USD).

Phase II: Fusion of spleen cells with the myeloma cell line, plating of the fusion product into six 96-well plates, and identification by ELISA of wells expressing antibodies against the antigen. All antibody-secreting colonies are isolated, transferred to a 24-well plate, expanded, and partly cryopreserved. Antibody-containing medium (3 ml) from up to 12 ELISA-positive preclones^a is sent to the customer for evaluation in the customer's specific application. Duration: ~3–4 weeks. Cost: \$2,900 (USD).⁹

Phase III: Cloning of positive (IgG-secreting) wells. A maximum of 5^b of the positives selected by the customer will be cloned by limiting dilution and isotyped. Clones will be expanded, and 10 vials of each selected clone will be frozen. Medium (10 ml) from each clone, together with the frozen vials, will be shipped to the customer. Duration: ~1.5–3 months. Cost: \$2,800 (USD).⁹

Total cost for each project is \$7,500 (USD). Additional clones can be isolated and processed as in Phase III for \$350 (USD) per clone. The success of a custom hybridoma project depends on the antigenicity of the material supplied by the customer. Cell Essentials cannot guarantee that the material supplied by the customer will elicit an immune response sufficient to warrant proceeding to Phase II; Cell Essentials guarantees only that the antibodies generated by the resulting hybridomas will recognise the antigen supplied by the customer for immunisation.

^a Preclones: bulk (uncloned) hybridoma cultures at the 96-well stage, prior to sub-cloning. ^b Selected by the customer from among the ELISA-positive preclones for progression to cloning.

CONCLUSION

In diagnosis, pregnancy can be detected by assaying hormone levels using monoclonal antibodies. Similarly, pathogens can be detected within a few hours, saving the several days otherwise required for culturing. Immunopurification involves the separation of one substance from a mixture of closely related molecules.

Antibodies are proteins synthesised in the blood against specific antigens to combat infection and confer immunity. When collected directly from the blood serum of an immunised animal, such antibodies are heterogeneous – a mixture of antibodies directed against different epitopes of the antigen – that is, polyclonal antibodies, and they therefore lack the defined specificity of monoclonal antibodies. By contrast, when a single antibody-producing lymphocyte is isolated, cultured in vitro, and (for practical production) fused to form a hybridoma, it is capable of producing a single, homogeneous type of antibody directed against one specific antigenic epitope. Such antibodies are known as monoclonal antibodies. Owing to their defined specificity, monoclonal antibodies are widely used in the diagnosis of disease.

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