

Mouse monoclonal antibodies in biological research: strategies for high-throughput production

Pieranna Chiarella · Vito Michele Fazio

Received: 8 February 2008 / Accepted: 12 March 2008 / Published online: 17 April 2008
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Abstract Mouse monoclonal antibodies have become key components in basic research as well as in the clinical laboratory. Being invaluable tools in many biological assays, they continue to be the primary choice in the research field, although the conventional technology used for hybridoma generation and screening is a still lengthy, time-consuming and low-throughput process. With the advent of genetic immunisation and the application of automation and microarray to the traditional biological assays, the monoclonal antibody field has been revolutionised. Here, we will briefly review the most relevant strategies which have made the manufacture of murine monoclonal antibodies a faster and high-throughput technology.

Keywords High-throughput · Hybridoma technology · Microarray · Monoclonal antibodies · Proteome

Introduction

Monoclonal antibodies are well-established tools for investigating the proteome with widespread

applicability in biomedical science. They have the peculiar characteristic that they are high-specificity and high-sensitivity binding reagents which makes them ideal tools for basic research as well as for cancer diagnostic and therapeutics. In the research field they are used to detect proteins in cells in their native conformation (immune-localisation), in the denatured form (western blot), or as high affinity binders in immune-affinity based assays. In medical therapy, e.g. cancer and infectious diseases, monoclonal antibodies have significant advantages compared to conventional chemical compound-based therapies. They show favourable pharmacokinetics and pharmacotoxicity, have few side effects due to their high specificity and affinity for the disease target, and are able to recruit immune effectors to initiate destruction of the target cells.

In the last 30 years monoclonal antibody technology has developed enormously. Despite later development of alternative methods for monoclonal antibody production, such as recombinant DNA-based technology and expression in bacterial systems (Siegel 2002); construction of phage display libraries (Rader and Barbas 1997); chimerisation and humanisation strategies based on the use of transgenic animals (Lonberg et al. 1994); the demand for large-scale monoclonal antibody production is constantly raising. This has created a significant implementation of conventional methods for monoclonal antibody generation and screening.

P. Chiarella (✉) · V. M. Fazio
Laboratory of Molecular Medicine and Biotechnology,
CIR, Campus Bio-Medico University of Rome, Via
Alvaro del Portillo 21, 00128 Rome, Italy
e-mail: p.chiarella@unicampus.it

Hybridoma technology

The hybridoma technology to create monoclonal antibodies was first described by Kohler and Milstein (1975). Hybridomas are hybrid cells derived from the fusion of immortal myeloma cells with B-lymphocytes taken from the spleen of animals immunised with the target antigen. After limiting dilution cloning, hybridomas represent a pure and indefinite source of monoclonal antibodies with the desired target specificity. Once established in culture, the hybrid cell lines are tested for antigen specificity, expanded and permanently stored into liquid N₂ for collection into a hybridoma bank. In addition to murine antibodies, produced by hybridoma technology, it is now possible to make human antibodies. Unlike the original hybridomas, the spleen cells are taken from a transgenic mouse which has the murine immunoglobulin locus replaced by the human genes (Lonberg 2005). However, since human antibodies have been specifically designed for being used *in vivo* as therapeutic agents, due to their lower immunogenicity in the human host, mouse monoclonal antibodies remain the primary tools of basic research for investigating the proteome.

Although the hybridoma technology is still largely used and is the most widely employed method for making monoclonal antibodies, many investigators have searched for alternative, simpler time-saving strategies to increase the throughput of antibody production. The hybridoma-free generation of monoclonals, pioneered by Pasqualini and Arap (2004a, b), is an example. With this novel technology, which consists of generating immortal splenocytes as a source of monoclonal antibodies, researchers succeeded in by-passing the *in vitro* somatic fusion of splenocytes with myeloma cells. They created a transgenic mouse with a mutant temperature-sensitive simian virus large tumour antigen under the control of a murine major histocompatibility promoter from which splenocytes were derived. At a permissive temperature (33°C) the splenocytes were immortal and became capable of secreting monoclonal antibodies into the culture medium. However, although the immortal splenocytes showed a higher genetic stability in comparison to that of hybrid cells, the hybridoma-free technology was not likely to be effective at saving costs and time needed for monoclonal antibody production. The conditional

antibody-producing cells grow at low density and need long periods of culture, estimated to take approximately 2 months, with frequent medium exchange. Therefore, even though this strategy allowed the exclusion of the somatic fusion among splenocytes and myeloma cells, it did not reduce significantly the amount of cell culture work and the time required for maintenance of the conditionally immortal cells in culture (Pasqualini and Arap 2004a, b).

High-throughput hybridoma generation and culture

So far a limiting step in the throughput of mouse monoclonal antibodies is the laboratory capacity for high somatic fusion number and the downstream cell culture operations which follow hybridoma generation. Traditional methods in accomplishing these tasks are labour-intensive, time- and man-power-consuming. The simultaneous performance of a high number of fusions and the maintenance of thousands of hybridoma cell lines in culture would be an impossible goal to achieve if the use of robotics was not the primary choice for a laboratory.

A semi-automated method for hybridoma generation has recently been developed by using robotic technology (De Masi et al. 2005; Sawyer and De Masi 2003). With this strategy and the availability of a cell culture automatic workstation in the laboratory it was possible to carry out up to eight somatic fusions simultaneously in a controlled environment. Furthermore, all the hybridoma culture operations following somatic fusion were executed by a robotic machine: *i.e.* hybridoma seeding and plating-out, feeding and medium exchange, and harvesting supernatants for testing antibody specificity against the target antigen. The development of automated systems for controlling cell plating, medium change, growth factor addition, cell harvesting and seeding has already been described for the maintenance of several mammalian cell culture (Lutkemeyer et al. 2000) including embryonic stem cells (Terstegge et al. 2007) and it is a substantial help to a laboratory dedicated to large-scale monoclonal antibody production. Many industries specialised in liquid handling and laboratory automation have satisfied the demands of several investigators for fully automated off-the-shelf robotic solutions which can be

customised for specific applications (Chapman 2003). Robotic workstations are equipped with all the necessary devices and are supplied with software integrating the liquid handling system. Here, cells are dispensed into several plate-formats, they are processed in a controlled environment and moved around by robotic arms. In those stations which are connected to tissue culture incubators, cell flasks and plates undergo feeding and medium exchange by simply programming the robot software. With the help of automated systems, scale-up of monoclonal antibody production became a quicker and easier process. Among the advantages offered by robotics and cell culture automation there are consistency in procedure and sterility, which ensure less variability among batches, and process programming. The result is a dramatic reduction in time and workforce spent for routine cell culture operations.

Improving immunisation strategies

The first step in the monoclonal antibody production is the animal immunisation with antigen plus adjuvant. Choosing the best immunisation protocol is always critical as the overall results are influenced by many factors such as the antigen dose and concentration, the choice of adjuvant, the choice of time interval between inoculation and the immune response analysis as well as the choice of an appropriate detection method. Optimisation of immunisation protocols has been achieved considering all the following aspects: (a) antigen dose, (b) systems used for antigen delivery, (c) number of boosts, (d) time interval among boosts, (e) inoculation route, (f) use of multiplexing immunisation protocols and (g) use of alternative immunisation techniques such as genetic immunisation (Hu et al. 1989, 1990; Davis 1997). The optimal antigen dose can vary depending on the antigen immunogenicity. Minimal antigen dosage is required to induce sufficient humoral response in the mouse. A higher dose of antigen can initially induce a strong response in the animal with a large amount of antibodies which unfortunately might show lower affinity leading to immune tolerance. Small multiple doses of antigen have been preferred to higher doses, being effective in obtaining a good antibody titer (Nakashima et al. 1974). Also, excessive number of boosts is

detrimental for obtaining a high antibody concentration in the mouse serum since it induces immune tolerance instead of increasing responsiveness. Current immunisation protocols aimed at generating monoclonal antibodies are usually short, with a low antigen dose and a minimum number of boosts thereby minimising the animal immunisation schedule. Further improvements in shortening the immunisation schedule has been achieved upon the introduction of the single-step immunisation (Khan et al. 1994). According to this approach the need for multiple injections of antigens has been circumvented by a single shot-injection through the use of appropriate adjuvants capable of sustained delivery such as microparticles, polymers, liposomes and virus-like particles (Aucouturier et al. 2001). It has been shown that the humoral immune response stimulated by long lasting antigen delivery can be comparable to the secondary response induced by the same total dose of antigen emulsified in a strong adjuvant such as complete Freund's adjuvant (Preis and Langers 1979).

Genetic immunisation and delivery route

Among alternative high-throughput immunisation techniques it is worthy to discuss the DNA-based immunisation. This methodology, first described in the early 1990s, enabled researchers to obtain antibodies directed against proteins that could not be purified. The protein is synthesised *in vivo* in its native form with correct post-translational modifications and three-dimensional folding favouring production of antibodies against the native protein (Barry et al. 1994). DNA expression vectors are usually administered to animals by intra-dermal particle bombardment, e.g. gene gun, or by intramuscular injection (Chen et al. 2000; Wolff et al. 1990).

Genetic immunisation-based antibody production has been shown to offer several advantages, including *in vivo* antigen synthesis, high-specificity, high flexibility and high-throughput. This technique has proven valuable to achieve antibodies with high-specificity against antigens by using different strategies to increase the *in vivo* antigen expression and the animal immune response. Codon optimisation and antigen fusion to immunostimulating sequences have contributed to enhance both the gene expression and

the immune response. By genetic immunisation, useful antibodies have been raised against a wide range of antigens with a remarkable success rate. In the paper by Chambers and Johnston (2003), the authors succeeded in making antibodies against 84% of the antigens injected in animals (130 antigens in total). Antigen immunogenicity was improved by the linkage to various elements that stimulated the immune system and by co-delivering plasmids encoding genetic adjuvants (Chambers and Johnston 2003). The applicability of genetic immunisation has successfully been extended also to the monoclonal antibody production (Kilpatrick et al. 1998; Ny et al. 2004). One of the first applications of genetic immunisation to the production of monoclonal antibodies was tested by Barry et al. (1994) who produced different monoclonal antibodies against the human growth hormone that could recognise both denatured and native form of the protein.

The flexibility of genetic immunisation is strictly dependent on the deoxyribonucleic acid. DNA is a highly stable molecule, it can be designed to encode more than one antigen or epitope and it can be co-injected with a wide range of adjuvant molecules. The effectiveness of this technique in raising an adequate antibody response in mice has radically been improved by two technical approaches. One is the stimulation of skeletal muscle with electrical pulses following intra-muscular DNA injection (Mir et al. 1999), the other is the prime-boost strategy where an initial priming with DNA is followed by a single boosting with the same antigen given in the form of protein or viral vector (Moore and Hill 2004). Both strategies have been shown increase antigen expression and to create a pro-inflammatory environment at the injection site which aid immune activation. Recently, a high-throughput method for producing monoclonal antibodies by genetic immunisation has been licensed. Antibodies against different proteins were produced at the same time following immunisation with several nucleic acids encoding for different proteins or for all subunits of a polypeptide complex (Lohrmann et al. 2004).

Immunisation protocols, including those based on the use of DNA, have been optimised also through the choice of a specific inoculation route which is crucial for improving the animal immune response. By delivering the gene encoding the antigen as an alternative method to protein immunisation, many

scientists have obtained satisfactory results in evoking antibody production in mice. When several inoculation routes have been compared for optimal DNA delivery (i.e. intra-muscularly, intra-peritoneally, intra-venously and intra-splenically) it was observed that only the intra-splenic route was the most efficient at inducing specific antibody production after a single injection of DNA. Although it has been verified that a single intra-muscular immunisation with plasmid DNA was sufficient for antibody response (Bohm et al. 1996), in other reports at least two DNA injections were required (Davis 1997; Dale et al. 2006). These results were supported also by Kasinrerker et al. (2002) who demonstrated that only the intra-splenic route could induce a specific antibody response after a single DNA injection. This type of immunisation contributed to further reducing the time interval between mouse priming with antigen and selection of positive hybrid clones. With this approach, specific hybridomas secreting antibodies against a 18-kDa protein from *Brucella abortus* were generated only 25 days after single-shot (Velikovskiy et al. 2000). Therefore, the intra-splenic immunisation of DNA encoding for the antigen of interest has been proposed as a helpful tool for high-throughput production of monoclonal antibodies (Yu et al. 2005; Velikovskiy et al. 2000).

Multiplex immunisation

Large-scale production of monoclonal antibodies requires an extensive immunisation programme with a large number of mice, particularly when a single mouse is immunised with a single antigen. Thus, from an economic and also ethical perspective it would be desirable to develop efficient and high-throughput strategies in order to cut down on the number of animal used. The strategy of multiplex immunisation, i.e. the injection of more than one antigen into a single mouse during the same immunisation, has recently been evaluated for monoclonal and polyclonal antibody production and has proven to be successful (De Masi et al. 2005; Ning et al. 2006; Larsson et al. 2006). As far as monoclonal antibody production is concerned, multiplex immunisation minimises the number of animals needed for immunisation and the overall tissue culture load related to hybridoma generation. In the article by De Masi et al. (2005), mice were injected with five antigens in the

presence of adjuvant and boosted twice before undergoing somatic fusion. Hybridomas secreting antibodies against all the target antigens have successfully been isolated. In this trial, after the first monoclonal antibody screening carried out by Antigen Microarray Assay (AMA), an average number of 18 hybridomas per antigen were obtained. Following a second round of screening by Enzyme linked Immunosorbent Assay (ELISA), an average number of 9 clones out of 18 were confirmed positive against each target antigen, thus supporting the efficacy of multiplex immunisation. This monoclonal antibody platform has been scaled-up in a trial where 80 antigens were used. Here eight mice were immunised with 10 antigens per mouse and boosted only once before fusion to avoid immune-dominance among antigens. In this trial functional monoclonal antibodies were obtained against 67 of the 80 injected antigens showing a 83% success rate (De Masi et al. 2005). It is noteworthy that the higher the antigen number injected in the same mouse, the lower the variety of hybridoma clones obtained against the same antigen, indicating that the injection of too many antigens is discouraged if the main goal is to obtain a broad panel of antibodies functional in different biological assays and able to recognise different epitopes of the same target protein. A balance between cost and expected results always needs to be considered prior to designing immunisation protocols.

High-throughput monoclonal antibody screening

An additional major obstacle in the high-throughput production of monoclonal antibodies is the screening of thousands of hybridoma supernatants. The assays traditionally used in a laboratory for monoclonal antibody screening are ELISA and Western blotting. In general 96 supernatants are usually tested by ELISA with a single microtiter plate and up to 40 supernatants are tested on blots with a specific apparatus for multiple screening. However these assays are time-consuming and require large quantities of both sample and reagents, thus limiting the number of hybridoma cell lines that can be tested for antigen-antibody interaction in a single day. In the last few years implementation of robotics, where the liquid handling and cell supernatant transfer stages

are fully automated, allowed a single technician to process many ELISA plates simultaneously (Rohner et al. 2005). Automation is essential at this stage because screening of even a minimum number of hybridoma clones on the target antigen requires several plates that have to be manipulated manually. However, a significant drawback of ELISA automation is the time required for performing the assay and the cost of reagents which are used in large quantities. A solution to this problem is represented by the development of nanotechnologies such as protein microarray. The microarray technology permits the simultaneous ultra-sensitive measurement of many analytes in small sample volumes. From an economical perspective miniaturised assays are largely convenient. Given the high cost of many reagents used in biomedical experimentation, considerable savings can result by simply using lesser amounts of reagents and materials.

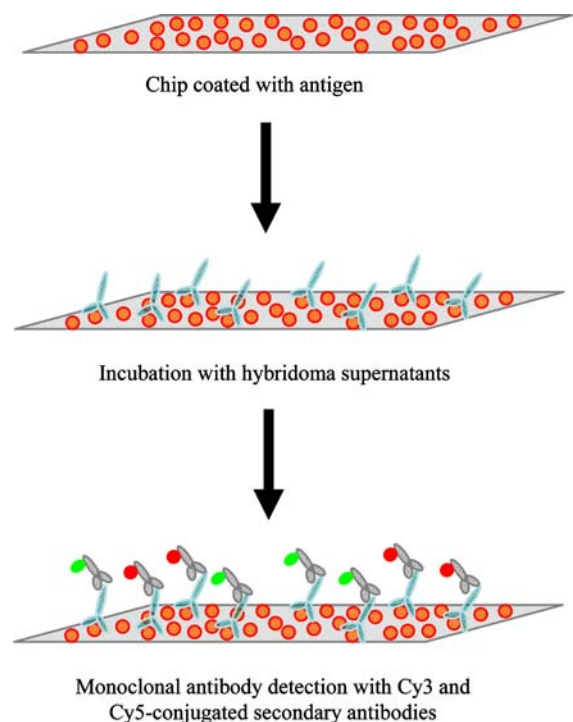


Fig. 1 Scheme of the antigen microarray assay. Antigen is immobilised on the chip surface. Hybridoma supernatants are printed on the antigen-coated chip as a microarray by a robot microarrayer. Anti-mouse IgM antibody conjugated to Cy5 and anti-mouse pan IgG antibody conjugated to Cy3 are used for monoclonal screening and isotype detection. Chips are scanned in a microarray laser to identify IgM and IgG monoclonal antibodies that specifically bind the target antigen

A significant application of miniaturisation to the monoclonal antibody screening is well represented by the AMA assay development. AMA is a high-throughput array-based immunoassay which exploits the multiplexing strategy offered by the microspot microarray technology in conjunction with hybridoma culture automation (De Masi et al. 2005). This method, developed at European Molecular Biology Laboratory and licensed in 2005, allowed for the maximising of the screening throughput of monoclonal antibodies and offered the advantage of obtaining reproducible results in a very short time effectively reducing both costs and human labour while also overcoming most of the current ELISA limitations (Walter et al. 2000). As it was conceived, the AMA technique allowed the simultaneous detection of thousands of antigen-specific monoclonal antibodies, associated with detection of the antibody isotype. In a trial where 80 antigens were used for the immunisation of ten mice, this system showed a 83% success rate in a single batch run within six week of primary immunisation (De Masi et al. 2005).

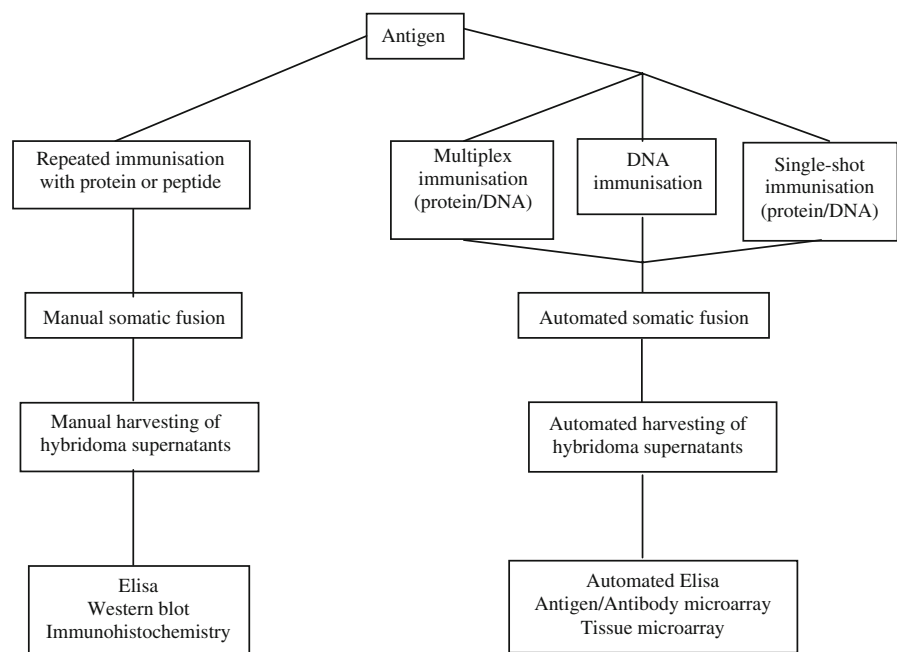
AMA is a miniaturisation of the ELISA assay where the detection step is characterised by the scanning of chips probed with secondary antibody conjugated to different fluorochromes instead of enzyme substrates (Mendoza et al. 1999; Huang

2001). The target antigen is fixed on the surface of a chip, a chemically treated slide, and following a saturation step, hybridoma supernatants are spotted onto slides in a microarray format. Antigen-coated chips are incubated with secondary antibodies conjugated to different fluorochromes in order to distinguish the IgG and IgM isotype and scanned into a microarray laser scanner, enabling detection of the monoclonal antibody of the right specificity (Fig. 1). Hybridoma supernatants that passed the first selection were tested by ELISA and western blotting to confirm their specificity in the binding of both the native and denatured antigen. A further evidence of the antibody affinity to antigen expressed *in vivo* could be rapidly accomplished by tissue microarray analysis, where each monoclonal antibody can be tested on an array of histological tissue sections.

Conclusions

Recent advances in the study of proteome underscore the insatiable demand for high-affinity binding ligands, namely monoclonal antibodies, which were historically generated with lengthy, expensive and low-throughput techniques. With the advent of genetic immunisation, nanotechnologies and the

Fig. 2 Diagram showing a comparison between conventional and high-throughput methods for production of monoclonal antibodies



application of automation and microarray to the conventional biological assays, the monoclonal antibody production field has been revolutionised.

In spite of these encouraging advances, the characterisation of monoclonal antibodies still remains a major bottleneck that may limit production, provided that the cost for large-scale manufacture undergo a substantial reduction and the investigators make a profitable use of these potent research tools. However, identification of the bottlenecks in the traditional murine hybridoma generation method and exploitation of automatic machines and miniaturised assays for large-scale culture and screening (Fig. 2), have turned the conventional monoclonal antibody production into a faster, high-performance and reliable high-throughput technology.

Acknowledgements A special acknowledgement goes to Ario de Marco, Emanuela Signori, Joseph Spalluto and Brendan Fox for critical reading of the manuscript and to Alan Sawyer and Federico De Masi for their profound expertise on high-throughput monoclonal antibody production and microarray technology. This paper was partially supported by MIUR-FIRB 2006: RBIP0695BB.

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