

REVIEW

Monoclonal and polyclonal antibodies production – preparation of potent biorecognition element

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Summary

Antibodies are macromolecular structures with a high affinity to a molecule called the antigen. Though the natural aim of antibodies is the protection of the body from pathogens, they have been found to play a useful part in a number of specific treatments and diagnoses. This wider role for antibodies extends to assays where antibodies provide a recognition capability improving available physical methods. Photometrical methods such as ELISA or flow cytometry and electrochemical, optical or piezoelectric immunosensors (biosensors) are typical examples. The present review summarizes facts about the parameters and the production of antibodies. The structure of immunoglobulins, and the production and purification of monoclonal and polyclonal antibodies are described in four chapters. The review was written in order to collect the available knowledge on antibodies into one study improving orientation in this field for anyone wanting to construct immunoassays.

Key words: antibody; immunoglobulin; biosensor; polyclonal; monoclonal; immunoassay

INTRODUCTION

Antibodies play an important role not only *in vivo* but also *in vitro* in the detection of a large number of analytes; they represent recognition elements able to selectively recognize multiple analytes. Antibodies are employed in a large number of methods from chromatographic and electrophoretic to classical immuno-methods such as enzyme-linked immunosorbent assay (ELISA), dot blot

immunobinding assays, electrochemiluminescence, time-resolved fluorescence, and several microscopic techniques. Flow cytometry is a method suitable for the measurement of immune response associated cells (Pasupathi et al. 2008). Actually, the construction of immunosensors is another promising way of employing antibodies for analytical purposes (Pohanka and Skládal 2008). This novel dimension of the analytical role of antibodies arose from the association of antibodies with nanoparticles (Tejral et al. 2009).

Some properties of antibodies, such as sensitivity, cross-reactivity and specificity, are important. These characteristics of a given antibody can predict the properties of methods employing it or the arrangements in which the antibody will be included. The sensitivity of an immunoassay is probably the most important parameter depending on antibody properties. Better sensitivity is logically achieved with higher affinity antibodies (Nimmo et al. 1984).

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Cross-reactivity is the interaction between the paratope of an antibody and a similar epitope or identical epitope presented on different antigens, and it plays a qualitative role in immunoassays. The ideal antibody exhibits minimal or no cross-reactivity and maximal sensitivity. The last important parameter is specificity. It is a function of affinity (avidity for polyclonal antibody) and cross reactivity, and the lack of specificity may lead to false positive or negative results. An antibody with high cross-reactivity and low specificity will predict false positive results with a consequent impact on assay performance.

THE STRUCTURE OF ANTIBODIES

Five types of immunoglobulins are normally present in the human body: IgG (above 75% from all immunoglobulins; in healthy human serum it is present in concentrations of 8–18 g/l), IgM (5–10%), IgA (10–20%) and a minor fraction of IgE and IgD. IgG is the antibody type most frequently used for immunoassay. It occurs in four different forms: IgG1–IgG4. IgG is produced in the human body by B-lymphocytes approximately one week after exposure to the antigen. In comparison, IgM is produced shortly after exposition and it is typically specific to lipopolysaccharides (LPS) and other glycosylated macromolecules (Pohanka 2007). IgG has a molecular weight of 155 kDa and consists of two heavy (approx. 50 kDa) type chains γ and two light (22–25 kDa) chains type κ or λ . The light chains κ and λ are common to the whole group of immunoglobulins. The structure of IgG is schematically similar to the shape of letter Y. The structure of a typical IgG is clearly depicted in Fig. 1. IgG has two binding sites – paratopes – on the two so-called Fab regions including the variable and constant part of the light chain (V_L and C_L) and the variable plus one from three parts of the heavy chain (V_H and C_H1). The constant part from the two heavy chains Fc (C_H2 and C_H3 domains) plays an important role in the immune response and allows binding to tissue cells, complement and/or T and B lymphocytes. The most important role of the Fc part in immunoassays is the convenient orientation of the paratope due to its fixation in the Fc region. Fc and even Fab regions are glycosylated but the most glycosylated is the C_H2 domain (Dwek et al. 1995). The IgG molecule can be selectively fragmented by some enzymes. Papain cuts the heavy chains between the C_H1 and C_H2 domains before the hinge region. Two Fab and Fc fragments are formed. Pepsin is able to degrade the Fc part behind the hinge region so that

the $F(ab)_2$ fragment remains (Parr 1977).

Although not commonly used for analytical purposes, other immunoglobulins besides IgG, would be suitable. IgM is one of the greatest immunoglobulins. This is a pentameric structure where the Fc parts are bound in a circle by the J chain resulting in a final weight of 900 kDa. The heavy chain is the μ type. On the other hand, because of a pentameric structure offering ten binding sites, IgM can initiate the creation of highly crosslinked precipitates. Because of this pentameric structure, the inactivation of the paratope by immobilization is not common compared to the immobilization of IgG. Immunoglobulins A are produced by plasmatic cells near mucous membranes especially the intestine. It has a dimeric structure (occasionally monomeric and trimeric) connected by the J chain and including secretion particles for membrane penetration. The heavy chain is of the α type. The molecular weight of IgA increases in the range from 160 (monomer) – 370 (dimer) – 1000 kDa (trimer) with secretion particles. Immunoglobulins A have only minimal importance as recognition elements in analytical devices but some immunization schemas for IgA secretion have appeared in model systems (Externest et al. 2000). IgE is present in human serum in very low concentrations; its main targets are multicellular parasites and it frequently participates in allergic reactions. The heavy chain is ϵ type and it is largely glycosylated. IgE has a monomeric structure with a molecular weight of 190 kDa. IgD is a receptor on the surface of B-cells and although free in serum is in only relatively low concentrations; its molecular weight is 180 kDa. Both IgE and IgD have minimal importance in immunoanalytical methods even though a large glycosylation in their Fc parts could be an advantage for labeling.

PRODUCTION AND PURIFICATION OF ANTIBODIES

Antibodies can be distinguished according to the number of B-lymphocyte lines that produce them. Polyclonal antibodies are produced from different B-lymphocyte lines as a mixture of immunoglobulins. The monoclonal antibody is a product of one clone of B-lymphocyte. A specific issue is the preparation of recombinant antibodies (Emanuel et al. 2000) where genetic manipulation is applied and the producing cell would be of different origin.

Many purification methods have been developed for the production of crude antibodies over past decades. Since the molecules of immunoglobulins are

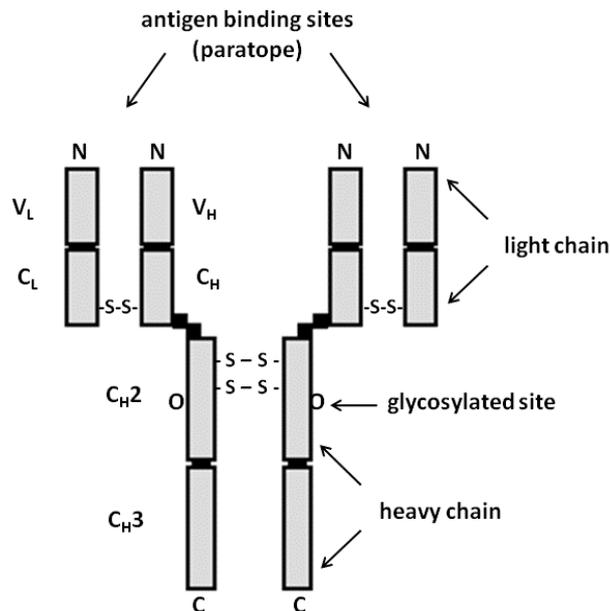


Fig. 1. Schematic structure of immunoglobulin G.

typical proteins, current methods for protein purification are suitable also for the purification of immunoglobulins. For example gel chromatography is one convenient method for separating IgG from the IgM present in polyclonal antibody samples. Precipitation by ammonium sulfate is able to divide the isotypes of immunoglobulins (Bergmann-Leitner et al. 2008). Compounds using pathogenic bacteria for their defense against a hostile immune system are convenient for both affinity chromatography and solid phase extraction (Akerström et al. 1987, Ventury et al. 2000). Proteins A, G and L are very common and commercially supplied either free or bound to supports such as agarose. Antibodies can be simply purified by solid phase extraction using only a matrix with the intercepted protein A, G and/or L (Pohanka et al. 2007). Another suitable bio-ligand is an antibody specific against either a whole Ig group or to one class or subclass of immunoglobulins. Compounds prepared by organic synthesis such as mercaptoethyl pyridine are successful alternatives to biologically derived ligands.

PRODUCTION OF POLYCLONAL ANTIBODIES

For a laboratory allowed and equipped to work with living animals, the preparation of polyclonal antibodies remains the simplest method in comparison

with alternative approaches (Pohanka et al. 2008). Antibodies against pathogens are produced naturally in organisms after the start of an infection but many different antigens are non-pathogenic organisms or simpler macromolecular structures, so there is a risk of inducing tolerability or a hypersensitive reaction. For these reasons, the application of adjuvants is necessary in typical immunisation schemas. Among classical options are Freund's adjuvants, including Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA). FCA is a water-in-oil emulsion with surfactant mannide monooleate and extracts from *Mycobacterium tuberculosis* and *Mycobacterium butyricum*. It is a highly toxic substance and in the immunization protocol, may be used only once. FIA does not include extracts from *M. tuberculosis* and *M. butyricum*, is less effective than FCA, but is less toxic to the immunized organism. A combination of FIA and FCA is usually optimal. Another widely known adjuvant is the Ribi adjuvants system. It is an oil-in-water emulsion including squalene, surfactant Tween 80 and some parts from *Mycobacterium* sp. This system is less effective than FCA, but on the other hand it is less toxic. Titermax is another interesting alternative. It was developed by team headed by Rober L. Hunter in the early 1980's. The main advantage of titermax is the presence only of definable organic compounds with reduced toxicity. This adjuvant titermax includes no biological materials. It is based on the organic copolymers polyoxypropylene and polyoxyethylene,

squalene and some other ingredients. Titermax is comparably effective to FCA but it lacks acute toxic side effects.

An important issue in the production of a polyclonal antibody is the selection of a convenient animal. Outbreeding animals are cheaper but there is poor reproducibility in comparison with inbreeders. The specificity of polyclonal antibodies can be improved when specific pathogen free (SPF) animals are used. The immune system of SPF animals is “tabula rasa”. On the other hand, SPF animals may be inclined to high mortality due to the naivety of their immune system. For polyclonal antibody production, animals such as goats, pigs, hamsters, horses, rats, mice, and sheep should be chosen; however, rabbits seem to be the most convenient for these purposes (Morris and Stanley 2003). Chickens or eggs may be used but avian antibodies have a slightly different structure from mammalian ones. The required amount of antibody and the availability of a menagerie are also important parameters of choice.

Polyclonal antibodies can be prepared against whole microorganisms; their preparation has also been reported using rabbits immunized by peptides such as two fragments – bacteriocin and pediocin PA-1 from *Pediococcus acidilactici* conjugated to the carrier protein keyhole limpet hemocyanin (Martínez et al. 1998) and fragments of human chaperonin 10 bound to ovalbumin (Somodevilla-Torres et al. 2000). The polyclonal antibody can even be prepared against small molecules such as organophosphate when haptened; for example organophosphate paraoxon conjugated with BSA was used for the immunization of New Zealand rabbits (Heldman et al. 1985). The conjugate BSA-paraoxon was emulsified in a complete Freund’s adjuvant and was administered at four doses at 2–4 weeks intervals resulting in production with hyperimmunized serum.

PRODUCTION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies are identical antibodies produced by the hybridized cloning of immortalized B-lymphocyte originating from one parent cell. Hybridoma technology was used for the production of monoclonal antibodies by Köhler and Milstein (1975). Scheme of hybridoma technology is depicted in Fig. 2. At the beginning of the process of antibody production, the laboratory animal is immunized. The B-lymphocytes from either spleen or lymph nodes from the laboratory animal are isolated and are fused with mutant myeloma cells in the presence of

polyethylene glycol. For the selection of fused cells from the original unfused ones the selective HAT (hypoxanthine-aminopterin-thymidine) medium is used. Normal cells include the main pathway for nucleotide synthesis and an alternative pathway based on the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). The myeloma cells employed have damaged genes for HGPRT; the 8-azaguanine will kill cells translating the HGPRT enzyme. If the main pathway for nucleotide synthesis is inhibited by aminopterin, only fused cells (hybridomas) including genes for HGPRT from B-lymphocytes are able to grow and metabolize hypoxanthine. The non-fused B-lymphocytes are not able to grow at all and non-fused myeloma cells are not able to employ hypoxanthine so they do not have a sufficient pool of nucleotides for growth.

The hybridomas thus obtained produce specific antibodies. The culture of hybridomas is diluted up to a single cell per microplate well and colonies resulting from each individual cell are consequently cultured. Antibodies come from one clone. The antibody thus prepared is monospecific. Every colony should be tested and the colonies producing antibodies with the desired properties can be cultivated, allowing the production of antibodies in large quantities. Stocks of the cell line can be kept frozen for an unlimited time. The monoclonal antibody can also be produced in live animals. When the hybridoma cells are injected into the laboratory animal, they produce tumours containing an antibody rich solution – ascites fluid. This technique is very painful for animals, is often considered unethical, and thus the production of monoclonal antibodies in fermentation chambers is currently preferred.

Monoclonal antibodies have been prepared for large number of antigens including small molecules such as the organophosphate soman (Erhard et al. 1990) or larger structures such as the LPS surface antigen O157 from *E. coli* (Laegreid et al. 1998). Monoclonal antibodies could be prepared also for the assay of whole cells such as *Listeria monocytogenes*; Crowley et al. (1999) used prepared antibodies for the construction of an amperometric immunosensor as well as ELISA. In other studies, monoclonal antibodies against the cell wall proteins of *Bifidobacterium longum* (Amrouche et al. 2006) and *B. anthracis* lethal toxin (Little et al. 1990) were prepared in suitable amounts and quality. A technique for the production of human monoclonal antibodies of an appropriate isotype against selected antigens is possible by employing mice and standard hybridoma technology (Mendez et al. 1997); the original Ig loci in mice are reconstituted with human ones. The antibodies thus prepared are tolerated by the human

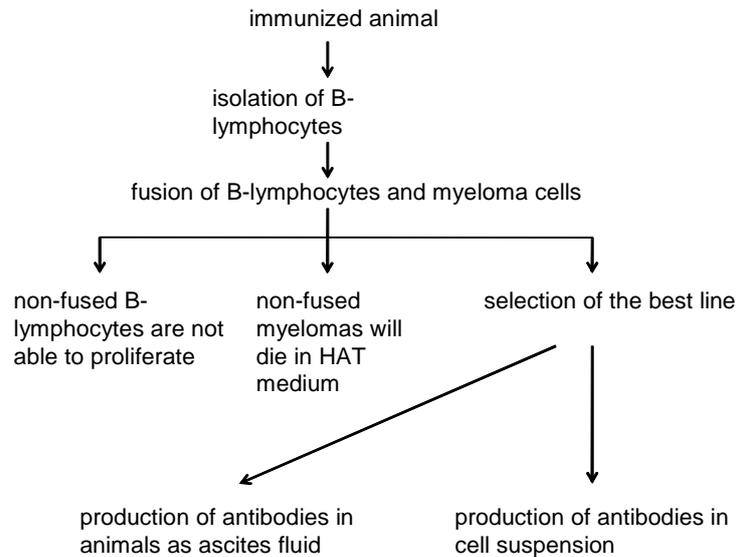


Fig. 2. Blocking scheme of monoclonal antibody production.

body and this could be promising for some specific treatments as well as diagnoses. The antibody with specificity to the LPS O side chain antigen from *Pseudomonas aeruginosa* generated from mice is a practical example of a humanized monoclonal antibody (Hemachandra et al. 2001).

EXAMPLES OF THE APPLICATION OF ANTIBODIES

Antibodies are a necessary part of many devices. ELISA kits for the diagnosis of infectious diseases, the levels of specific markers such as the marker of inflammation cyclooxygenase 2 are typical examples (Kowal-Bielecka et al. 2005). An example of a complex technical instrument using antibodies as a recognition element is the RAPTOR device developed in the Naval Research Laboratory (Washington DC, USA) in the 1990s. This portable device is based on a completely automated fiber optic biosensor for simultaneous detection of four different biological agents in 3–10 minutes. The assay scheme was classical: fibers were covered by primary antibodies; the complex with analyte is closed by the second antibody labeled Cy-5. The system was used, for example, by Anderson et al. (2000) for the laboratory detection of the following biological

warfare agents (in brackets are shown the limits of detection): *Bacillus globigii* (5×10^4 CFU/ml; mimetic of *B. anthracis*), *Francisella tularensis* (5×10^5 CFU/ml), and staphylococcal enterotoxin (SEB; 10 ng/ml) and ricin (50 ng/ml).

Pohanka and Skládal (2007) used the polyclonal antibody immobilized via protein A to construct a piezoelectric biosensor based on quartz crystal microbalance (QCM). The biosensor was found useful for the label-free detection of *F. tularensis*. A limit of detection of 10^5 CFU/ml in buffer and 10^6 CFU/ml was achieved when *F. tularensis* was attenuated by 0.5% phenol. Antibodies can be widely used for assays of mycotoxins as an alternative to the more elaborate instrumental techniques (Kolossova et al. 2006, Pohanka et al. 2008b); for example, two mycotoxins, aflatoxin B1 and ochratoxin A, were assayed by a tandem immunoassay column and competitive ELISA (Goryacheva et al. 2007). They reached a limit of detection of 5 (aflatoxin B1) and 10 (ochratoxin A) $\mu\text{g}/\text{kg}$.

CONCLUSIONS

Antibodies are a wide group of molecules suitable for specific interaction with analytes. A continuous effort to prepare antibodies is necessary to develop

immunoassay. This study describes techniques necessary for the production of antibodies and their purification. Monoclonal as well as polyclonal antibodies seem to be promising for analytical purposes as can be seen from the examples given. Though the production of antibodies is quite cheap, it is still necessary to use laboratory animals and thus the ethical aspect of antibodies production should be also taken into account.

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