

Immunization, immune sera and the production of antibodies

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The overall characteristic of antibody molecules is the uniqueness with which they bind selectively antigens. Thus, the antigen-binding specificity make antibodies important tools in research, in analytics and diagnostics as well as in therapeutic strategies. Antibodies can be produced *in vivo* or *in vitro* (Table 1); they can be of polyclonal or monoclonal origin.

Table 1: Production and features of polyclonal and monoclonal antibodies

Procedure	Type of antibody	Advantage	Disadvantage
Animal immunization	Polyclonal antibodies (hyperimmune sera)	Fast and not expensive	Unwanted and cross-reacting antibodies
Animal immunization followed by fusion of antibody producing cells with myeloma cells (e.g. mouse hybridomas)	Monoclonal antibodies (hybridoma)	Mass production of homogenous antibody populations	Restricted conformational epitope specificity
Human hybridomas	Monoclonal antibodies (hybridoma)	Clinical applications	No routine production For research application No significant advantage
Immunization <i>in vitro</i> followed by fusion of antibody producing cells with myeloma cells (e.g. mouse hybridomas)	Monoclonal antibodies (hybridoma)	Small amount of antigen required	Mainly research area
Fusion techniques, e.g. electrofusion and antigen-directed fusion (hybridomas)	Monoclonal antibodies (hybridoma)	Enhanced efficiency in production of hybridomas compared to classical fusion technique	Mainly research area
Cloning of antibody genes in bacteria	Recombinant antibodies (gene technology)	Selection of antibody specificities which may not emerge <i>in vivo</i>	No affinity maturation as <i>in vivo</i>
Retroviral vectors, transformation of antibody-secreting cells	Recombinant monoclonal or polyclonal antibodies (gene technology)	Immortalization of antibody secreting plasma cells	Mainly research area
Proteins and peptides derived from primary sequence (phage display libraries)	Recombinant antibodies (gene technology)	High selectivity	Mainly research area

Cloning of antibody genes in plants (cDNA from immunized animals; phage display libraries)	Recombinant antibodies (gene technology)	High selectivity	Mainly research area
Antibody construction by fusion of Fab with Fc of other species (CDR-grafting)	Humanized monoclonal antibody	Reduced immunogenicity of mAbs from xenogeneic sources for clinical application	Mainly research area
New developed culture media (hybridomas)	Monoclonal antibodies (new culture conditions)	Easy purification of antibodies due to low levels of foreign proteins in culture media	Mainly research area

Polyclonal antibodies

Immunization of laboratory animals with an antigen results in the generation of a pool of antibodies, with each antibody species produced by a specific B cell clone and recognizing a specific epitope of the antigen. These antibodies are collectively called polyclonal antibodies and are used as affinity reagents for *in vitro* diagnostics. Since these antibodies can be directed against different epitopes on the same target, they can result in cooperative binding. The use of a population of different antibody types increases the chance that at least one type of antibody will bind the target. However, the major disadvantage is that polyclonal antibody production is not exactly reproducible, even if the same type of animal is immunized with the identical antigen. This phenomenon is due to the way in which B cells mature during the immune response.

For the production of antibodies, one can start with purified proteins, cDNA or PCR fragment for the antigen, peptides or haptens. Even today, polyclonal antibodies are usually obtained by immunization of animals. Hyperimmunization is achieved according to injection schedules in which animals are repeatedly boosted with the same antigen. Hyperimmune sera contain many antibody populations specific for a broad range of epitopes of a given antigen (including denaturation resistant epitopes). Polyclonal antibodies not only differ with respect to the epitopes they recognize on the immunizing antigen, but may also differ in their affinity for the same determinant. Hence, specificity and affinity must be considered together when characterizing polyclonal antibodies in hyperimmune sera.

Antibodies from hyperimmune sera usually work well on fixed tissue samples, and, thus are a good source for immuno-stainings of aldehyde fixed tissues and paraffin sections. However, apart from the above objections, polyclonal immune sera may contain irrelevant antibodies of unknown specificity which can create troublesome problems for immunohistology. Even sera from nonimmunized animals can give background when used at high concentration. Part of this background will come from nonspecific antibody binding to the tissue preparation, another part will be due to specific interaction of irrelevant antibodies. Thus, immunoaffinity purification of the specific antibodies should be performed for clear cell staining patterns.

Immunogenicity is the ability of a molecule to induce an immune response, and this is determined by the structure of the injected molecule and by the fact whether or not the host can recognize this substance. With respect to protein antigens, one has to consider that a single gene may generate different protein isoforms, for example by alternative splicing of the

primary gene transcript giving multiple different mature transcripts coding for (immunologically) different proteins. Furthermore, complexity to proteins derived from a single gene is added by posttranslational steps such as glycosilation and proteolytic processing.

Proteins including peptides, carbohydrates, carbohydrate-protein complexes (glycoconjugates), nucleic acids, lipids and many other natural or synthetic compounds are to be considered as immunogens. For a successful immunization, the compound must contain an epitope that can bind to the surface receptor of a B cell (cell surface antibody) and, in general, it must promote cell-to-cell communication between B cells and helper cells.

Source and purity of antigens are critical points in immunization. Before starting an immunization protocol, the major decision to be made is how pure should the antigen need to be. When highly specific antibodies are an essential prerequisite, then the antigen must be of purified to homogeneity; alternatively, the antigen preparation should be used to prepare monoclonal antibodies. Standard techniques for purification include affinity chromatography, differential extraction, subcellular fractionation, or if the protein of interest can be seen as a unique band on a SDS-polyacrylamide gel, the gel can be used as a suitable purification step.

Small changes in the antigen structure can largely affect the strength of antigen-antibody interaction. Also, changing the amino acid residues that form the binding site of an antibody molecule can alter the strength of an antigen-antibody interaction (low and high affinity antibodies) as to be observed in the hypervariable regions of the antibody (the actual binding sites for the antigen which are referred to as the complementarity determining regions or CDRs). In the course of B cell differentiation and maturation, CDR residues undergo extensive mutation, yielding antibodies that differ in the microstructure of their antigen binding sites. This process continues during reexposure to antigen (secondary immune response) which results in a stronger and more specific antibody response.

Immunization of animals

A wide range of species can be used for immunization purposes, rabbits, mice, rats, hamsters and guinea pigs are most common. When large volumes of immune sera are needed, sheep, goats, donkeys, horses, pigs can be used as well. For practical purposes, rabbits are the most frequently used animal. This popularity is mainly attributed to the fact that rabbits can be easily and repeatedly bled. Moreover, rabbit antibodies precipitate proteins over a wide range of antigen or antibody excess. Then, pools from several rabbits are readily made which are less likely to give major batch-to-batch variations than pools prepared from few larger animals (e.g. goat, sheep, horse).

For successful immunization, the following characteristics have to be fulfilled:

- Antigens for immunization must not be part of the host self-repertoire.
- The size of antigen should be not smaller than about 3000-5000 daltons. Otherwise small molecules may be coupled to larger substances such as bovine serum albumin or keyhole limpet hemocyanin to overcome the problem of immunogenicity (e.g. hapten coupling with carrier protein).
- The immunizing substance must carry B- and T-cell epitopes.

- Molecules for immunization must be degradable when they enter the B cell. Degraded fragments will migrate to the surface where they must bind to receptors (HLA class II protein). This complex is then bound by a receptor on the surface of helper T cells (T-cell receptor). The physical link between these two cells is formed by the antigen fragment and is essential in the differentiation of a B cell into a plasma cell. Similar types of cell-to-cell contact are needed in other stages of the immune response (in activating helper T cells; for interactions between helper T cells and cells known as antigen-presenting cells).

The solubility of an antigen is not important for immunization. In contrary, precipitated antigens are usually good immunogens. Of course, the route of antigen inoculation will influence the immune response such as the cell type involved in antigen internalization and processing and, also, the lymphoid organ in which the antibody response will take place.

The amount of pure antigen being necessary for primary immunization depends on its nature (high molecular or low molecular antigens) and its solubility. Typically, 50-100 µg of soluble antigen are sufficient per injection. Primary immunization is usually done with small amount of antigen mixed with an adjuvant. The antigen is often injected in small volumes (0.1-0.5 mL) intradermally or subcutaneously; injections into the foot pad muscle or intraperitoneally are also possible.

After primary injection, immune sera will have substantial amounts of IgM antibodies, whereas hyperimmune sera contain mostly IgG antibodies. Furthermore, hyperimmune sera have higher levels of IgG, and the average affinity of specific antibodies for a given antigen will increase with repeated injections. Thus, after primary immunization, booster injections are needed to obtain good hyperimmune sera. The first booster injection will follow 3-5 weeks after primary immunization with further boosters (3-6 injections are often needed) at appropriate intervals (1 to 3 weeks) and depending on the antibody titer.

In order to enhance antibody responses, especially to low antigen doses or to poor immunogens, the immunizing antigen is mixed with appropriate adjuvants which *per se* are not immunogenic. The enhancing effect is due to more efficient antigen capture and to a prolonged antigen presence after inoculation depot effect). Furthermore, significant nonspecific stimulation of the immune system is achieved when bacterial components are included in the adjuvant. The choice of the adjuvant has some effect on the generated isotype of the antibody response. A number of adjuvants have been successfully used such as (a) the complete and incomplete Freund's adjuvants, (b) alum hydroxide, (c) *Bordetella pertussis* toxin. Some adjuvants are harmful when introduced into animals, and one has to comply with the local legislation for handling laboratory animals.

It is important to control the rise of antibody titers at different time intervals. Depending on the type of study, the animal may be left alive or exsanguinated. The bleeding method depends on the animal species, the amount of serum needed, the frequency of sampling and, of course, whether the animal should survive. Peripheral blood can be harvested by different procedures, f.e.

- Marginal ear vein: mainly rabbits and guinea pigs.
- Jugular vein: goat, sheep, donkey and other larger animals.
- Cardiac puncture: mouse, rat, chicken, fish; cardiac puncture is a risky method and often associated with sacrificing the animal.
- Orbital sinus: mainly small rodents.

- Caudal tail vein: rat and mouse.
- Brachial vein: mainly birds.

Collected blood is allowed to clot, then spin down the clot by centrifugation and transfer the serum into appropriate tubes for further processing. For the use in immunohistology it is not important whether antibodies are precipitating (e.g. polyclonal antibodies) or nonprecipitating (e.g. monoclonal antibodies) because reaction with immobilized antigen entails capture onto the tissue preparation rather than precipitation. Yet, prior to employ immune sera in immunohistological studies, specificity and antibody titers must be examined by a number of immunochemical techniques which include electrophoretic analyses, gel diffusion techniques, ELISA or RIA methods and others. The analysis of antibodies with proteome microarrays is another promising approach to test antibody specificity because an array will contain a great number of antigenic substances for the relevant tissue and, thus, will allow simultaneous screening for possible cross-reactivity at the same time.

An alternative method to the use of purified antigen as immunizing substance is DNA immunization. The procedure is based on cloned cDNA (coding for an antigen) which is directly injected in the animal (usually mouse). The method implies that the cDNA is under control of a strong promoter. In favorable conditions, the clone will be taken by the host cells and integrated into the nucleus. The transcribed antigen may be secreted or expressed on the cell surface, thus eliciting an immune response.

Monoclonal antibodies

Normal sera and immune sera as well as hyperimmune sera contain different types of antibodies that are specific for many different antigens. The lack of specificity, however, can create a variety of problems in immunochemical techniques, and the preparation of homogenous antibodies with defined specificity is often needed. This goal can be achieved with the hybridoma technique developed by G KÖHLER and C MILSTEIN (1975). In this technique, an antibody secreting B cell (isolated from an immunized mouse) is fused with an immortal cancer cell (myeloma cell, B cell tumor) which is called hybridoma. These immortal somatic cell hybrids (a clonal population) are grown *in vitro* where they continue to secrete antibodies of defined specificity, the so-called monoclonal antibodies.

The myeloma cells provide the correct genes for continued cell division *in vitro*, and the antibody-secreting cells provide the appropriate immunoglobulin genes. The main characteristics of monoclonal antibodies are (a) their binding specificity, (b) their homogeneity, and (c) their ability to be produced in unlimited quantities. Finally, the hybridoma production is of great advantage as compared to animal hyperimmunization inasmuch as impure antigens can be used for the production of specific antibodies.

Because monoclonal antibodies are derived by descendants of one hybridoma cell, those antibodies are extremely useful in testing for the presence of a desired epitope. The reproducible specificity make monoclonal antibodies especially valuable in immunohistological studies. Any substance that can elicit a humoral immune response can be used for the preparation of monoclonal antibodies. In practical work, monoclonal antibodies may be used either as single antibody preparations or as pools.

Hybridomas can be prepared by fusing myeloma cells and antibody secreting cells from different species, but the ratio of viable hybridomas is greatly increased when closely related species are used. Furthermore, fusion of cells from the same species allows the resulting hybrids to be grown as tumors in this strain. When derivatives of BALB/c myelomas are used as fusion partner, then immunizations are normally done in BALB/c mice.

In practice, polyethylene glycol (PEG) is the fusing agent of choice for hybridoma production. PEG fuses the plasma membranes of adjacent myeloma and antibody secreting cells, forming a single cell with two or more nuclei. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells, but segregation does not always give identical sets of chromosomes. When chromosomes that carry immunoglobulin heavy- and light-chain genes are lost, antibody synthesis will stop and result in unstable lines.

Even in efficient fusion experiments, only about 1% of the starting cells are fused, and only 1 of 10^5 will form viable hybrids. Cells from the immunized animal will not grow in culture while the myeloma cells continue to grow, and, thus must be killed. This is achieved by drug selection. Commonly, myeloma cells have a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis. Selection with 8-azaguanine often yields cell lines harboring a mutated hypoxanthine-guanine phosphoribosyl transferase gene (HPRT). The addition of any compound that blocks the *de novo* nucleotide biosynthesis will force cells to use the salvage pathway; cells containing a nonfunctional HPRT protein will die in this condition.

Hybridoma production involves the following steps,

- Immunization of the animal (mice): primary immunization with an antigen followed by a booster injection 2 weeks later. About 10 days later, the humoral immune response is tested, and if necessary, the animal is further boosted.
- Screening procedure: animal bleedings are used to develop and validate the antibody screening.
- Production of hybridomas: several days prior to the fusion experiments, animals are boosted again with the antigen (the final boost is intravenously). About 3-6 days later, antibody secreting cells are prepared from the animal and mixed with myeloma cells for
- fusion. After fusion, cells are diluted in selective medium and plated in multiwell culture dishes.
- Hybridoma screening: about 1 week after fusion, hybridomas are tested for antibody.
- Selection by single cell cloning: cells from positive wells are expanded and single-cloned by limiting dilution.
- Subcloning: once positive clones have been selected, cloning and recloning of positive hybridomas is necessary in order to avoid overgrowth by nonproducing cells; up to 6 or more clonings may be necessary. Positive hybridomas may be frozen for later experiments and subcloning. Hybridomas can be maintained *in vitro* and will secrete antibodies with defined specificity.
- Expansion of clones: propagation of hybridomas in culture (or storage in low-temperature freezer at -80°C) for mass production of monoclonal antibodies. Alternatively, monoclonal antibodies at high concentration can be obtained by production of ascites. For this purpose, clones are injected intraperitoneally into mice of the same strain.

Apart from using mice for the production of monoclonal antibodies, other species including rat, rabbit, human or else may be useful, too. This can be of special interest and advantage when other species than mice are able to recognize antigens which are non-immunogenic in the mouse system.

Recombinant antibodies, fragments and alternative affinity ligands

Insights into the molecular mechanisms of cellular antibody synthesis enables adaptation of the same principles to the creation of “synthetic” antibodies. The isolation of cDNA from hybridoma or other sources allows for the transfer and engineering of the antibody genes into alternative expression hosts. In the mean time, technology for the production of various antibody constructs has become significantly developed. Thus, full-length hybridoma derived antibodies are used side by side with recombinant antibodies or fragments of antibodies.

Chemically, *Fab fragments* (antigen binding fragments) are obtained by enzymatic cleavage of whole antibodies with papain. The smaller *single chain Fv* (scFv) which consist of the variable region’s light and heavy chains are genetically created by joining both fragments with a flexible linker (f.e. 15 amino acids). Thus, light and heavy chain fragments are obtained as a single polypeptide chain. It can be expected that even smaller recombinant antibody fragments than the scFvs or engineered variants will be useful alternatives in a variety of applications. A multitude of engineered fragments of antibodies are now available by recombinant technology, f.e. dimers and trimers of identical Fab fragments, so that binding benefits from the avidity effects of many proximal binding sites. ScFvs have been expressed as non-covalently linked dimers (diabodies), trimers (tribodies) and tetramers (tetrabodies), and these multimeric antibody fragments have been produced as multispecific ligands with different specificities for each domain (HOLLIGER P and HUDSON PJ, 2005). Single-chain variable fragments of antibodies (scFvs) displayed on phage provide an alternative to classical hybridoma technology and the isolation of antibodies *in vitro*.

Since 1990, phage display is utilized for display and selection of antibody fragments expressed on the surface of a phage (MCCAFFERTY J et al., 1990). This technology that physically links antibody DNA with antibody protein (i.e. genotype with phenotype) allows for the construction of large libraries of antibody fragments from which clones capable of interacting with a desired target can be “fished” out for later identification and recombinant production. Libraries of antibody fragments can be constructed using antibody genes from different sources. Library designs and selection concepts are able to identify monoclonal antibodies with virtually any specificity (HOOGENBOOM HR, 2005). During the last decade a number of large scFVs libraries have been constructed and displayed using methods such as phage display (MARKS JD et al., 1991; KNAPPIK A et al., 2000) or ribosome display (HANES J et al., 2000). Moreover, improved screening and selection methods have been developed which are now exploited to build human antibodies with high affinity and specificity (MARKS JD et al., 1992).

One can choose from more than 15 billion unique, functional antibody specificities (in Fab format) by use of a synthetic human library mimicking the natural diversity and selection of the immune system. In the library, the structural diversity of the human antibody repertoire is represented by seven heavy chain and seven light chain variable region genes which give rise to 49 frameworks in the master library. Then, highly variable genetic cassettes (CDRs, Complementarity Determining Regions) are superposed to mimic the entire human antibody repertoire, and more than 15 billion human antibody specificities in Fab format are readily

prefabricated in *E. coli* or phage libraries. Selection of the antibodies occurs in an automated panning process where the antigen is immobilised on a solid surface and brought into contact with the antibody specificities in the library. Finally, unique candidates are then expressed in bacteria and affinity purified.

With the development of recombinant technology, an interest in alternative non-immunoglobulin based binding elements emerged. For example, libraries of random linear peptides have been used in epitope mapping and affinity ligand development (LABROU NE, 2003). Alternative protein architectures are now under consideration for the generation of artificial receptor proteins with prescribed ligand specificities. Thus, proteins of the lipocalin family are thought to be powerful candidates (SKERRA A, 2000; WEISS GA and LOWMAN HB, 2000; SKERRA A, 2003). Despite differences in fold and shape, antibodies and anticalins share much in common. The monomeric anticalin is similar in size to a single chain variable domain (V_L - V_H) of an antibody. Both types of molecules form small molecule binding sites or CDRs by forming binding surfaces from flexible loops. In first experiments with CTLA-4, an immunomodulatory molecule expressed on the surface of T cells after activation and overexpressed on a number of lymphoma subtypes, anticalins could be successfully employed for flow cytometry and immunohistochemistry. These findings suggest that anticalins are in principle suited as detection reagents in diagnostics including the localization of target molecules on fixed cells.

Lipocalins represent a class of natural proteins with more than 200 known members. They are found in a variety of organisms where they have evolved for diverse physiological functions. Lipocalins are a family of “professional binding proteins” that specialize in binding small molecules (e.g. hydrophobic, insoluble or chemically sensitive organic compounds) for transport and storage. Retinol-binding protein is one of the first characterized lipocalins. Lipocalins with novel binding specificities can be engineered by subjecting a set of amino acid residues that surround the natural ligand to genetic random mutagenesis, followed by selection at the protein level (BESTE G et al., 1999). This novel class of engineered binding proteins is called anticalins. This strategy may result in a combinatorial protein library with more than 10^{10} different members, comprising binding sites with diverse shapes. The library is then employed for selection experiments against the prescribed ligand using phage display methods. An affinity-enriched pool of typically 10^3 to 10^5 anticalins is prepared which can be further narrowed down by colony screening techniques (SCHLEHUBER S et al., 2000) or automated high throughput screening.

Other examples of engineered affinity ligands based on alternative scaffolds include *affibody* molecules (NILSSON B et al., 1987; NORD K et al., 1997; WIKMAN M et al., 2004), *ankyrin* repeats (BINZ HK et al., 2003), *knottins* (CRAIK DJ et al., 2001) and *aptamers* (TUERK C and GOLD L, 1990; BRODY EN et al., 1999; PROSKE D et al., 2005). A discussion of all these molecules, however, is beyond the scope of the paper.

Selected publications for further readings

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Full citation of publications is given in chapter *References*

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