The Use of Antibodies in Immunoassays

Introduction

Immunological reagents are the backbone of every immunoassay system. Immunoassays can be utilized to quantitatively and qualitatively measure minute quantities of analytes in complex biological systems. Although the concepts behind the development of an immunoassay seem straightforward, development of assays that provide false or misleading information is common when proper selection of immunoreagents is not a critical part of the assay design. Immunoassays use many types of supports and detection systems. However, at the heart of every immunoassay is an antibody that determines the level of sensitivity and specificity.

Antibody Structure

The γ-globulins were recognized as a distinct group of serum proteins by Tiselius in 1937. Immunoglobulins (also known as antibodies) are glycoproteins synthesized and secreted by B-lymphocytes and plasma cells. They are separated into classes based on physical characteristics including the structure of the heavy chain, size and valency (number of antigen binding sites). Mammalian species produce 5 classes of immunoglobulins: IgG, IgM, IgA, IgE and IgD. The basic structure of an immunoglobulin secreted by a single B cell is a heterodimer consisting of 2 identical light chains and 2 identical heavy chains arranged such that there is a bifold axis of symmetry in the molecule and a valency of two. The light chains consist of approximately 110 amino acids referred to as the variable region at the N-terminal end of the molecule and approximately 110 amino acids referred to as the constant region at the C-terminal end. The heavy chain has a similar structure, of an approximately 110 amino acid variable region, but it is followed by an approximately 330 amino acid constant region. The variable regions serve as the antigen binding part of the molecule and provide each different antibody molecule with its binding specificity.

Each heavy and light chain is linked by disulfide bonds in their constant regions and hydrophobic interactions in both their variable and constant regions. The heavy and light chain pairs are also linked by disulfide bonds and
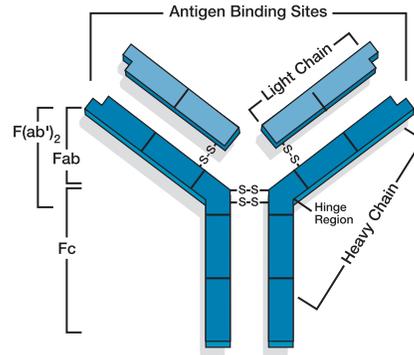
Structure of an IgG Antibody

Figure 1.

hydrophobic interactions, both of which occur in the heavy chain constant region. The basic structure resembles a "Y", in which the two arms that contain the light and heavy chain variable regions bind antigen. This is referred to as the F(ab) (antigen binding) region The arm containing only heavy chain has physiological functions such as complement binding and placental transfer properties and is referred to as the Fc (fragment crystallizable) region (see Figure 1).

Within the antigen binding arms the variable regions of the heavy and light chain fold to form the antigen binding pocket. Within the heavy and light chain variable regions are areas of increased variability referred to as hypervariable regions that serve as contact areas with the antigen and provide the high degree of three dimensional complementarity and the noncovalent interactions with which an antibody and antigen interact.

The five immunoglobulin classes are divided according to the type of heavy chain found in the molecule: IgG has gamma chains (γ), IgM has mu chains (μ), IgA has alpha chains (α), IgE has epsilon chains (ε) and IgD has delta chains (δ). Antibodies also have two types of light chains known as kappa (κ) and lambda (λ). Kappa and lambda light chains may associate with any of the heavy chains (see Table 1). IgG can be further subdivided into subclasses based on slight variations in the amino acid sequence of the heavy chains. For example, human IgG can be divided into IgG1, IgG2, IgG3, IgG4 and mouse can be divided into IgG1, IgG2a, IgG2b and IgG3. Other species have similar subdivisions.

Antibody Class Characteristics

Class	Heavy Chain	Light Chain	Approximate Molecular Weight	Molecular Formula
IgG	γ	κ or λ	150,000	$\gamma 2\kappa 2$ or $\gamma 2\lambda 2$
IgM	μ	κ or λ	950,000	$(\mu 2\kappa 2)5$ or $(\mu 2\lambda 2)5$
IgA	α	κ or λ	150,000-500,000	$(\alpha 2\kappa 2)n$ or $(\alpha 2\lambda 2)n$ where n=2,4 or 6
IgE	ε	κ or λ	190,000	$\varepsilon 2\kappa 2$ or $\varepsilon 2\lambda 2$
IgD	δ	κ or λ	180,000	$\delta 2\kappa 2$ or $\delta 2\lambda 2$

Adapted from Harlow E. and Lane D., Antibodies, A Laboratory Manual, Cold Spring Harbor (1988)

Table 1.

Antibodies can be enzymatically digested into $F(ab')_2$, Fc, and F(ab) components. The Fc fraction does not contain a specific antigen-binding domain but contains regions to which cell surface receptors, serum and bacterial proteins may bind. When IgG is digested with pepsin, enzymatic cleavage occurs on the C-terminal side of the disulfide bonds that hold the two heavy chains together. This results in two F(ab) regions that are held together by disulfide bonds and is referred to as $F(ab')_2$. $F(ab')_2$ has the same valency as normal IgG and a molecular weight of approximately 110,00 daltons. Papain digestion cleaves on the N-terminal side of the disulfide bonds that hold the two heavy chains together yielding two molecules of F(ab) and one Fc. The F(ab) fragment is half the molecular weight of the $F(ab')_2$ and has a valency of only one.
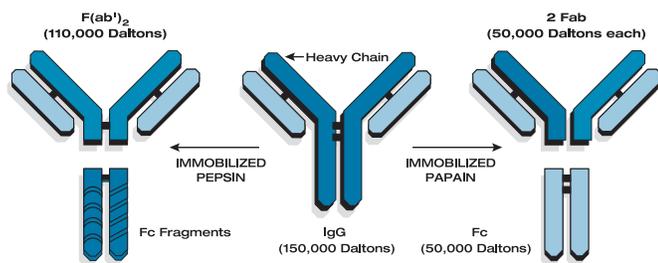

Figure 2.

Digestion of an antibody with pepsin releases the two antigen binding domains bound together by disulfide bonds, resulting in the F(ab')$_2$ fragment. Digestion of an antibody with the enzyme papain splits the immunoglobulin molecule into three fragments of similar size: two F(ab') fragments and one Fc fragment.

Antibody Class Functions

IgG Antibodies: IgG is the dominant antibody found in blood. Functionally, IgG promotes the uptake of microorganisms by immune cells and is responsible for long-term protection from disease as a secondary response. For example, this antibody is used in the detection of blood viruses.

IgM Antibodies: IgM is the first antibody produced as a primary response to infection and is very effective in activating complement and destroying bacteria. It is a pentamer of the basic (two light chain two heavy chain) structure. The pentamer is held together by extra disulfide bonds formed near the C-terminal end of the heavy chains plus a short polypeptide referred to as J chain. IgM is often used in assays for early detection of infection.

IgA Antibodies: IgA is known as the secretory antibody. It is found in mucous membrane secretions as a dimer associated with a secretory chain. It protects the respiratory and gastrointestinal tracts from infection. In some mammals IgA is a major factor in milk that passively protects the newborn.

IgE Antibodies: IgE binds to the Fc receptors on mast cells and basophils and triggers the allergic response. It is also associated with defense against parasites. It is present in blood in extremely small concentrations. Typically, this antibody is used in allergy testing.

IgD Antibodies: IgD is a minor blood component. It is typically bound to the surface of B-lymphocytes.

Antibody-Antigen Interactions - *Some useful definitions*

Immunogen - What is injected into an animal to induce an immune response.

Antigen - The molecule that an antibody binds with high affinity and specificity. Often these molecules are large, sometimes they are proteins that are even larger than an antibody molecule. In fact, in some cases they are not a single molecule, but may be an organism such as a bacteria or virus.

Epitope- What an antigen binding site actually envelops and binds to. A large protein or an organism may have many epitopes. In some cases each one is different in shape. In some cases the same epitope is repeated several times.

Antibodies are defined functionally by the epitope they specifically recognize. Binding to an epitope occurs through weak noncovalent interactions such as: hydrophobic bonding; van der Waals forces; hydrogen bonding and ionic interactions (in order of increasing strength). Each of these interactions alone are respectively 1000-100 times weaker than a covalent bond. It is the sum of many of these interactions between an epitope and an antibody binding site that give this complex a very low Kd (dissociation constant). In addition, these types of interactions only occur over very short distances so it is the complementarity of shape between an epitope and an antibody binding site ("lock and key" or "hand and glove") that allows the two to come close enough together for these forces to exert their strength.

The shape of an antigen binding pocket can vary tremendously depending on the shape of the antigen being bound. Small molecules or short peptides typically bind in a pocket or groove lying between the heavy and light chain variable regions and there may only be contact (noncovalent interactions) between 1-2 amino acids in the antibody molecule and the epitope. Other antigens such as large proteins or organisms can be larger than the antibody molecule itself. In these cases the complementary area of interaction may be an extended surface with an area of 500-1000 A^2 . Within this area, 15 - 20 amino acids in the antibody may contact the same number in the epitope.

Antibody affinity is determined by the rate of formation of an antibody-epitope complex relative to the rate of its dissociation.

The rate of formation (ka) of [Antigen Antibody] complexes depends on the rate of diffusion of the two molecules in the solution while the rate of breaking the complexes (kd) depends on the strength of the interaction (complementarity + noncovalent bonds).

Thus, affinity is a thermodynamic property indicative of the likelihood that the epitope and the antibody will be in a complex. Affinity is measured between one epitope and one antibody binding site, but as described above an antibody molecule has two or more binding sites. Avidity, another measure of antigen-antibody interaction, takes this into account. Affinity is one determinant of avidity; others include the valency of the antibody (number of antigen binding sites), valency of the antigen, and conformational changes in the antibody or antigen upon binding. Thus an antigen with multiple repeating epitopes will more strongly interact with an antibody than the same antigen if it did not have repeating epitopes. In addition this antigen would more strongly interact with an IgM antibody (higher valency) than an IgG antibody with the same binding sites. Avidity is not a thermodynamic property, rather, it can only be described functionally under given assay conditions. It is for this reason that many researchers utilize antibody titer to characterize antibody activity and concentration in a biological sample.

Secondary Antibodies

While antibodies have antigen binding specificity, they can also serve as antigens themselves. For instance, the constant regions of both heavy and light chains in an IgG from a mouse or a human are different in amino acid sequence and thus the three dimensional structure from the heavy and light chains of IgG from a goat. These differences do not create dramatic changes in the structure of the antibody but they do create epitopes that the goat does not have on its own IgG. Therefore, if we inject mouse IgG into a goat it will produce IgG that will react with mouse IgG. This would be referred to as goat anti mouse IgG. Since a mouse antibody is a large molecule it will have many epitopes, some on the heavy chain and some on the light chain, mostly in the constant regions.

Heavy and Light Chain Cross-Reactivity

Antibodies consist of both heavy and light chains. Thus the goat anti-IgG referred to above will actually be anti IgG (H+L), and recognize sites on both the heavy and light chains. These antibodies are widely used to detect IgG and may also recognize IgA and IgM due to common light chain epitopes between the classes. The heavy chains of antibody classes each possess distinct regions that are specific for that class. They also have regions that are similar to each other, which can cause cross-reactivity in immunoassays. Polyclonal antibodies produced against the heavy chain of IgG contain many populations of antibodies that recognize different regions on the IgG heavy chain. Some of these regions are also

similar to the IgA and IgM heavy chains. Therefore, this antibody consists of subpopulations that also recognize IgM and IgA. To make the antibody specific for the IgG heavy chain, these subpopulations must be removed or reduced. To do this, the purified antibody is further adsorbed by passing it over affinity columns with immobilized whole IgM and IgA antibodies containing both heavy and light chains. The antibody populations which recognize IgM and IgA are adsorbed onto the columns, reducing reactivity to IgM and IgA heavy chains, as well as common light chain activity. Antibodies specific for the IgG heavy chain are collected and used for the final product. Heavy chain specific antibodies, i.e. anti-IgG(γ), are useful for the quantitation of the heavy chain antibody only in samples containing other immunoglobulins and proteins such as serum or tissue culture media.

Species Cross-Reactivity

Immunoglobulins of related animal species often share similarities in structure and sequence. For example, affinity purified antibodies against mouse IgG may recognize similar epitopes on human IgG. To minimize cross-reactivity between mouse and human species, antibodies to mouse immunoglobulins are adsorbed against immobilized human serum on an affinity column. The resulting antibody (Anti-Mouse IgG, Human Serum Adsorbed or HSA) is highly specific to mouse IgG and will have only minimal reactivity with human IgG or any other human serum components. Select antibody preparations are further adsorbed against multiple animal species to reduce reactivity to shared regions among these species. KPL's extra-serum adsorbed (XSA) antibodies are cross-adsorbed to as many as 9 different species for the greatest possible specificity. These highly specific antibodies are ideal for use in microwell ELISA, membrane blotting, immunohistochemistry, flow cytometry, immunoprecipitation and hybridoma screening.

Antibodies are commercially available in a variety of forms, such as antiserum, ascites containing monoclonal antibody, purified immunoglobulin, and affinity purified antibody. These may vary significantly in antibody concentration, purity and heterogeneity. The antibody may be provided as a whole molecule or as an antibody fragment: F(ab')$_2$, Fc or F(ab). Which antibody is chosen and in what form depends on the particular application. The goal is to choose an antibody system that provides the greatest sensitivity with the least amount of non-specific activity.

Monoclonal Antibodies

Monoclonal antibodies are produced following the fusion of myeloma cells with antibody secreting B-cells. The resultant continuous cell line (hybridoma) produces large quantities of homogeneous, well defined, single epitope antibody. The availability of large quantities of continuously produced antibody allows for greater standardization and quality control of the antibody reagent. Therefore, monoclonal antibodies are more precisely characterized, legally protected and have greater acceptance by regulatory agencies when used in diagnostic applications.

Because monoclonal antibodies are the result of cell fusion, these proteins may have peculiar differences from other immunoglobulins. They may not precipitate under standard conditions. They may also demonstrate unpredictable binding patterns to Protein A and Protein G. These antibodies may also not purify under ionic exchange conditions as one might expect. These properties may make the purification of monoclonal antibody from ascites a difficult and expensive proposition. Monoclonal antibodies are also very sensitive to conjugation, and often lose activity once conjugated to enzymes such as horseradish peroxidase or alkaline phosphatase. However, one can usually conjugate monoclonal antibodies with small molecular weight molecules such as biotin with minimal risk of losing antigen-binding activity.

The advantages of using monoclonal antibodies in specific applications are numerous. Because monoclonal antibodies can be selected based on affinity during production, high affinity antibodies may be obtained. Monoclones make excellent primary antibodies in heterogeneous ELISA and other immunoassays. In competitive assays for drug, hormone or other small analytes, monoclonal antibodies are the best choice for quantitative and reproducible assays. Because of the defined specificity of the antibody reactivity these reagents can be used in epitope mapping and characterization of fine antigenic structure.

Polyclonal Antibodies

Polyclonal antibodies are obtained from the serum of animals immunized with a particular antigen. The antibody pool obtained from serum is the result of many B-cell clones, each secreting one specific antibody. Antiserum refers to a pool of serum containing all of the antibody fraction plus other serum proteins. Due to serum proteins other than immunoglobulins, immunoassays using unpurified antiserum components usually exhibit high background, poor dynamic range, and low sensitivity. Antibody purified from antiserum is obtained by selective precipitation and various forms of chromatography. An IgG fraction may only contain 10% specific antibody and is only slightly more purified than antiserum. However, this fraction is void of many serum proteins that can interfere with immunoassays.

An affinity purified antibody (see "How KPL Purifies Its Antibodies") is one that has been purified from the IgG fraction by affinity chromatography to a selected antigen. Affinity purified antibodies exhibit the highest specificity and sensitivity that can be obtained from a circulating serum antibody pool. These antibodies exhibit specific activity to a population of antigenic determinants including continuous and discontinuous antigenic sites. Affinity purified antibodies are useful as primary and secondary antibodies in heterogeneous immunoassays and make excellent secondary anti-species (e.g., Goat anti-human IgG) antibody conjugates. Anti-bacterial membrane or viral coat proteins are directed to multiple antigenic determinants. Therefore, unlike monoclonal antibodies, affinity purified antibodies can be used as both the capture and detection antibody in capture immunoassay systems.

Although affinity purified polyclonal antibodies have many advantages, they are not the antibody of choice for some immunoassay systems. In competitive assays designed for measuring drugs or small molecular weight analytes, polyclones are not as reliable as monoclonal antibodies. Since an affinity purified polyclonal antibody contains multiple individual antibodies with varying affinities for an epitope, the affinity constant can not be accurately determined. Multiple antibody affinities may increase the assay variation about a standard curve in comparison to an assay designed with a single monoclonal antibody. Affinity purified antibodies require consistent antiserum quality. Due to the normal variation in the animals producing the antibody, there is greater variation in the final activity of an affinity purified polyclonal antibody than a monoclonal antibody. For systems that require exact reproducibility, monoclonal antibodies may be a better choice than polyclonal. For assays requiring broad spectrum specificity to large molecular weight antigens, affinity purified polyclonal antibodies are the clear choice.

Antibody Fragments

$F(ab')_2$ may be very useful tools for certain situations. They can be coupled as easily as whole Ig with enzymes and fluorophores, but the lack of an Fc region may decrease background from unwanted interactions with Fc binding proteins found on tissue cells and bacteria. In addition, the reduced size may permit more rapid diffusion of the molecule into complex samples such as tissues. Higher sensitivity may be obtained from $F(ab')_2$ than whole molecule antibodies due to the total decrease in mass while maintaining the same antibody valency. Because the F(ab) antibody has only a valence of one, there is decreased stability of the antigen-antibody interaction due to decreased avidity (the cooperative effects of having two identical binding sites in close proximity). In applications where nonspecific activity due to other serum proteins is of major concern, the $F(ab')_2$ is the antibody of choice over a whole molecule.

Enzyme Conjugates

Many immunodetection methods use secondary antibodies conjugated to enzymes in order to amplify the signal via the catalytic properties of the enzyme. The enzymes most commonly used for this purpose are horseradish peroxidase (HRP), alkaline phosphatase (AP), and to a lesser extent, β-galactosidase. Each enzyme offers unique features that, under the right conditions, makes it the optimal choice. Historically, HRP substrates have been shown to be more sensitive in immunoassays as compared to AP substrates. This is primarily due to the faster catalytic rate of HRP. Thus, more product is generated in a shorter incubation time. However, these products tend to fade after development, and they can be hazardous. Additionally, H_2O_2, a cosubstrate in the reaction, ultimately limits the activity of HRP by oxidizing heme iron. This can result in a shorter linear incubation period than seen for AP. In contrast, AP exhibits a slower catalytic rate, but is not self-limiting. Reaction rates remain linear over longer periods of time; therefore, sensitivity can be improved by allowing the reaction to

proceed for longer incubations. AP substrates also tend to be less toxic and thus easier to handle. β-galactosidase conjugates are limited in application by a relative lack of substrate choices and because the size and structure of the enzyme make it difficult to conjugate to antibodies while maintaining activity and solubility. Another factor influencing the choice of conjugate is the presence of endogenous enzyme activity in the sample which may interfere with the assay and/or increase background signal[2]. Mammalian tissues often exhibit peroxidase activity, particularly in cells of hematopoietic lineage (blood cells, macrophages, etc.). In these tissues, AP conjugates present a distinct advantage. Alternatively, different isoenzymes of alkaline phophatase are expressed in many tissues. AP conjugates are typically prepared using the intestinal isoenzymes, which are not inhibited by levamisole. Thus, it is often possible to inhibit endogenous AP activity by levamisole pretreatment. The obvious exception is in intestinal tissue, where HRP would be the conjugate of choice.

Summary

A wide variety of antibody products are available to the researcher designing an immunoassay. Each has advantages and disadvantages in specific applications. The goal of the researcher is to find the best antibody product for each application, minimizing non-specific reactions while increasing sensitivity and dynamic range. To accomplish this goal, one must be cognitive of the underlying physical chemistry involved in antibody-antigen interaction during each phase of the immunoassay to obtain meaningful and reliable results.

References:

1. Porstmann, B. et al. (1985). J Immunol. Methods 79:27-37.

2. Bratthauer, G. L. (1994). in Jovois, L.C., ed. Methods in Molecular Biology, Vol. 34: Immunocytochemical Methods and Protocols. Humana Press Inc., Totowa, NJ, 155-164.

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