Antibody molecules and the antigen-antibody interaction

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The immune system is a diffuse organ dispersed in the body and basically constitutes lymphoid cells, cytokine and antibody molecules. The latter are the product of lymphoid cells. In the past, the cellular basis for antibody formation was the subject of several theories, e.g., the selective theory according to P Ehrlich (1900), the instructive antigen-template theories as suggested by L Pauling (1940) and F Haurowitz (1967) and the clonal selection theory of FM Burnet (1959). There is now sufficient evidence from numerous experiments to support the clonal theory. In the case of antibody synthesis, the necessary mechanisms and pathways of cellular cooperation and B cell maturation are quite well understood.

Mammalian antibody molecules

Immune sera for experimental work are primarily obtained by immunization of mammals. The ability of a substance to cause specific antibody production when introduced into a host is called immunogenicity. Plasma cells and their progenitors (B lymphocytes, B cells) are responsible for the synthesis and secretion of of humoral antibodies (review by Nossal GJV and Ada GL, 1971). Within the immune system, the biosynthesis of antibodies is regulated by sequential steps of cellular and molecular cooperation and involves (1) recognition and processing of the antigenic/immunogenic information, (2) cellular cooperation *via* antigen-specific receptors, (3) transformation of committed lymphocytes into blast cells with subsequent differentiation and maturation into plasma cells, (4) the expression of antibody genes, and (5) immunoglobulin class switching, the mechanism whereby an IgM producing B cell switches the isotype to begin to synthesize IgG molecules instead.

The very first antigen challenge of an animal will elicit a primary immune response with a typical lag phase during which components of the adaptive immune system must "learn" (with help of the innate immune system) to react against the foreign substance with specific antibody formation. First, the structural variety of antigen binding sites relies on the somatic repertoire of immunoglobulin genes which arises from statistical recombination of germline gene segments. In this way, the B lymphocyte encodes an individual antibody with a novel antigen-binding site initially displayed as antigen receptor on the cell surface. Second, if this B lymphocyte encounters the antigen and if its antibody exhibits sufficient affinity to form a membrane-bound cluster with the antigen, this cell will become stimulated for clonal proliferation whereby the B cell and the antibody will undergo maturation.

Genetic mechanisms create antibody diversity at the somatic level in a step-wise process by recombination of a set of gene segments (VL and VH domains) and hypermutation events during which the B cell maturation occurs. In the course of differentiation and maturation processes, antibody genes are transcribed into messenger RNA, and, specifically, antibodies are tailored by multiple gene splicings and gene rearrangements. Hence, the finally obtained antibodies are the product of affinity maturation with respect to clonal selection and somatic maturation by which B cell clones secreting high-affinity antibodies are selected for

proliferation. This process is of advantage when the immune system is challenged again with the same antigen, and the subsequent secondary immune response (even after a long time and then recruiting memory cells) will react faster and more efficiently than at the time of first antigen contact.

Antibodies are serum proteins which are called immunoglobulins. In most species, immunoglobulins comprise five main isotypes (listed in order of decreasing quantities): immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin D (IgD) and immunoglobulin E (IgE), differing for example in the number of constant domains (CH-domains), glycosylation and properties related to the formation of higher valency forms (i.e. dimers or pentamers). Immunoglobulins are found in the γ-globulin fraction which is called as such according to its electrophoretic mobility. All antibodies have the same basic structure; the regions of the antigen binding site are formed by the variable light chains (VL) and the variable heavy chains (VH). Every antibody molecule has two identical light chains and two identical heavy chains. The antibody isotypes are determined by their heavy chains: the five types of antibody classes are defined by five different heavy chains. Major structural and functional details of the human immunoglobulins are summarized in Table 1.

Table 1: Human immunoglobulin classes and their major characteristics

Immunoglobulin	IgG	IgA	IgM	IgD	IgE
H chain	Gamma (γ)	Alpha (α)	My (μ)	Delta (δ)	Epsilon (ε)
L chain	Kappa (κ) or lambda (λ)	Kappa (κ) or lambda (λ)	Kappa (κ) or lambda (λ)	Kappa (κ) or lambda (λ)	Kappa (κ) or lambda (λ)
Structure (secretory form)	One Y (monomer)	One, two or three Y (monomer, dimer or trimer)	Five Y (pentamer)	One Y (monomer)	One Y (monomer)
Formula	$\gamma_2\,\kappa_2$ or $\gamma_2\,\lambda_2$	$(\alpha_2\,\kappa_2)_n$ or $(\alpha_2\,\lambda_2)_n$	$(\mu_2\,\kappa_2)_5$ or $(\mu_2\,\lambda_2)_5$	$\delta_2\,\kappa_2$ or $\delta_2\,\lambda_2$	$\varepsilon_2\,\kappa_2$ or $\varepsilon_2\,\lambda_2$
Subtype	IgG_1, IgG_2, IgG_3, IgG_4	IgA_1, IgA_2	IgM_1, IgM_2 (?)	-	-
Mol. weight	154 kDa	160 kDa	970 kDa	185 kDa	190 kDa
Valency	Two	Two, four or six	Ten	Two	Two
Serum concentration	8-16 mg/mL	1-4 mg/mL	0.5-2 mg/mL	0-0.4 mg/mL	0-100 kU/mL (<0.001 mg/mL)
Total immunoglobulin	85%	5-15%	5-10%	<1%	<1%
Half-life	20-25 Days	6 Days	10 Days	2-8 Days	1-5 Days
Function	Resistance Opsonin Secondary response	Resistance Secretion Mucous membrane barrier	Resistance Precipitin Pimary response	Receptor for B cells Immune regulation	Anaphylaxis (type I) reaction Reaction against parasites
Complement	Yes	No	Yes	No	No

fixation

Binding to mast cells, basophilic granulocytes	No	Yes	No	No	Yes
Placental transfer	Yes	No	No	No	No
Mucosal transfer	Yes	Yes	Yes	No	No
Protein A and G binding *(Staph. aureus* and *Strept. group G)*	Yes	No	No	No	No

In hyperimmune sera which are used f.e. in immunohistology, specific antibodies are almost all IgG globulins. Antibodies consist of two pairs of identical heavy and light chains, and they can be divided into two parts based on functional differences: (a) the Fc-region with effector binding sites for complement activation and attachment points for receptor proteins; and (b) the Fab-regions which are variable regions consisting of a heavy chain-part and light chain-part that make up each "arm" of the IgG's *Y* structure; the Fab contains the Fv-regions which determine the specificity of the antibody.

Structure and shape of IgG molecules have been studied extensively (PORTER RR, 1959; HILSCHMANN N and CRAIG LC, 1965; PUTNAM FW et al., 1967; EDELMAN GM et al., 1969, EDELMAN GM and GALL WE, 1969; PUTNAM FW, 1969). Briefly, the IgG globulin has a molecular weight of about 154,000 daltons and consists of 4 polypeptide chains: 2 identical heavy chains (H chains) and 2 identical light chains (L chains). The two heavy-chain polypeptides in the *Y* structure are about 55,000 daltons, and the two light chains (which are also identical) are about 22,000 daltons. One light chain is associated with the amino-terminal region of one heavy chain to form an *antigen binding site*. The carboxy-terminal regions of the two heavy chains fold together to make the Fc domain. The four polypeptide chains are held together by disulfide bridges and noncovalent bonds.

All chains possess constant and variable regions. The N-terminal domain at the tip of the arms of the "*Y*" on both heavy and light chains are variable in amino acid sequence. These regions are called the variable domains, i.e. VL for the variable region of the light chain and VH for the variable region of the heavy chain. All other domains are the so-called constant domains: one constant domain on the light chain (CL), and three constant domains on the heavy chain (CH1, CH2, CH3).

Only the variable regions account for the specificity of the antibody reaction, the sites which bind specifically with the antigen. The varibale domains possess three regions of hypervariability in sequence (hypervariable loops). They are called the *complementarity determining regions* (CDRs). CDRs differ in length and sequence between different antibodies, they are mainly responsible for the specificity and affinity of the antibodies.

Proteolytic digestion of antibodies results in different fragments which are termed Fv (Fragment variable), Fab (Fragment antigen binding) and Fc (Fragment crystallization). For example, when an IgG globulin is treated with papain, it splits into three parts: one Fc fragment which is inactive in antigen binding and two Fab fragments, each containing one combining site (MW 40000). Upon pepsin treatment, the two Fab units are left united in a bivalent fragment while cleaving the Fc fragment. After reduction with mercaptoethanol, the Fab'$_2$ splits into two univalent fragments. For various work, whole antibody molecules as well as biochemical split products such as Fab and Fab'$_2$ fragments or "mini-antibodies" (VH-

region of H chain) may be suitable. Antibody engineering can join the separate segments of the heavy and light chains.

In addition to IgG, serum contains other classes of antibody molecules, i.e. IgA, IgM, IgD and IgE (see Table 1), which are distinguished by their type of heavy chains and which may vary in the number of *Y*-like structures that join to form the complete molecules; each *Y* unit has two antigen binding sites. Differences in heavy chain polypeptides allow those immunoglobulins to function in different types of immune responses including the particular stages of maturation. The protein sequences which are responsible for the differences are mainly localized in the Fc fragment. While there exist five different types of heavy chains for the five different immunoglobulin classes, there are only two light chains, i.e. kappa (κ) and lambda (λ). Any antibody molecule will have only one type of heavy chain and one type of light chain. However, there are no restrictions as to the types of heavy or light chains to the form antibody molecules. Thus, antibodies (with the different heavy chains) will contain either kappa or lambda light chains.

Numerous pitfalls can be encountered in the preparation of immune sera. During immunization with proteins, relatively minor contaminants (depending on their immunogenicity) in the immunizing mixture may produce significant amounts of antibody apart from those antibodies against the primary immunogen. Hence, careful analysis of immune sera by immunochemical methods is always necessary. Also, unwanted antibodies such as natural antibodies or antibodies which have been formed upon previous stimulation e.g. with infectious agents and which share common antigenic determinants with the studied material will give rise to unexpected reactions. Natural antibodies are also those with anti-A and anti-B reactivities in normal human sera.

Furthermore, cross-reacting antibodies can arise upon immunization. Cross-reactivity may be due to certain antibody heterogeneity as a result of immunization of an animal with a given antigen containing identical epitopes. This is the case when for example molecules used for immunization possess in addition to their "specific" determinants further determinants which are also shared by other molecules in other tissues. Furthermore, cross-reactivity must be expected with glycoepitopes of proteins. Typically, glycoproteins contain one or more oligosaccharide chains bound to the polypeptide structures. Since glycoepitopes possess structural homologies beyond the limits of protein classes, these structures are responsible for significant cross-reactivity and, thus, are called cross-reactive carbohydrate determinants (CCD).

Avian IgY antibodies

For the majority of research, diagnostic or clinical purposes, polyclonal and monoclonal antibodies are traditionally derived from mammalian sources. Yet, for many of those applications avian antibodies can be also used or are even more suitable than mammalian antibodies. Several publications have shown the great potential of IgY technology (NARAT M, 2003). Nevertheless, IgY technology is quite rarely used. Several advantages of avian antibodies can be denominated, f.e.

- Chicken housing is inexpensive as compared with housings for mammals.
- The yolk of eggs laid by immunized chickens is the source of antibodies.
- Collection of blood samples is not needed, egg collection is noninvasive.
- Eggs from immunized chicken provide a continual, daily source of antibodies.

- A single egg yolk contains as much antibody as an average bleed from a rabbit.

- The major antibody is called IgY, its overall structure is similar to mammalian IgG.

- Chicken IgY does not cross-react with mammalian IgG, and chicken IgY does not bind bacterial or mammalian Fc receptors.

- Due to the phylogenetic distance, conserved mammalian proteins are often more immunogenic in oviparous (egg laying) animals than in mammals.

The chicken immune systems has significantly contributed to a general understanding of the immune response including the separation of T- and B-cell lineages.The chicken immune system is made by the Bursa of Fabricius, bone marrow, spleen, thymus, Harderian gland, lymph nodes, lymphoid tissue in the gastrointestinal tract and circulating lymphocytes. The bone marrow is the source of bursal and thymic stem cells. The thymus is a center where stem cells differentiate into T-lymphocytes, while the antibody synthesizing B-cells are derived from the Bursa of Fabricius with the spleen as center for plasma cell proliferation and the generation of memory B-cells (TOIVANEN P et al., 1974). The mechanism of antibody diversity in chicken is somewhat different from that of mammalians and is mainly due to somatic hyperconversion (REYNAUD CA et al., 1985; WEILL JC and REYNAUD CA, 1987).

Three immunoglobulin classes have been shown in chicken: *IgY* (immunoglobulin of egg yolk), *IgA* and *IgM*; antibodies homologous to mammalian IgE and IgD have been also proposed but not yet proven. Molecular weights, structure and immunoelectrophoretic mobility of chicken IgA and IgM are similar to mammalian IgA and IgM. IgY is the major serum immunoglobulin in egg laying animals and can be found in other secretions such as tracheal washings, duodenal contents and seminal plasma. The highest concentrations of IgY are measured in the yolk of eggs. IgY is called to distinguish this immunoglobulin from its mammalian counterpart IgG (LESLIE GA and CLEM LW, 1969)

The structure of IgY is similar to that of mammalian IgG, with two light (L) and two heavy (H) chains. The molecular mass is about 167 kDa, and, thus, slightly larger than IgG (160 kDa). The H-chain is called upsilon (υ, capital letter Y) with one variable (V) region and four constant (C) regions. Domains Cυ3 and Cυ4 of the IgY are closely related to Cγ2 and C3 domains of IgG, respectively; Cυ2 domain is absent in the γ chain of the IgG molecule (SUN S et al., 2001; WARR GW et al., 1995). The Fc region of IgY mediates most of the biological effector functions such as complement fixation and opsonization. Furthermore, IgY is a skin-sensitizing antibody that can mediate anaphylactic reactions similar to IgE in mammalians (FAITH RE and CLEM LW, 1973). Hence, IgY combines in many ways the functions being associated with mammalian IgG.

IgY molecules are transported from the serum to the egg yolk in analogy to the cross-placental transfer of IgG antibodies in mammals. The egg white does not contain IgY. IgY concentration in the yolk is quite constant through oocyte maturation. At maturity, the yolk will contain about 10-20 mg IgY/ml, and about 100-400 mg IgY are packed in the egg.; IgA and IgM are not present in the yolk (ROSE ME et al., 1974; KOWALCZYK K et al., 1985). The amount of antigen specific antibodies within the pool of antibodies in an egg was reported to be up to 10% (THALLEY BS and CARROLL SB, 1990). The actual concentration of specific antibody synthesized will mainly depend on immunization procedures, immunogenicity of the antigen and the animal itself (inbred or outbred strains).

The production of chicken monoclonal antibodies was first reported in 1991, and, later on, recombinant chicken antibodies were produced by phage display technology and from antibody libraries (NISHINAKA S et al., 1991; DAVIES EL et al., 1995; YAMANAKA HI et al., 1996; NAKAMURA N et al., 2000).

It is known that with increasing difference between the antigen and the immunized animal its immune response will usually increase. There is a greater phylogenetic difference between avian and mammalian species compared with the difference between two mammalian species. This evolutionary spread means less immunological cross-reactivity between chicken IgY and mammalian IgG. Due to the evolutionary difference, chicken can be regarded as a better choice than f.e. rabbits for the production of antibodies against conserved proteins. It could be shown that chicken antibodies will bind more epitopes on a given mammalian antigen than the corresponding mammalian antibody, and chicken antibodies also recognize other epitopes than mammalian antibodies (HADGE D and AMBROSIUS H, 1984; HORTON JJ et al., 1985; LARSSON A et al., 1993; OLOVSSON M and LARSSON A, 1993).

Lack of cross-reactivity between IgY and IgG's from different mammalian species and lack of binding with mammalian Fc receptors is of practical interest in many techniques. For example, increased background binding may result in assays using secondary anti-mammalian IgG antibodies, or the IgG antibody may cross-react with IgG (of another mammalian species) present in a histological tissue section. Thus, the use of chicken IgY can be of great advantage in certain conditions including immunohistology.

Antigen and antibody interactions

Antibodies possess the ability to react *in vitro* and *in vivo* specifically with the antigen eliciting their synthesis. The region of an antigen that interacts with an antibody molecule is defined as an epitope. The epitope is an antigenic determinant which represents the smallest structural area on a complex antigen molecule that can combine with an antibody or a T-cell receptor. Multiple epitopes may be found on large antigenic molecules. X-ray crystallography has shown that the epitopes are relatively small and consist of exposed "hill and ridge" regions that manifest surface rigidity; the antigen bindings sites of an antibody molecule was visualized as a pocket into which the epitope docked. Because antibodies can recognize small regions of antigens, they can find similar epitopes on other molecules. This phenomenon is the molecular basis for cross-reactivity.

Binding of an antibody to an antigen is entirely due to noncovalent interactions. Major contributors to the intrinsic affinity between the antibody and its antigenic determinant are ionic interactions, hydrophobic bonds (surface tensions lower than that of water), van der Waals forces (weak electrostatic interactions between dipolar molecules or atoms) and electrostatic (coulombic) interactions between one or more ionized sides of the antigen determinant and oppositely charged ions on the antibody binding site. Also, hydrogen bonds (dipole interactions between OH and C=O, NH and C=O, and NH and OH groups) can be involved in antigen-antibody-binding. The formed immune complex is stabilized by the combination of interactions that depend on the precise alignment of the antigen and antibody; antigen-antibody complexes are in equilibrium with the free components. The association constant (K_a) of the binding between an antibody molecule and its antigenic determinant is a measure of the antibody's affinity which can range from 10^3 to 10^9 liters per mole (the reciprocal of concentrations in moles per liter). The higher the affinity of the antibody, the lower the concentration of antigen which is needed for available binding sites to become saturated. The term avidity describes the sum of all intrinsic affinities.

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 molecules 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.

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