The “IgG-like” subunit of all human antibodies consists of two identical LIGHT CHAINS and two identical HEAVY CHAINS, and proteolytic digestion can be used to establish the different functions of distinct portions of antibody molecules. The nine different CLASSES and subclasses (ISOTYPES) of human immunoglobulins exhibit different physical and biological properties, determined by the amino acid sequence of the CONSTANT REGION of the heavy chains. On the other hand, the VARIABLE REGION of heavy and light chains together form the ANTIGEN-BINDING SITES of antibodies.

The DOMAIN organization of antibodies, and an understanding of the COVALENT and NON-COVALENT interactions involved in their structure and in their interaction with antigen, form the basis for understanding their distinct roles in PRIMARY and SECONDARY IMMUNE RESPONSES, and the key features of IMMUNOLOGICAL MEMORY.

ANTIBODY HEAVY AND LIGHT CHAINS: PROTEOLYTIC FRAGMENTS

In order to examine the structure of antibodies, we will again start with a "typical" IgG molecule, such as the rabbit antibody we discussed at in the last chapter. A schematic diagram of an IgG molecule, together with its proteolytic fragments, is shown below:
Intact IgG has a molecular weight of ~150,000.

Each IgG molecule consists of two identical heavy chains (mol.wt.~50,000) and two identical light chains (mol.wt.~25,000). The two heavy chains are linked to each other by one or more disulfide bonds (shown as lines in the diagram), and each light chain is linked to one heavy chain by a single disulfide bond. Immunoglobulin heavy and light chains, as we will see later, are encoded by evolutionarily related members of a large multigene family.

This molecule can be broken down into smaller fragments by a wide variety of enzymatic and chemical treatments. Of particular importance has been the use of two proteolytic enzymes, papain, and pepsin. Treatment with papain yields two kinds of fragments, a pair of F(ab) fragments, and one Fc fragment. Treatment with pepsin yields a single fragment, the F(ab')$_2$ (the Fc region is degraded by this enzyme).

If we begin with an antibody molecule of known antigen specificity, we can study the fragments generated in this way not only with respect to their physical properties, but also for their ability to interact with antigen and complement. Their properties are as follows:

- **Fab**
  - size ~50,000 Da
  - binds antigen (ab = antigen-binding)
  - does not show precipitation or agglutination
  - will not fix complement or opsonize

- **F(ab')$_2$**
  - size ~100,000 Da
  - binds antigen
  - can show precipitation and agglutination
  - will not fix complement or opsonize

- **Fc**
  - size ~50,000 Da
  - cannot bind antigen
  - is crystallizable
  - does not fix complement (except very poorly)

- **[Fd]** that portion of the heavy chain which is included in the Fab fragment

Thus we see that proteolytic cleavage can separate IgG into fragments with different functional properties. The F(ab) and F(ab')$_2$ fragments are capable of binding antigen (they contain the antigen binding sites), but only the F(ab')$_2$ can exhibit agglutination or precipitation; this is because these functions require bivalent binding for cross-linking. Each F(ab) fragment has one antigen-combining site that requires the presence of both the heavy chain and the light chain. In addition, we see that without the Fc piece, complement fixation does not take place.

The Fc piece, on the other hand, does not bind antigen; and while it is required for complement fixation, it cannot significantly bind complement on its own. The fact that it can be made to form crystals was recognized very early as a striking property of the Fc (hence its name). Antibodies in general do not form crystals, as a result of their characteristic heterogeneity. The Fc fragment does not share the major source of this heterogeneity (variation in the antigen combining site) since it does not contain a binding site.
IMMUNOGLOBULIN CLASS (ISOTYPE) IS DETERMINED BY THE HEAVY CHAIN

So far we have discussed the properties only of a "typical" rabbit IgG molecule. If we look at human immunoglobulins, we see that a variety of different forms exist, and this variety is typical of mammals in general. Five major classes of immunoglobulin are known, IgG, IgM, IgA, IgD and IgE; in addition, there are four subclasses of IgG, namely IgG1, IgG2, IgG3 and IgG4, and two subclasses of IgA, namely IgA1 and IgA2, yielding a total of nine different classes and subclasses (="isotypes"; see Chapter 6) of human immunoglobulin.

**PROPERTIES OF HUMAN IMMUNOGLOBULINS**

<table>
<thead>
<tr>
<th>Class</th>
<th>subclasses</th>
<th>serum conc. mg/ml</th>
<th>sedim. coeff.</th>
<th>size</th>
<th># binding sites</th>
<th>H-chain class</th>
<th>Biological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>4</td>
<td>12</td>
<td>7S</td>
<td>150 KDa</td>
<td>2</td>
<td>γ (γ1, γ2, γ3, γ4)</td>
<td>C-fixing, placental X-fer</td>
</tr>
<tr>
<td>IgM</td>
<td>-</td>
<td>1</td>
<td>19S</td>
<td>900 KDa</td>
<td>10</td>
<td>μ</td>
<td>C-fixing, B-cell surface Ig</td>
</tr>
<tr>
<td>IgA</td>
<td>2</td>
<td>2</td>
<td>7,9,11S</td>
<td>(160 KDa)n</td>
<td>2,4,6</td>
<td>α (α1, α2)</td>
<td>secretory Ig</td>
</tr>
<tr>
<td>IgD</td>
<td>-</td>
<td>0.03</td>
<td>7S</td>
<td>180 KDa</td>
<td>2</td>
<td>δ</td>
<td>B-cell surface Ig</td>
</tr>
<tr>
<td>IgE</td>
<td>-</td>
<td>0.0003</td>
<td>8S</td>
<td>200 KDa</td>
<td>2</td>
<td>ε</td>
<td>&quot;reaginic&quot; Ig</td>
</tr>
</tbody>
</table>

The **class** (and subclass) of an immunoglobulin is determined by the kind of heavy chain it bears. Nine different heavy chains define the nine classes and subclasses (isotypes) of human Ig.

All immunoglobulins share the same pool of light chains; there are two types of light chains, kappa and lambda. Thus, an IgG1 molecule has two gamma-1 heavy chains, and may have. Likewise for an IgM molecule which has mu heavy chains, and may have either kappa or lambda light chains, but not both...etc.

All immunoglobulins share the same basic "IgG-like" structure consisting of two linked heavy chains and two light chains. IgG, IgD and IgE have precisely this structure, and differences in their molecular masses (seen in the table above) are due to differences in the size of the heavy chain polypeptides and their carbohydrate content.

We can represent this basic structure by the general empirical formula, H2L2. Thus, a particular IgG1 molecule may be represented as either γ12 κ2, or γ12 λ2, depending on whether it contains kappa or lambda chains.

IgM and some IgA consist of multiple "IgG-like subunits". IgM consists of a pentamer of five such subunits, each of which consists of two mu chains and two light chains (either kappa or lambda); it therefore contains ten antigen combining sites, and is the largest of the immunoglobulins. IgA can exist either as a monomer or dimer (rarely a trimer) of
the basic H₂L₂ subunit, and therefore can bear two, four or six combining sites. IgM and polymeric IgA have an additional polypeptide associated with them, the J-chain (for "joining"). The J-chain is thought to participate in the process of polymerization of these molecules. (See also Chapter 9, Ig BIOSYNTHESIS)

DIFFERENT Ig ISOTYPES HAVE DIFFERENT BIOLOGICAL FUNCTIONS

The structures of the different immunoglobulin heavy chains are quite different from one another, in amino acid sequence, in chain length and in carbohydrate content. These differences result in marked differences in biological properties of the different isotypes (classes and subclasses) of Ig.

IgM  Most efficient Ig at complement fixation.
      Produced early in immune responses.
      Serum IgM bears an additional polypeptide, the J-piece (for “joining”), as does polymeric IgA.
      Serve as membrane receptors of B-cells (as “IgMs”, an “IgG-like” monomer.

IgG  Most abundant serum Ig.
      Various subclasses can all fix complement (via classical or alternate pathway).
      Can be transferred across the placenta into the fetal circulation.
      Produced late in immune responses.
      Opsonizing activity via Fc receptors present on macrophages and PMNs.

IgA  While IgA is the second-most abundant serum Ig, it is most characteristically a secretory immunoglobulin, and is the most abundant Ig in exocrine secretions (saliva, bile, mucus of the gut and respiratory tract, milk, etc.).
      IgA in serum is mostly an α₂L₂ monomer; IgA in secretions is polymeric (mostly dimer, i.e. [α₂L₂]₂, with some trimer), includes an S-piece (for “secretory”), and like IgM also bears the J-piece.

IgD  Antigen-binding receptor on B-cells (together with IgMs).
      Rare in serum; no other known biological function.

IgE  Extremely low levels in serum.
      Reaginic" antibody, responsible for allergic reactions following binding to surface of tissue mast cells (see Chapter 21).
      While antibodies are generally very stable, IgE is an exception and is characteristically heat labile.

HOMOGENEOUS IMMUNOGLOBULINS: MYELOMA PROTEINS

We have already noted that heterogeneity is one of the most striking features of antibodies; this is understandable in that the primary function of antibodies is to bind to a wide variety of epitopes. This heterogeneity, however, makes it difficult to carry out conventional structural studies on these molecules, particularly by amino acid sequencing or crystallization, which both require a homogeneous protein.

Our understanding of immunoglobulin structure resulted largely from the existence of MYELOMA PROTEINS, homogeneous forms of immunoglobulins produced by tumors of antibody-forming plasma cells. Since the tumors are monoclonal in origin, so are their protein products; in any particular patient, the monoclonal protein (also referred to as "M-component") is of a single heavy chain class and bears a single kind of combining site. One
reflection of their homogeneity is the fact that myeloma proteins are often crystallizable, unlike conventional Ig. In some cases the homogeneous protein consists of free light chain without an associated heavy chain; these free light chains are rapidly cleared by the kidneys and appear in the urine as BENCE-JONES PROTEINS. In other cases a tumor might produce only heavy chains which accumulate in serum, with no associated light chain.

A variety of pathological conditions can result in the presence of monoclonal Ig in serum, collectively referred to as Monoclonal Gammopathies. While some of these conditions are benign, many represent malignant tumors (cancers) of antibody-forming cells. Depending on their clinical presentation, these malignancies may be termed Multiple Myeloma, Plasmacytoma, Immunocytoma, Waldenstrom's Macroglobulinemia, Heavy Chain Disease or Light Chain Disease. We will use the general term “myeloma protein” to refer to any such monoclonal immunoglobulin.

IMMUNOGLOBULIN HEAVY AND LIGHT CHAINS HAVE VARIABLE AND CONSTANT REGIONS

Amino acid sequencing studies of Bence-Jones Proteins first showed that light chains consist of a variable region and a constant region. If several kappa-type B-J proteins (each from a different patient), are subjected to amino acid sequencing, one finds that the amino terminal half of the polypeptide (about 110 amino acids) shows substantial variation from one protein to another, while the carboxy-terminal half is essentially constant (except for allelic variants discussed in Chapter 6). Similarly, the heavy chains of different myeloma proteins have an amino-terminal variable region (also about 110 amino acids long), while the remainder of the polypeptide remains constant within a given isotype.

The antigen combining site of an antibody is made up of the variable regions of one light chain and one heavy chain. As can be seen in the diagram above, the two variable regions (\(V_H\) and \(V_L\)) are located in the F(ab) regions, precisely where we would expect them to be if they are responsible for antigen binding.

Within the variable regions, typically comprising 105-110 amino acids, some positions show more sequence variation than others. The highest degree of variability between different monoclonal proteins exists in three small sub-regions within the entire V-region, which were named the Hypervariable Regions; the four segments outside these hypervariable regions are termed "framework" regions, and show considerably less diversity (although they are still “variable”). The hypervariable regions form the antibody combining site in the native three-
The dimensional structure of the antibody, and are now commonly known by the more descriptive name of COMPLEMENTARITY-DETERMINING REGIONS (CDRs).

THREE FAMILIES OF V-REGIONS EXIST, one for kappa light chains, one for lambda light chains, and the third for heavy chains. In spite of their variable nature, one can readily determine from its amino acid sequence (even from the first few N-terminal residues) to which of these three families a given V-region belongs, kappa, lambda or heavy chain. However, in the case of VH regions, one cannot predict which of the heavy chain isotypes it will be associated with; all heavy chains, regardless of class or subclass, draw from a single pool of VH-regions. We will examine the cellular and genetic basis for this phenomenon in later chapters.

IMMUNOGLOBULIN HEAVY AND LIGHT CHAINS CONSIST OF GLOBULAR "DOMAINS"

Up to now we have diagrammed Ig molecules as consisting simply of linear polypeptides connected to one another by disulfide bonds. However, the polypeptides making up Ig molecules (and proteins in general) are not normally stretched out like pieces of spaghetti, but are folded into complex three-dimensional structures. In the case of a light chain, the V-region and C-region are each folded into separate, compact globular DOMAINS, the two of which are connected to each other by a short stretch of extended polypeptide chain. The heavy chain likewise has a single V-region domain, but has several C-region domains, a total of three in the case of IgG. The diagram in Figure 4-3 incorporates this more sophisticated view of immunoglobulin:

![Figure 4-3](image_url)

Every heavy or light chain is made up of one V-region domain and one or more C-region domains. In the case of light chains these are named VL and CL (Vκ and Cκ for kappa chains, etc.). In the case of heavy chains, these are termed VH for the variable domain, and CHI, CHII etc. for the constant domains; for a particular heavy chain, mμ for instance, the constant domains become CμI, CμII, etc.
Light chains have a single constant domain, heavy chains have three (IgG, IgD, IgA) or four (IgM, IgE) constant domains.

Each domain consists of a compact globular unit, and they are linked to one another by short stretches of extended polypeptide chain.

Different domains share varying degrees of amino acid sequence similarity and a high degree of similarity in their three dimensional structures. These patterns of similarity indicates that the different domains, and the three different immunoglobulin gene families (encoding the heavy, kappa and lambda chains), are all the product of a series of gene duplications of a primordial gene resembling a single domain.

Heavy chains have a stretch of extended polypeptide chain which is not part of any domain, between the C_HI and C_HII domains. This segment is known as the HINGE REGION, and is the region containing the cysteine residues involved in the H-H and H-L disulfide linkages.

COVALENT AND NON-COVALENT FORCES STABILIZE ANTIBODY STRUCTURE

Many kinds of molecular interactions contribute to the extraordinary stability of antibody structure. We have already seen that the chain structure is held together by a series of covalent disulfide linkages between the two heavy chains, and between each light chain and one heavy chain.

In addition, powerful non-covalent interactions exist between various adjacent pairs of domains, specifically between V_L and V_H, between C_L and C_HI, and between the two C_HIII domains; these are indicated by heavy lines between these regions in Figure 4-3. Thus, even if all the disulfide bonds holding the chains together are broken, the overall structure of the Ig molecule will generally be maintained.

Each domain forms an extremely stable globular structure, held together by powerful non-covalent forces as well as a single internal disulfide bond. The compactness of these structures confers a degree of resistance to proteolytic cleavage; it is for this reason that much proteolytic cleavage of immunoglobulins (as for instance with papain) takes place in the extended chains between domains.

ANTIBODIES BIND ANTIGENS BY POWERFUL NON-COVALENT FORCES

The basis of antibody-antigen binding is steric complementarity between the combining site and the epitope or antigenic determinant ("lock and key" concept). The combining site itself is made up by both V_L and V_H, most directly involving the hypervariable regions or CDRs ("complementarity determining regions") of both.

A variety of non-covalent forces are involved in this binding:

1) **Van der Waals interactions.** These are very weak and extremely short-range forces (they decrease with the seventh power of the distance), but they become important when many such interactions over the large area of an interactive surface are added together.

2) **Hydrophobic interactions.** Exclusion of water molecules between hydrophobic surfaces can also be a major contributor to antigen-antibody binding.
3) **Hydrogen bonds** and **ionic interactions** can also provide considerable stabilization of antibody-antigen binding, in those cases where the epitope is capable of participating in such interactions.

![Diagram](image)

**Figure 4-4**

The single most critical factor in the strength of binding between antibodies and their antigens is the *degree of complementarity* ("goodness of fit") between the antibody combining site and the epitope (Fig. 4-4, left). A very slight change in the shape of either one can turn extremely strong binding into *no binding at all* (Fig. 4-4, center). Alternatively, a different kind of shape variation might turn strong binding into weaker binding, without eliminating it altogether; this is part of the basis of **cross-reactivity** between different but related antigens (Fig. 4-4, right).

**IMMUNOLOGICAL MEMORY: PRIMARY VERSUS SECONDARY RESPONSES**

One of the hallmarks of immune responses is their ability to display **memory**; the immune system in some way "remembers" what antigens it has seen previously, and responds **more effectively** the second time around. This is the basis, for instance, of acquired resistance to smallpox - having once recovered from the disease (or having been vaccinated), a person’s immune system responds more rapidly the next time it is exposed to the virus, and eliminates it before the disease process can be initiated.

Having discussed the variety of known Ig **isotypes** and the concept of **affinity**, we can now identify several of the key features of immunological memory, illustrated in Figure 4-5 below, which shows the results of immunizing an animal with a "typical" antigen. The graph on the left represents the results of a *primary* immunization; plotted on the X-axis is the time after immunization in weeks, while the Y-axis represents a measure of the amount of antibody in the serum (which could be determined by any of the techniques we have described, agglutination, precipitation, ELISA, etc.). In the graph on the right we see the results following re-immunizing the same animal with the *same antigen* at some later date, termed a *secondary* immunization. The differences between the two graphs represent the features which define **immunological memory**.
Outlined in the table below are the most important features which distinguish primary and secondary humoral immune responses.

<table>
<thead>
<tr>
<th>PRIMARY RESPONSE</th>
<th>SECONDARY RESPONSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>• SLOW</td>
<td>• FAST (kinetics of clonal selection)</td>
</tr>
<tr>
<td>• LOW Ab LEVELS</td>
<td>• HIGH Ab LEVELS</td>
</tr>
<tr>
<td>• SHORT-LIVED</td>
<td>• LONG-LIVED</td>
</tr>
<tr>
<td>• MAINLY IgM</td>
<td>• MAINLY IgG (Ig structure)</td>
</tr>
<tr>
<td>• LOW AFFINITY</td>
<td>• HIGH AFFINITY</td>
</tr>
</tbody>
</table>

The first three features are a consequence of the cellular events in immune responses, while the last two relate to the physical properties of the antibodies produced.

1) **Speed.** In a “typical” primary response we see a lag of about 4 days, followed by a slow rise of antibody to a peak at about two weeks. In the secondary, the lag is only two days, and antibody levels rise very rapidly to a peak within 4-6 days.

2) **Antibody levels.** The secondary response reaches peak antibody levels which are very much higher than in the primary.

3) **Duration.** Primary responses not only appear more slowly, they disappear fairly rapidly. Antibody titers in secondary responses remain at high levels for longer periods of time, and in humans may persist for many years.

4) **Ig class.** The majority of the antibody in a primary response is IgM, with some IgG appearing later in the response; in a secondary, IgG is the predominant antibody throughout the response. IgM also appears during a secondary response, but at levels
and with a time course comparable to the primary and is therefore swamped out by the higher levels of IgG. **IgM does not exhibit immunological memory:** it does not appear more rapidly or at higher levels in secondary responses, and does not undergo significant affinity maturation.

5) **Affinity.** The average affinity of antibody in a secondary response is higher than in a primary response. Even during the course of a particular secondary response, it can be shown that the affinity of "late" antibody is higher than that of "early" antibody. This progressive increase in the average affinity of antibody is known as AFFINITY MATURATION.

**The net result of immunological memory is an immune response of greatly increased effectiveness.** The antibody is made more rapidly, it is made in higher amounts, it lasts longer, and it is made with higher affinity so that it binds more effectively to its target. The mechanisms involved in all of these phenomena will become clearer when we have discussed the CLONAL SELECTION THEORY (see Chapter 7).

NOTE: It is important to keep in mind that immune responses vary greatly in their time course, intensity and persistence. While the figure above illustrates the antibody responses to a "typical" antigen in a "typical" organism, any particular response may be very different in many of its features. One antigen may result in a much slower or more rapid response than another, or may be unable to produce a secondary response at all. Nevertheless, the fundamental differences between primary and secondary responses are important and instructive generalizations.

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**CHAPTER 4, STUDY QUESTIONS:**

1. Describe the structure and biological properties of the pepsin- and papain-generated PROTEOLYTIC FRAGMENTS of antibodies.

2. What are the distinguishing **structural** and **biological** features of each of the five CLASSES of human Ig?

3. What are the common structural features of all antibodies?

4. Why did the discovery of VARIABLE and CONSTANT regions of immunoglobulin heavy and light chains pose such a serious puzzle for molecular biologists?

5. How do the globular DOMAINS of immunoglobulins contribute to the remarkable stability of antibodies?

6. What features of SECONDARY antibody responses make them more effective than PRIMARY responses?