1. PRECIPITIN CURVE

    Let’s examine a typical precipitin curve, in which we take a fixed amount of antigen (say BSA), and add to it increasing amounts of antibody. We then measure the quantity of the resultant precipitate.

![Precipitin Curve Diagram]

We find that the precipitate increases as we add more antibody (as expected), but that after a certain point the total precipitate decreases again. This is due to the formation of soluble complexes of two different kinds, one in the region of large antigen excess, the other in region of large antibody excess; these are illustrated in the diagrams above the precipitin curve. The antibody-antigen ratio near the peak of the curve is known as the region of equivalence, which results in the maximum formation of large, insoluble complexes. Since the binding of antibody with antigen is a reversible process, changes in the concentration of either component can drive the reaction in one direction or the other.

Figure A1-1

    We find that the precipitate increases as we add more antibody (as expected), but that after a certain point the total precipitate decreases again. This is due to the formation of soluble complexes of two different kinds, one in the region of large antigen excess, the other in region of large antibody excess; these are illustrated in the diagrams above the precipitin curve. The antibody-antigen ratio near the peak of the curve is known as the region of equivalence, which results in the maximum formation of large, insoluble complexes. Since the binding of antibody with antigen is a reversible process, changes in the concentration of either component can drive the reaction in one direction or the other.
2. LABELING OF ANTIBODIES (AND OTHER PROTEINS)

The ability to label proteins with radioactive markers led to the development in the 1950s of the RIA (RADIOIMMUNOASSAY), which provided an extremely sensitive approach to detecting and quantitating antibodies and antigens. Other methods for labeling proteins have also been widely used, including fluorescent dyes and enzymes.

**Radiolabeling - RIA.** While radioactive isotopes of iodine ($^{125}$I and $^{131}$I), are not the only radioactive markers used for proteins ($^{3}$H tritium and $^{35}$S sulfur have also served, among others), they have been the most widely used. A variety of different reaction schemes can be used to attach either free iodide or small iodine-containing molecules to antibodies, resulting in a highly radioactive but still biologically active antibody molecule. These radioactive antibodies can be detected in extremely small quantities, either in precipitates or bound to a solid state antigen. For many years RadioImmunoAssays (RIA, see APPENDIX 5) have served as sensitive and widely used assays in both clinical and research settings. Beginning in the 1970’s, however, RIA's have progressively been replaced by equally sensitive and less expensive Enzyme-Linked Immunosorbent Assays (ELISA, see below, and APPENDIX 5).

**Fluorescent labeling - IF.** Fluorescent dyes can be coupled to antibodies, and the binding of such labeled antibodies to bacteria (or other particulate antigens), or to antigens in histological sections, can be readily detected by fluorescence microscopy, a technique known as IMMUNOFLUORESCENCE (IF). The two classical dyes used have been Fluorescein (which fluoresces green) and Rhodamine (red), although a large number of newer dyes such as Texas Red and others have since been added to this list.

One major feature of IF is its ability to determine the localization of antibodies and antigens within histological sections; such studies were the first, for example, to demonstrate that plasma cells were the major site of antibody production. More recently, IF has been a major contributor to the power of Fluorescence-Activated Cell Sorters (FACS), machines which can analyze and separate complex populations of cells, one cell at a time. The basis of such separation is often the labeling of one or another cell type in a complex mixture with a fluorescent antibody specific for that cell type, but cells can also be sorted on the basis of size, DNA content and a variety of other physical and biological parameters.

**Electron-dense labels.** A variety of electron-dense markers can be attached to antibodies, including Ferritin (which has an extremely high content of iron atoms) and colloidal gold particles. The resulting antibodies can be visualized in the electron microscope just as fluorescent antibodies can localized by light microscopy, but, of course, with much higher resolution.

**Enzyme labeling - ELISA.** A variety of enzyme molecules can be coupled to antibodies, which can then be sensitively detected by their associated enzymatic activity. The resulting ELISAs (ENZYME-LINKED IMMUNOSORBENT ASSAY) are very widely used, and have replaced RIA's in most modern applications. Commonly used enzymes have included horseradish peroxidase, alkaline phosphatase, , β-galactosidase, and, more recently, luciferase.

ELISA's can equal or exceed the sensitivity of RIA's, and have the major advantage of not requiring the use of radioactivity with its associated costs and hazards. The detection method often involves the addition of a colorless substrate which the enzyme converts into a colored (or fluorescent) product, and the results can be tabulated photometrically in fractions.
of seconds per sample as opposed to minutes per sample for typical RIAs. Another detection method which has become increasingly widely applied involves the use of enzymes which cause their substrates to emit photons of visible light (“luminescence”). Such luciferase/luciferin based assays can be considerably more sensitive than the more traditional colorimetric assays.

**Enzyme-based histochemistry.** Enzyme-labeled antibodies can also be used for microscopy in the place of fluorescent-labeled ones (see IF, above). In such cases a substrate is used which yields a visible and insoluble product which is deposited at the site of enzyme (and therefore antibody) localization. The fact that a single enzyme molecule can yield many molecules of product makes such immunohistochemistry potentially more sensitive than immunofluorescence, and the resulting microscopic preparation is more permanent and easier to photograph than fluorescent material.
3. OUCHTERLONY ANALYSIS

The Ouchterlony assay was widely used in myriad research and clinical contexts for many years after its development in 1948, although it has been largely superceded by other assays which are more sensitive and suitable for quantitation (see APPENDIX 5). Nevertheless, this technique provides a very useful tool for illustrating and clarifying the principles of antibody heterogeneity and specificity, which is why we cover it in some detail here.

In the Ouchterlony assay, the antigen and antibody solutions are placed in nearby wells cut out of a thin layer of agarose, and allowed to stand for a few hours or a day or two. During that time they diffuse toward each other, and where they meet they will form a visible line of precipitation.

The pattern in which adjacent lines cross one another yields considerable information about the antigenic relationships between different antigens. Let’s illustrate this with a pattern generated by a rabbit antiserum made against whole human serum, using three purified protein antigens as targets, namely HSA (human serum albumin), BSA (bovine serum albumin) and HTf (human transferrin). The well labelled “Ab” contains the antiserum, and the resulting pattern is shown below.

Several conclusions can be drawn from the pattern of precipitation shown here:

1) The antiserum (in the well labelled “Ab”) contains antibodies against all three antigens, since each one shows a precipitin line.

2) The lines produced by the two adjacent wells containing HTf join completely, in a pattern of complete identity, or simply "identity." This indicates that the antigens in the two wells (which in this case we know are exactly the same) are antigenically indistinguishable by this antiserum. We can’t tell from the pattern whether the antiserum is detecting just one epitope or twenty, but we do know it is detecting all of them in both wells.

3) The two antigens HSA and HTf show a pattern of non-identity - the precipitin lines cross each other without joining at all. This indicates the two antigens are antigenically unrelated, they have no epitopes in common which are
recognized by this antiserum. This is not unexpected, since these two molecules are not related in structure or function.

4) The two antigens HSA and BSA show a pattern of **partial identity** - the lines join together, but not completely; there is a "spur" of the HSA line over the BSA line. This indicates that the two antigens are related, but not identical, with respect to this antiserum. More specifically, it means that there are at least **two epitopes** recognized by the antibody on HSA, one of which is also present on BSA. This is illustrated in the two cartoons on the right, showing HSA as bearing two epitopes ("X" and "Y"), while BSA bears only one of the two ("X").

We can be more precise by stating that there are two **classes** of epitopes that the antiserum detects on HSA, and only one of the two classes is present on BSA. That is, what is shown as epitope “X” in the cartoons might actually be, say, eight separate epitopes which are all present on both HSA and BSA, while what is indicated as “Y” might be three epitopes present only on HSA.
4. ABSORPTION AND AFFINITY PURIFICATION

Affinity Purification of anti-DNP Antibody

Antigens and antibodies can be readily analyzed and purified by **affinity chromatography**, a technique widely used in chemistry. When applied to antigen-antibody systems, this procedure is often referred to as **immunoabsorption**.

Suppose we immunize a rabbit with DNP-OVA (where the protein ovalbumin is the carrier for the hapten DNP), and collect the resulting antiserum, which contains antibodies both to DNP and to the OVA protein carrier. We can separate the two kinds of antibody by preparing an immunoabsorption column as illustrated in Figure A4-1. We start by covalently coupling DNP alone, or DNP-BSA (DNP coupled to an unrelated carrier) to an insoluble matrix such as cellulose, and place it in a column. We pass our rabbit antiserum over this column; and the antibodies to DNP will bind to the DNP while everything else (including the anti-OVA antibodies) flows through. We refer to this “pass-through” fraction as an “absorbed antiserum”; the anti-DNP antibodies have been removed (“absorbed”) leaving behind only anti-OVA antibodies, together with all the normal serum proteins, of course.

![Affinity Purification of anti-DNP Antibody](Figure A4-1)
We can then recover the anti-DNP antibodies by releasing them from the column, using either a solution of free DNP (which will compete with the bound DNP for the antibodies' combining sites), or a denaturing agent such as urea or guanidinium salts. After removing the eluting agent by dialysis, this “affinity purified” fraction contains pure anti-DNP antibodies. If, on the other hand, we use a column containing insolubilized OVA, we can recover a pure preparation of anti-OVA antibodies in the same manner.

Absorption and Affinity Purification of αHSA antiserum

This general procedure can be used to modify the specificity of antisera in more subtle ways. An antiserum prepared in a rabbit against human serum albumin (HSA), for example, will cross-react with bovine serum albumin (BSA), since the two proteins are closely related. Since distinct populations of antibodies bind to different epitopes, and binding of antibody to its antigen is reversible, we can use affinity chromatography to separate and purify the antibody populations as illustrated in Figure A4-2.

We begin with an antiserum generated by immunizing a rabbit with purified human serum albumin (HSA). When we analyze this antiserum “A” by Ouchterlony (bottom left) we see that there are two distinct populations of antibody present, one which binds to both HSA and BSA (bovine serum albumin), and another which binds only to HSA.

We then pass this antiserum over a column containing covalently bound BSA. All of those antibodies capable of binding to BSA will be retained on the column, and everything else will pass through, yielding preparation “B”, which we refer to as an “absorbed antiserum” (more specifically, “αHSA[absBSA]”). Ouchterlony analysis of this fraction (bottom center) shows the presence of a single population of antibodies which bind only HSA (since all those antibodies capable of binding BSA have been retained in the column).

We can now recover the antibodies bound to the column by washing it with 3M guanidium. We can remove the guanidium by dialysis and test this “affinity purified antibody” fraction by Ouchterlony (bottom right). The resulting pattern tells us there is a single population of antibodies which bind to both HSA and BSA.

All three of these preparations can be useful in research and clinical settings, and it will be instructive to distinguish their properties.

- Whole antiserum

Whole antiserum can be used without further treatment in many assays, such as precipitation (as we have seen), agglutination, etc., so long as cross-reactivity between related molecules is not important. For example, a researcher might want to detect the presence of HSA in a set of samples which do not contain BSA or other cross-reactive proteins; in such a case absorption and affinity purification are unnecessary. While only a small percentage of the total protein in whole serum is specific antibody, the presence of other proteins does not interfere in most assays.
ABSORPTION AND AFFINITY PURIFICATION OF $\alpha$HSA ANTISERUM

Original antiserum

"A"

Pass-through: Absorbed antiserum

"B"

Eluate: Affinity-purified Ab

"C"

Partially cross-reactive

Non-cross-reactive, "Monospecific" (but not monoclonal)

Completely cross-reactive

3M guanidinium

ABSORPTION AND AFFINITY PURIFICATION OF $\alpha$HSA ANTISERUM

Figure A4-2

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● **Absorbed antiserum**

If a forensic immunologist needs a reagent to determine if a blood stain is human or animal, then absorption of the original antiserum becomes necessary. Fraction “B” in our example will do the job in many assays (Ouchterlony, for example), showing a positive reaction only with human albumin.

● **Affinity-purified antibody**

There are a number of applications, however, for which affinity purification of antibody is either required or highly desirable. One example would be in developing an RIA or ELISA (see APPENDIX 5) to measure mammalian serum albumin. In this case we need to be able to label *only the antibody molecules* (with radioactivity or enzyme), and it is impossible to do this in the presence of the large excess of irrelevant proteins in whole or absorbed antiserum. Our *affinity-purified* fraction “C”, however, contains only specific antibody molecules. This fraction could be labelled and would provide a reagent to detect mammalian serum albumin by RIA or ELISA, reacting equally well with human and bovine albumin (and presumably other related species). [QUESTION: How would you go about preparing an affinity-purified antibody for use in an RIA specific for human serum albumin?]

Another example would be for the therapeutic use of antibodies which are to be injected into a patient. The vast majority of irrelevant protein in either whole serum or absorbed antiserum would expose the recipient to a far greater risk of SERUM SICKNESS than affinity-purified antibody (see Chapter 5, COMPLEMENT)
5. RADIOIMMUNOASSAY - RIA

Principles of the assay

The development of the radioimmunoassay in the 1950’s revolutionized the field of immunochemistry in both research and clinical applications. The RIA relies on the one property of antibody function which is a universal feature of all antibodies, namely the ability to bind specifically to an antigen. The degree of binding can be very accurately quantitated by making one of the components radioactive, in this example the antibody.

Our sample assay is designed to determine the concentration of insulin in some unknown samples (blood serum, urine, cell culture supernatants, etc.). We begin by coating a glass or plastic tube with a set quantity of insulin in such a way as to be permanently fixed in place. As illustrated in the top row of Figure A5-1, we then add a fixed amount of anti-insulin antibody which we have tagged with the radioactive isotope $^{125}$Iodine. Most of the antibody will bind to the insulin stuck to the tube, and we then rinse the tube with saline to wash away all unbound material. We can now measure the amount of radioactivity in the tube, representing the amount of bound antibody; we determine that there are 5,000 counts per minute of radioactivity in the tube, which represents “maximum binding”.

We now carry out the same exercise (shown in the second row of figures), but this time we add a large amount of soluble insulin (“inhibitor”) together with the radioactive antibody. In this case, the antibody can bind either to the fixed or soluble insulin, and since there is more soluble than fixed insulin, most of the antibody will bind to the soluble form. This time when we rinse the tube with saline, we wash away most of the radioactive antibody together with the soluble insulin. The small amount of radioactivity which still remains behind, namely 400 counts per minute, represents some antibody (or contaminating radioactive material) sticking nonspecifically to the assay tube, or “background binding”.

This defines the principles of the RIA – specific binding of antibody to antigen can be sensitively measured, and this binding can be inhibited by the addition of soluble antigen. A small amount of soluble antigen will inhibit binding only slightly, a large amount will inhibit more, and we can use this relationship to determine the concentration of inhibitor in any unknown sample.

Inhibition curves

If we have a series of experimental tubes to which we add increasing amounts of inhibitor, we can generate inhibition curves such as those shown at the bottom of Figure A5-1. One common practice is to make serial dilutions of the inhibitor from an original stock (say 1:2, 1:4, 1:8, etc.) and add a fixed volume of each dilution to each assay tube. There are a variety of methods for plotting such data, and the one we will use is shown at the right, plotting increasing inhibition (from 0 to 100%) on the Y-axis, versus increasing dilution of inhibitor on the X-axis. Using our standard volume of a low dilution (i.e. a large amount) of inhibitor, we see 100% inhibition. As we increase the dilution (i.e. decrease the amount) of the inhibitor, we see less and less inhibition, until the curve finally flattens out at 0% inhibition. In this example we see 50% inhibition at a dilution of about 1:40, and we refer to this curve as exhibiting a “titer” of 1:40.
**RadiolmmunoAssay (RIA)**

**ASSAY:** Fixed quantity of *bound* Ag (insulin= ○)
- Fixed quantity of $^{125}$I-Ab (= *)
- Variable quantity of *soluble* inhibitor (insulin= ○)

*Maximum total binding* = 5000 cpm.
*Maximum inhibition* yields 400 cpm bound (=100% inhibition).
*Maximum specific binding* = 4600 cpm (=0% inhibition).

**VARIOUS WAYS TO PLOT RESULTS...**

*Note: Display and interpretation of data are identical for RIA and the more commonly used ELISA*

**Figure A5-1**
How do we use this assay to determine the concentration of insulin in an unknown sample? Consider the inhibition curves shown below in Figure A5-2, where our standard insulin solution shows a titer of 1:40, while the unknown sample has a titer of 1:160. We conclude that the unknown sample (in its original, undiluted form) contains insulin at a concentration four times greater than our standard. If our standard insulin stock has a concentration of 20 pmol/L (picomoles per liter), which we can determine by looking at the manufacturer’s label, then the unknown sample must be at 80 pmol/L.

![Figure A5-2](image)

**ENZYME-LINKED IMMUNOADSORBENT ASSAY (ELISA) versus RIA**

As mentioned in APPENDIX 2, RIA’s have been superseded in many of their applications by Enzyme-Linked Immunosorbent Assays (ELISA), which can be as sensitive than RIA’s while avoiding the hazards and expense associated with the use of radioactive materials. We have introduced this class of binding assays using the RIA as the primary example because it is conceptually simpler, but the principles of the ELISA are the same as those described above, and the data are plotted and interpreted in precisely the same manner as RIA’s. In fact, there is no way to know simply by examining an inhibition curve whether it was generated in an ELISA or RIA.
6. ANALYSIS OF THE ANTIBODY COMBINING SITE BY EQUILIBRIUM DIALYSIS

Equilibrium dialysis was the first technique used to determine the thermodynamic properties of antigen-antibody binding. It revealed that this reaction was a **non-covalent and reversible reaction**, that the basic IgG-like unit had exactly **two combining sites**, and it determined the values of the equilibrium constant (**affinity**) of the binding reaction.

**Requirements for the Analysis**

1) We must have a *pure preparation of antibody*, not just a gamma-globulin preparation or an ammonium sulfate fraction; the reason for this is that we must know the **molar concentration** of antibody. We must, therefore, also know the molecular weight of IgG antibody, which is about 150,000.

2) We must carry out the analysis using a *monovalent, low molecular weight antigen* such as a hapten. The reasons for this are twofold: first, the hapten must be **small** enough to pass across a dialysis membrane (the antibody, of course, is too large); and second, it must be **monovalent** to fulfill the assumptions we will make regarding independent binding to multiple antibody combining sites on the antibody.

**Dialysis Apparatus**

The apparatus consists of two small chambers connected only through a semipermeable membrane, as diagrammed below. In one chamber we place a known amount of total antibody (Ab$_T$), in the other a known amount of total hapten (H$_T$); the starting condition is shown on the left of the figure below.

![Dialysis Apparatus Diagram](image-url)

**Figure A6-1**

The two chambers are then allowed to reach thermodynamic equilibrium, which may take one or two days. During that time, the hapten will diffuse across the membrane, and some of it will be bound by the antibody in the other chamber, as indicated on the right side of the diagram.
The key point is that the concentration of free hapten on both sides of the membrane will be the same at equilibrium, indicated as H'. In addition, there will be free antibody (Ab) and antibody bound to hapten (AbH).

Since $H_T = 2H' + AbH$, we can determine the value of AbH ($H_T$ is known, and $H'$ is measured). Then, since $Ab_T = Ab + AbH$, we can also determine Ab, or the concentration of free antibody.

**Analysis**

The experimental data are collected by measuring the concentration of free hapten at equilibrium, having started with a series of different initial hapten concentrations. The basic rationale is that at any given hapten concentration, a higher affinity antibody will bind a higher proportion of hapten.

We can rewrite the reaction as a series of reactions, as follows:

\[
\begin{align*}
Ab & + H \leftrightharpoons AbH \quad K_1 \\
AbH & + H \leftrightharpoons AbH_2 \quad K_2 \\
AbH_2 & + H \leftrightharpoons AbH_3 \quad K_3 \\
\text{etc...}
\end{align*}
\]

Each reaction represents the binding of one additional hapten molecule to an antibody molecule, up to some unknown maximum (which depends on the total number of sites actually present on each antibody molecule); each reaction is governed by an equilibrium constant, $K_1$, $K_2$, etc.

**Assumptions:** (1) All binding sites on a single Ab molecule have the same affinity, and (2) binding of H by different combining sites on the same antibody molecule is independent; i.e. binding of H to one site does not help or hinder binding of H to another. If these assumptions are correct, then all the reactions above can be represented by a single reaction with a single equilibrium constant, where "S" represents a single antibody-combining site.

\[
S + H \leftrightharpoons SH \quad K_{eq}
\]

By the definition of the equilibrium constant:

\[
K_{eq} = \frac{[SH]}{[S][H]}
\]
This can be shown to be equivalent to the following equation:

\[
\frac{r}{c} = Kn - Kr
\]

where:
- \( r \) = moles H bound per mole of antibody
- \( c \) = concentration of free H, or \([H]\)
- \( K \) = \( K_{eq} \), defined above
- \( n \) = number of binding sites per Ab molecule (or \([S]/[Ab]\))

*This equation is that of a straight line* of the form \( Y = b + mX \), where \( Y \) is replaced with \( R/c \), the constant \( b \) is replaced with \( Kn \), and \( mX \) is replaced with \(-Kr\). If we plot our data, we expect to get the straight line labeled "theoretical", as shown below:

![Diagram](image)

**Figure A6-2**

Two important values can be determined from this straight line; first, *the slope* (actually the negative slope) gives us \( K_{eq} \), the equilibrium constant, which we have defined as the affinity; second, *the X-intercept* gives us the value of \( n \), the number of combining sites per antibody molecule. In this graph, the theoretical line is drawn so that it crosses the X-axis at a value of 2.

So far we have been dealing with a theoretical straight line; but when we actually carry out such an analysis with the rabbit anti-DNP antibodies and plot the results, we get *not a straight line, but a curve* such as shown in the figure above (labeled "actual").

- **The curvature indicates that the antibody preparation is heterogeneous with respect to affinity.** There are antibodies with high and low affinities, and everything in between. (The degree of curvature can, in fact, be used as a measure of the degree of antibody heterogeneity.)
• If we extrapolate the curve to the X-axis, we can estimate a value for \( n \) of 2; this means that there are two combining sites for DNP on each IgG antibody molecule.

• If we carry out this analysis with homogeneous IgG anti-DNP antibodies (for instance with a human DNP-binding myeloma, or an anti-DNP hybridoma), we do get a straight line with an X-intercept of 2, and a slope which defines the affinity of that particular antibody (as shown by the "theoretical" line in Figure A5-2).

The shape of the curve can be understood intuitively. At very high values for \( r/c \), that is, at very low levels of free hapten, those antibodies with high affinities will more effectively compete for what little hapten is present; therefore, binding by the high affinity antibodies predominates and the slope of the curve is high (the curve is steep) in that region. At low values of \( r/c \) there is plenty of hapten to go around, and the lower affinity antibodies can contribute a larger proportion of the binding; the curve is shallower in this region, representing a lower overall affinity.

One can define the "average affinity" (\( K_{av} \)) of a heterogeneous antibody population as the slope of the curve where \( r = 1 \), that is, at the point where half of the available sites are occupied; this is indicated as \( K_{av} \) in Figure A6-2.

To better understand the relationship between these experimental data and the "real-life" functions of antibodies, let's define two terms which are distinct, but easily confused, namely AFFINITY and AVIDITY.

AFFINITY: The strength of binding of a single antibody combining site with its epitope; the equilibrium constant of the binding reaction. Conventional antibodies have measurable affinity constants in the range of \( 10^4 \) to \( 10^{10} \); higher and lower values certainly exist in nature, but are generally difficult to measure accurately. Many methods other than equilibrium dialysis exist for determining affinity constants, but they are all measures of the interaction of a single combining site with its antigen.

AVIDITY: The strength of binding of a multivalent antibody to a multivalent antigen; a measure of the ability of antibodies to form stable complexes with their antigens. The avidity of an antibody depends not only on the affinity, but also on the valency of both antibody and antigen, and on various physical properties of both. Avidity does not have a standard thermodynamic definition, although it can be defined in a relative and ad hoc manner within a specific given experimental context.
The difference between affinity and avidity may be illustrated by the following diagram, showing the two-step binding of a bivalent antibody to a multivalent antigen molecule:

![Figure A6-3](image)

The first step of binding occurs at a rate (shown as $k_1$ above) determined by the affinity of the combining site for its epitope (note that we are now discussing reaction rates, not equilibrium constants). The second step, however, occurs with a much higher rate constant, $k_2$; once the first site has bound to the antigen, the likelihood of the second site binding is much higher, since the second site is held in close proximity to another epitope. This cooperativity between binding sites on an antibody molecule makes the effective binding to antigen much stronger than would be predicted simply by the equilibrium constant; avidity is the term used to describe this greater strength of binding.

Antigens in nature are often polyvalent and highly repetitive structures. The strength of binding by antibodies to such antigens (microbial cell wall components, for instance) is much higher than the antibodies' affinities would predict, because of the effect of cooperative binding. This effect becomes even more important in the case of polymeric IgA, and of IgM which can have up to 10 combining sites in a single antibody molecule (although all of the sites may not necessarily be capable of binding antigen simultaneously).
7. CROSS-REACTIVITY

We’ve seen examples of serological cross-reactivity illustrated by Ouchterlony precipitation patterns. Adding the use of RIA allows us to distinguish two different modes of cross-reactivity.

Figure A7-1 shows the kind of cross-reactivity we have already seen, due to the presence of multiple distinct epitopes which may be unique or shared between antigens. At the left we see the structural basis for such cross-reactivity - antigen “A” bears two epitopes labeled “X” and “Y”, antigen “B” also has epitope “X”, together with a different epitope “Z”. Using an antiserum prepared against antigen “A” shows the Ouchterlony pattern we recognize as partial identity - a shared line of precipitation due to the presence of epitope “X” on both antigens, but with a “spur” of “A” over “B” because epitope “Y” is present only on antigen “A”.

Cross-Reaction due to Multiple Distinct Epitopes

If we carry out an analysis by inhibition of RIA (at the right), we also see evidence of partial cross-reactivity. The assay consists of antigen “A” bound to the tube incubated with affinity-purified radiolabeled antibody to the same antigen. Thus we have labeled antibodies to both epitopes “X” and “Y”. If we inhibit with a sufficient amount of purified antigen “A”, we can inhibit 100% of the antibody binding, as expected. However, when we use antigen “B” as inhibitor, we find that no matter how much we add we can only inhibit 50% of the binding. This is because some of the anti-A antibody (50% in this example) is directed against epitope “Y”. Since antigen “B” doesn’t have this epitope, it cannot inhibit this binding. (Note that the putative presence of epitope “Z” on antigen “B” is irrelevant in our example, since there are no antibodies to this epitope in the system.)
Let’s consider another situation, where two antigens each have one epitope which is related, but not quite identical, to that on the other (indicated as X and X’ below). In this case the pattern we see in Ouchterlony is one of complete identity, despite the fact that we expect antibodies to epitope X to bind less strongly to X’. But as long as the antibodies bind X’ sufficiently strongly to cause precipitation, Ouchterlony analysis will not distinguish the two antigens - they appear identical.

**Antigens:**

- A
- B

**Ouchterlony:**

- αA

**RIA Inhibition:**

- "A" bound to plate,
- 125I-labelled anti-A

![RIA Inhibition Graph]

**Cross-Reaction due to Related Epitopes**

**Figure A7-2**

When we analyze these antigens by RIA, however, we see something different. Antigen B can, indeed, completely inhibit the binding of anti-X to epitope X, but since its own epitope X’ is recognized by the antibody with lower affinity (therefore resulting in lower avidity), it has a more difficult job inhibiting. This is reflected in the inhibition curve for antigen “B” which shows a shallower slope than that for “A”, and a greater amount of antigen necessary to finally achieve complete inhibition.

Recognizing this property of cross-reactive antigens is of practical importance in interpreting RIA results. One explicit assumption in using RIA to determining the concentration of an antigen (as, for example, insulin in APPENDIX 5), is that the antigen in the unknown samples is identical to that in the standard. If we see an inhibition curve which is not identical in shape to that of the standard, then we know that the material in the unknown sample is structurally different from that in the standard, and we cannot draw any conclusion regarding relative concentrations.
8. COMPLEMENT FIXATION ASSAY (see Chapter 5, COMPLEMENT)

Many different methods exist to measure the levels and degree of activation of the various complement components, most of which are used only in highly specialized labs devoted to complement research. The classical Complement Fixation (CF) assay, however, has long been widely used by biologists of all kinds as a simple and sensitive way to detect and quantitate a wide variety of antigens and antibodies. While it is still sometimes used in both research and clinical situation, many of its applications have been replaced by ELISA or other more sensitive and quantifiable methods.

Let’s illustrate the principles by constructing a complement-fixation assay to detect a hypothetical viral antigen (VA) in several unknown serum samples.

We begin by making "sensitized" erythrocytes, which are simply RBC coated with anti-red blood cell antibodies; we call these EA (Erythrocyte + Antibody). In the absence of complement these cells remain intact, but they will be lysed if exposed to any source of complement; these EAs are our indicator cells.

Next, we prepare a standard source of complement, usually fresh guinea pig serum diluted appropriately. The dilution is such that it contains just enough complement to completely lyse a standard amount of EA.

We also prepare a standardized antibody to the antigen, by immunizing a rabbit with purified VA. Before use the rabbit antiserum is heat-treated (56°C for 30 min) to remove its inherent complement activity.

We then take a series of test samples (for example, heat-treated serum samples from various patients), mix them with a standard amount of our antibody, and incubate the mixture for an hour (allowing the antibodies to react with the antigen, if it is present). To this mixture we then add a standard amount of the guinea pig serum as a source of complement, and after a short time (to allow the AgAb complexes to interact with the complement) we add a standard amount of the sensitized erythrocytes (EA). We incubate the samples at 37°C for a half hour and examine them visually (or spectrophotometrically) for lysis; a suspension of intact RBC is cloudy, but it becomes transparent when the cells are lysed.

The protocol would be as shown on the table below (where "unk" refers to an "unknown" or test sample).

Tube 1 tells us that the erythrocytes have been properly sensitized and that our guinea pig serum (as a source of complement) is effective. Tube 2 shows us that the antibody alone does not interfere with the complement-dependent lysis of the sensitized RBC. In tube 3, the antibody will form complexes with the added VA protein, and these complexes will consume ("fix") the complement; therefore, there will not be enough complement left to subsequently lyse the indicator cells. Repeating this test with two unknown samples in tubes 4 and 5 tell us that sample No. 2 must contain the viral antigen VA, while sample No. 1 does not. We carry out one additional negative control, by re-testing unk sample number 2 in the absence of specific antibody. As expected (tube #6), no complement is fixed, and the indicator cells are lysed.
<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Sample</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>(saline alone + C) + EA</td>
<td>lysis</td>
<td>neg control</td>
</tr>
<tr>
<td>2)</td>
<td>(Ab + saline + C) + EA</td>
<td>lysis</td>
<td>neg control</td>
</tr>
<tr>
<td>3)</td>
<td>(Ab + VA + C) + EA</td>
<td>no lysis</td>
<td>pos control</td>
</tr>
<tr>
<td>4)</td>
<td>(Ab + unk No. 1 + C) + EA</td>
<td>lysis</td>
<td>no VA present</td>
</tr>
<tr>
<td>5)</td>
<td>(Ab + unk No. 2 + C) + EA</td>
<td>no lysis</td>
<td>VA present</td>
</tr>
<tr>
<td>6)</td>
<td>(unk No. 2 + C) + EA</td>
<td>lysis</td>
<td>neg control, OK</td>
</tr>
<tr>
<td>7)</td>
<td>(Ab + unk No. 3 + C) + EA</td>
<td>no lysis</td>
<td>VA present??</td>
</tr>
<tr>
<td>8)</td>
<td>(unk No. 3 + C) + EA</td>
<td>no lysis</td>
<td>anti-complement activity</td>
</tr>
</tbody>
</table>

However, the last two tubes (7 and 8) illustrated a possible complication in interpreting these results. Tube 7 shows no lysis, suggesting the presence of viral antigen in sample #3 (as was the case with sample #2). However, when we run this same sample again in the absence of specific Ab, there is still no lysis, although we expect the same result as in our negative controls (tubes 2 and 6). This shows that serum #3 inhibits complement activity by itself, a property referred to as "anti-complementary," we therefore can say nothing about the presence or absence of viral antigen in this sample unless we find a way to remove this activity. Knowing of this possibility, it is clear that we must repeat this control test for every positive sample (as we did for sample #2 in tube 6) to confirm that the positive result is not the result of anti-complementary activity. (Such activity might be due to the presence of unrelated immune complexes in the serum, the presence of antibodies which happen to recognize components of the guinea pig complement, or the presence of drugs which inhibit complement.)

Despite these cautions, complement fixation assays are still used in a variety of specialized applications. They can provide very sensitive tests for any antigen to which an antibody is available, or, by changing the variables, can be used to test for the presence of antibody to a standard antigen. They can also be used to quantitate the level of antigen (or antibody) by carrying out the tests with varying dilutions of the unknown, and accurately determining the level of lysis of the indicator cells by colorimetric means.
9. ANALYSIS OF KAPPA-CHAIN GENE REARRANGEMENTS BY SOUTHERN BLOTTING

Determining the nature of gene rearrangements in tumor cell populations can be a powerful tool to aid in diagnosis of a variety of cancers. Let’s examine how the Southern blotting technique can be applied to examining kappa-chain rearrangements in cells of the B-cell lineage.

At the top of Figure A9-1 (#1, germline) is a schematic representation of the genomic configuration of the human kappa-chain gene complex, showing the constant region with its five associated J-segments at the right, and one of the many V-regions at the left. Also indicated are the positions of “EcoRI” sites, short DNA sequences which are specifically cleaved by this restriction enzyme. If this un-rearranged configuration of DNA were treated with EcoRI, the only fragment containing Cκ sequences would be the 2.5kb fragment indicated.

If we look at the configuration of this region of DNA in B-cells which have rearranged their κ-chain complex, we might see any one of a large number of different results, two of which are illustrated in the figure. In the first case (line #2), the rearrangement has removed the DNA containing the EcoRI site just to the left (3’ side) of J1, so that the the Cκ region now happens to be on an EcoRI fragment 3kb in length. In the second case (line #3), the Vk gene segment that was used happens to have an EcoRI site near it’s 5’ end, so the Cκ gene is now on a fragment 2kb long. The hundreds of different possible Vk/Jκ rearrangements will yield many different results; in unrearranged DNA the Cκ gene segment will exist only on a 2.5kb EcoRI fragment, in rearranged DNA it could end up on any sized fragment.

How can we use this information to analyze cell populations and help diagnose B-cell tumors? Let’s use the Southern blotting technique to examine DNA from various sources. We extract DNA from a cell population, treat it with EcoRI, then separate the resulting fragments according to size by electrophoresis in an agarose gel. We then transfer the separated fragments from the gel onto a sheet of nitrocellulose, incubate the sheet with a...
radioactive DNA probe which will hybridize with its complementary sequences, and we can determine the location of the radioactivity by autoradiography.

A schematic showing the results of such an experiment is shown in Figure A9-2. Various samples of EcoRI-treated have been separated in an electric field from top to bottom, the smaller fragments moving the farthest. The size of fragments at each position is indicated on the left.

**Southern Blot Hybridized with Cκ Probe**

Sample #1 consists of DNA from a cell population which has *not* rearranged Cκ, for example skin, liver, kidney, brain, or any source *other* than cells of the B-cell lineage. Since no rearrangement has taken place, the only place we see Cκ-hybridizing sequences is at the position of the germline 2.5kb fragment.

Samples #2 and #3 have been prepared from two different plasmacytomas, Ig-producing human tumors. Since cancers are monoclonal in their origin, all the plasma cells in a particular tumor have undergone exactly the same rearrangement(s). In this case, each tumor has rearranged *one* of its two copies of Cκ, but *not the other*, resulting in the appearance of two bands in each lane, the familiar 2.5kb germ-line band (non-rearranged) and a single, unique new band (rearranged).

Sample #4 is what we would expect with DNA prepared from a population of normal B-cells, taken (for example) from spleen, lymph node or blood. Each B-cell in the population has rearranged at least one Cκ gene, some have rearranged both. But each cell has rearranged in a unique fashion, resulting in a heterogeneous mix of many different fragments which contain the Cκ segment; this is seen as the “smear” of radioactivity in the right-hand lane. Since some of the B-cells have rearranged only one of their two alleles, all the non-rearranged alleles contribute to the visible band at the germ-line position.

We can apply this approach, for example, to help distinguish a B-cell tumor from more benign forms of lymphoid hyperplasia in a particular patient. In the case of a tumor, Southern blotting will show a pattern indicating monoclonal rearrangement (one or two discrete non-germline bands as in Lanes #2 and #3 above), whereas a pattern of polyclonal rearrangement (as in Lane #4) would indicate non-cancerous form of B-cell hyperplasia. The same approach can be used to help in the diagnosis of T-cell tumors, in that case using probes specific for T-cell receptor sequences rather than immunoglobulins.
10. GENETICS OF INBREEDING

"...the development of inbred strains has constituted probably the greatest advance in all cancer research." (W.E. Heston, 1963)

"The introduction of inbred strains into biology is probably comparable in importance with that of the analytical balance into chemistry." (Hans Grüneberg, 1952)

IMPORTANCE OF INBRED STRAINS OF MICE

One of the major obstacles in biological research of all kinds is the genetic variation inherent in most natural populations of organisms. Individual humans as well as experimental animals vary widely in their physiological properties and biological responses, and much of this variation (although not all of it) is genetically determined. One particular manifestation of such variability was recognized by cancer biologists more than a century ago, namely the inability to reliably transfer growing tumors from one mouse in a laboratory colony to another. It was also recognized that occasional successful transplants generally involved closely related animals, and depended on their degree of genetic relatedness; this is the basis for the special relevance of inbreeding to cancer research alluded to by Heston, above.

Shortly after the turn of the twentieth century, the geneticist C.C. Little developed the earliest inbred strains of mice, which for the first time provided genetically uniform mammals for research. The importance of such animals cannot be overstated, as attested to by the two quotations above. While their development was originally stimulated by their need in tumor transplantation, inbred mice have provided essential tools for research in genetics, physiology and behavior, as well as in all aspects of immunology. They have provided animal models for the study of many human diseases, including amyloidosis, polyuria, diabetes, muscular dystrophy and many others. In an immunological context, the use of inbred strains has been indispensable for the success of adoptive transfer systems, of which we have discussed several examples, as well as for the elucidation of the biological roles and chemical structure of products of the Major Histocompatibility Complex (MHC).

PRODUCTION OF INBRED MICE: BROTHER-SISTER INBREEDING

We can illustrate the basic features of the process of inbreeding, and of the resulting inbred strains, by the protocol originally used by Little. Let’s begin with two randomly chosen mice taken either from the wild, or, as was more commonly done, from the colonies of mouse "fanciers" who kept them for amusement or for sale. The two mice we start with may or may not be related, but in either case will differ in the allelic forms of genes they carry at many genetic loci.

Let’s consider a randomly chosen genetic locus which has two allelic forms, which we name A and a. NOTE: We do not have to know what this locus is, nor do we need to be able to distinguish its genotypes in order to understand the analysis that follows.
Suppose we happen to start with two individuals heterozygous at this locus; the resulting cross can then be diagrammed as follows:

Parents: \( Aa \times Aa \)

Offspring (\( F_1 \)) \( AA \ Aa \ aA \ aa \)

Frequencies: 
- \( AA \): 25%
- \( Aa \): 50%
- \( aa \): 25%

Using simple Mendelian genetics, we can see that there are three possible genotypes for the offspring of such a cross, and their frequencies are given above. If we then randomly choose two of these offspring to mate for the next (\( F_2 \)) generation, there are six possible crosses which may result. These six possibilities, together with the probability of having chosen each, are shown below:

<table>
<thead>
<tr>
<th>probability of cross</th>
<th>next generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA X AA</td>
<td>1/16</td>
</tr>
<tr>
<td>AA X Aa</td>
<td>1/4</td>
</tr>
<tr>
<td>AA X aa</td>
<td>1/8</td>
</tr>
<tr>
<td>Aa X Aa</td>
<td>1/4</td>
</tr>
<tr>
<td>Aa X aa</td>
<td>1/4</td>
</tr>
<tr>
<td>aa X aa</td>
<td>1/16</td>
</tr>
</tbody>
</table>

For example, the probability of our having randomly chosen two animals of genotype \( AA \) is simply \( 1/4 \times 1/4 \), or \( 1/16 \) (= 6.25%). The probability of the second combination (AA X Aa) is \( 1/4 \times 1/2 \times 2 = 1/4 \) (we multiply by 2 to take into account the fact that there are two possible choices which will yield this cross, namely AA X Aa, and Aa X AA; the same situation holds for all crosses in which the two individuals differ from one another).

The first and last possibilities in this list (AA X AA, and aa X aa) are special cases: for both of these crosses all offspring will be identical to their parents. If we add the probabilities for these two cases, we find that there is a 12.5% likelihood (\( 1/16 + 1/16 = 1/8 \)) that we will happen to have picked a pair of identically homozygous animals in this generation. *If this happens, regardless of which of the two possibilities we have chosen, all subsequent generations will be homozygous at this locus, as long as we keep to the brother-sister mating scheme.*

This is equivalent to stating that *12.5% of all genetic loci will be homozygous* in this generation, based simply on chance. Adhering to the brother-sister mating scheme ensures that once we happen to have chosen two animals homozygous for a given locus, no new heterozygosity can ever be introduced. Thus, as we continue this mating scheme, generation after generation, the resulting animals progressively approach a condition of *uniform homozygosity at all loci*. It is important to recognize that this analysis does not depend on our knowing anything about any of the genetic loci involved, nor does it rely on any deliberate selection on our part.
After 20 generations of such inbreeding, the overall homozygosity is about 98.5% (the "coefficient of inbreeding" = 98.5%), and we are entitled to call this line an inbred strain. Brother-sister inbreeding is continued throughout the maintenance of all inbred strains, and the overall level of homozygosity continues to increase, until that point at which it is counterbalanced by the appearance of spontaneous mutations (at a very low rate).

The oldest inbred strains of mice and rats date back to about 1909, and many have gone through hundreds of generations of brother-sister mating since then. Commonly used strains of mice include C57Bl/6, C3H, BALB/c, DBA/2 and AKR. There exist well over 200 strains (and many substrains) of inbred mice and scores of inbred rat strains, as well as inbred strains of rabbits, chickens, hamsters and guinea pigs.

In addition to the random brother-sister mating scheme described above, additional selection can be imposed during the early stages of inbreeding for the establishment of desired traits. This has resulted in the production of inbred strains of mice and rats with high (or low) incidence of various tumors, dental caries, blood pressure, and many other physiological or behavioral characteristics.

**PROPERTIES OF INBRED STRAINS**

Inbred strains have several properties which distinguish them from conventional laboratory or wild animals.

1) All members of a particular strain are genetically uniform (or "isogenic"); they are as identical to each other as human identical twins.

2) Furthermore, they are homozygous at all loci (unlike human twins).

3) Inbred strains “breed true”; that is, if two members of an inbred strain are mated, their offspring are also members of that strain; they are uniformly homozygous and identical to all other individuals of that strain.

Individuals of inbred strains are often (although not always) fairly delicate and of limited fertility, which tends to make them difficult to maintain and expensive to use.

**PROPERTIES OF F₁ INBRED CROSSES**

Individuals of two different strains are often mated to produce “F₁ crosses”. For example, a female C57Bl/6 mouse might be mated with a male of strain DBA/2, yielding an F₁ cross named (C57Bl/6xDBA/2)F₁, or, more briefly, "B6D2F1".

Such F₁ crosses between inbred strains possess important and useful properties:

1) Like inbred strains, they are also genetically uniform ("isogenic"); they are homozygous at all those genetic loci for which the parents happen to be identical.

2) However, they are not homozygous at all loci, unlike their inbred parents - in this respect they are more similar to human identical twins than are their inbred parents. In fact, they are heterozygous at all those loci (but only at those loci) for which the two parental strains carry different alleles.
3) The property of being an F₁ hybrid *does not breed true*; a cross between two B6D2F1 mice does *not* yield another B6D2F1 mouse, but a mixture of genetically heterogenous offspring. A regular supply of F₁ hybrids therefore requires the continuous maintenance of *both* inbred parental strains.

4) The products of F₁ crosses between inbred strains are often very *hardy* and of higher fertility, exhibiting what is called "hybrid vigor". This generally makes them easier and less expensive to use, and such animals are widely used for many experiments which require genetic uniformity, but not homozygosity.
11. MIXED LYMPHOCYTE REACTION (MLR)

Allelic differences in the MHC complexes between two individuals can be detected by transplant rejection, and by specific antibodies to the transplantation antigens. They can also be detected by the MLR.

If lymphoid cells (spleen cells, for instance) from two different strains of mouse are mixed and allowed to incubate in culture for a few days, each population will be triggered by the foreign antigens of the other. One result of this triggering is a proliferative response which can be detected by increased uptake of tritiated thymidine.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Thymidine uptake, CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c + BALB/c</td>
<td>800</td>
</tr>
<tr>
<td>BALB/c + C57Bl</td>
<td>28,400</td>
</tr>
</tbody>
</table>

This is referred to as a "two-way" MLR, since both BALB/c and C57Bl1 cells can respond to the MHC antigens of the other. One can make this test more informative by turning it into a "one-way" MLR. This is done by treating one of the two cell populations with the drug Mitomycin C, or with a high dose of X-irradiation. In either case, the ability of the cell population to proliferate is eliminated, but its ability to act as a stimulator is not affected.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Thymidine uptake, CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c + BALB/c*</td>
<td>800</td>
</tr>
<tr>
<td>BALB/c + C57Bl</td>
<td>27,200</td>
</tr>
<tr>
<td>BALB/c*+ C57Bl</td>
<td>21,700</td>
</tr>
<tr>
<td>BALB/c*+ C57Bl*</td>
<td>950</td>
</tr>
<tr>
<td>BALB/c + (C57BlxBALB/c)F1</td>
<td>21,500</td>
</tr>
<tr>
<td>BALB/c + (C57BlxBALB/c)F1*</td>
<td>22,900</td>
</tr>
<tr>
<td>BALB/c*+ (C57BlxBALB/c)F1</td>
<td>1,100</td>
</tr>
</tbody>
</table>

Mitomycin treatment of either cell population in the first case reduces the response (since fewer cells are proliferating), while treating both eliminates the response altogether. In the second case, we are mixing BALB/c cells with F1 cells; this is already a one-way response, since the standard rules apply, and the F1 cannot respond to the parental cells (the F1 cells see nothing "foreign" on the parent). In this case, mitomycin-treating the F1 has no effect, while treating the BALB/c parent cells eliminates the response.

The MLR differs from the transplantation reaction in one major way, however, in that the antigens responsible for MLR are primarily Class II antigens of the MHC. Mixing cells from mice differing only at K or D will yield weak MLR reactions, while cells differing only in the I-region will give strong reactions. It is for this reason that the MLR has been of considerable importance in tissue typing for human transplantation. Antibodies against Class II antigens have generally been difficult to produce, and MLR has allowed transplants to be matched for Class II antigens as well as Class I antigens (which are readily typed serologically). It is for this reason that Class II antigens have generally been referred to as LD ("Lymphocyte determined"). antigens, as opposed to the Class I, SD ("serologically determined") antigens (see Figure 11-5).
12. PLAQUE-FORMING CELL (PFC) ASSAY

The development in the 1960's of the PFC (plaque-forming cell) assay by Jerne represented a major advance in the progress of cellular immunology. It provided, for the first time, a simple and reliable method for determining the number of individual antibody-forming cells (AFCs) in a cell population. The kinetics of antibody-forming cell production could then be studied without having to rely solely on the levels of serum antibody, which is related only very indirectly to the number of cells producing it.

This procedure relies on the use of red blood cells (usually SRBC) as antigen, and on their ability to undergo lysis in the presence of antibody and complement. Cells from an immune spleen are mixed with a suspension of SRBC in a warm agarose solution, which is spread in a thin layer on a microscope slide and allowed to solidify. The slides are incubated for one hour, and antibody to SRBC produced by any particular cell in this gel will diffuse and bind to nearby SRBCs. At the end of the hour, the slide is incubated in a suitable dilution of fresh guinea pig serum (as a source of complement), and any SRBCs which are coated with antibody will undergo lysis. The result is a small clear circle (a "hemolytic plaque") on a cloudy background, surrounding each AFC. The single Ab-producing cell in the middle of each plaque is not visible except under a microscope. These clear "plaques", schematically illustrated in Figure A12-1 below (middle slide) can be readily counted, and provide an accurate determination of the number of AFCs: each antibody-secreting cell produces a single clear plaque.

![Figure A12-1](image)

No C; 
continuous lawn of RBC

C only; 
direct (IgM) plaques

C + anti-IgG; 
indirect (IgG) plaques, 
also including direct plaques

Figure A12-1
Under the conditions described above, only those cells which produce IgM will create a visible plaque; IgG will not generally achieve high enough concentrations to yield two IgG molecules binding sufficiently close together on an SRBC to lyse the cell. In order to detect IgG-secreting cells, antibody to mouse IgG (in this example) is added to the complement solution. Two or more molecules of this secondary antibody will then bind to each single IgG bound to an RBC, and will result in lysis in the presence of complement.

The plaques produced in the absence of this secondary "developing" antibody are known as direct plaques, and represent the number of IgM antibody producers (middle slide above). The additional plaques developed in the presence of the anti-IgG antibody are known as indirect plaques and represent IgG producing cells (seen in the bottom slide in the figure).

In the example shown above, there are 5 AFCs producing IgM anti-SRBC (direct plaques), and an estimated 11 IgG-producing cells (i.e. 16 total indirect plaques, from which one must subtract an estimated 5 IgM-producing cells determined from the slide above).

As described here, this technique can only be used to measure those antibody-forming cells producing antibody to red blood cells (SRBC or other RBCs). However, it can be made more generally useful by coupling any desired antigen to RBCs, then using these coated cells as indicator cells for that antigen (similar to what is done for passive hemagglutination). AFCs producing antibody specific for DNP or HGG, for instance, can be detected as plaque-forming cells by using RBCs coated with either the hapten or the protein antigen.
13. HYBRIDOMAS: MONOCLONAL ANTIBODIES

THE PROBLEM: LIMITATIONS OF CONVENTIONAL ANTIBODIES, HETEROGENEITY AND BATCH-TO-BATCH VARIATION

Conventional antisera, as we have seen, contain complex and heterogeneous mixtures of antibodies with different antigen-binding specificity, as well as bearing different heavy and light chain isotypes. Immunization of a rabbit, for example, with a human kappa IgG3 myeloma protein will potentially result in formation of antibodies directed against kappa and $\gamma_3$ isotype epitopes, as well as allotypic and idiotypic epitopes on both heavy and light chains. Note that the rabbit antibodies may themselves consist of a mixture of classes (e.g. IgG and IgM) and light chain isotypes ($\kappa$ and $\lambda$), but this heterogeneity is not relevant for our discussion here.

In order to prepare a $\kappa$-specific reagent which could be used for a clinical laboratory assay for $\kappa$ chains, an elaborate and expensive procedure of absorption and/or affinity purification must be carried out, and the resulting antibody extensively characterized for its efficacy and specificity. Furthermore, when this one batch of antibody is used up, the entire procedure must be repeated from scratch, with no guarantee that the next batch will be the same as, or as good as the first, since every new rabbit may respond differently to immunization.

The problems of antibody heterogeneity and batch-to-batch variation are even more serious for those antibodies which are the result of fortuitous "natural" immunization rather than controlled laboratory immunizations, for example those antibodies used for human Ig allotype determination (e.g. from polytransfused patients) and for HLA-typing (e.g. from polytransfused patients or multiparous women).

THE SOLUTION: MONOCLONAL ANTIBODIES

In order to resolve these problems it clearly would be desirable to create a source of antibody which is monoclonal (and therefore monospecific), and can be produced in unlimited quantities with no batch-to-batch variation. This is precisely what the development of HYBRIDOMA technology in the mid-1970's has allowed us to do. In principle, we could simply choose a particular antibody-forming cell producing the antibody we desire, and "immortalize" it so that we can propagate it in culture and collect its secreted product indefinitely. Unfortunately these cells cannot ordinarily be grown in culture (although some success has been achieved in immortalizing such cells by transformation with Epstein-Barr virus).

AFC's AND FUSION PARTNERS

We begin with spleen cells from a mouse repeatedly immunized (in this example) with a human $\kappa$IgG3 myeloma protein. This population is enriched for AFC's producing the Ab we want (anti-$\kappa$), although many other irrelevant antibodies will also be represented.

We then harvest cells of a plasmacytoma cell line, the "fusion partner", which has been adapted to cell culture. Several such lines are commonly available, and they have several important properties. First, they have the capacity of unlimited growth in culture. Second, they have the required machinery for high-level expression and secretion of immunoglobulin; the commonly used lines have also been selected for loss of expression of
their own immunoglobulin, to avoid interference with the specific Ab we want. Third, they have been selected for loss of expression of one of the enzymes required for DNA synthesis by the "salvage pathway". One commonly used fusion partner is the mouse cell line SP2/0, which we will use in this example; it produces no Ig of its own, and is HAT-sensitive (see below).

"HAT" SELECTION

Figure A13-1 outlines the metabolic pathways involved in the "HAT" selection scheme. Cells can produce nucleosides required for DNA synthesis (and therefore cell proliferation) by either of two pathways. First, they can build them up from simple carbon sources such as glycine via the "de novo" pathway indicated as "1"; one step in this pathway requires the enzyme Dihydrofolate Reductase (DHFR), as indicated. Alternatively, if preformed purines and pyrimidines are made available to the cell, it can incorporate them via the "salvage pathway" indicated as "2", one step of which requires the enzyme Thymidine Kinase (TK).

However, if a cell has been selected for loss of TK expression (indicated in the figure as TK−), then the cell is completely dependent on the de novo pathway in order to synthesize DNA and proliferate. [Selection for loss of other enzymes in this pathway, e.g. Hypoxanthine-Guanine Phosphoribosyl Transferase, or HGPRT, can also be used for this purpose]. Such a cell is now exquisitely sensitive to the drug Aminopterin, which is a potent inhibitor of DHFR. This sensitivity is the basis for HAT selection, as discussed below.
MAKING HYBRIDOMAS: FUSION/SELECTION/SCREENING

Three basic steps are involved in the production of the hybridoma we desire.

1) FUSION: Having immunized a mouse with the purified κIgG3 protein, we mix its spleen cells (~10^8 cells) with cells of the fusion partner ("PC" in the figure; ~10^7 cells), in the presence of an agent which facilitates cell fusion (typically either polyethylene glycol [PEG], or Sendai virus). At low frequency (~10^-5) individual AFCs (indicated as "B" in the figure) will fuse with individual plasmacytoma cells.

2) HAT SELECTION: The mixture is then distributed into some 1000 separate culture wells (in a number of 96-well plates), and covered with HAT medium. This medium contains Aminopterin ("A"), which blocks the de novo synthesis pathway, as well as Hypoxanthine and Thymidine ("H", "T") which allow competent cells to utilize the salvage pathway. As indicated in the Figure A13-2, the only cells capable of growing under these conditions are the fusion products; the unfused plasmacytoma cells are TK^- and are killed by the drug, while the unfused AFCs inherently lack the ability to grow in culture (even though they are TK^+).

3) ANTIBODY SCREENING: After a week or two in culture, a few hundred (say 500) of the wells will contain growing cells, most of them the product of a single fusion event. Supernatants from these 500 wells are then assayed for the presence of Ab of the desired specificity, identifying perhaps ten positive clones. These clones are transferred into larger dishes, subjected to additional rounds of subcloning (to eliminate possible contamination by irrelevant clones or unstable variants), and eventually propagated for collection of the monoclonal antibody they produce, and frozen in liquid nitrogen for long-term storage.

![Diagram of Hybridoma Production](image-url)

Figure A13-2
In our example, the antibody screening might utilize an ELISA assay with purified κ chain as the antigen; using a variety of different monoclonal κ chains could enable us to identify mAbs with either isotypic or allotypic specificity. [NOTE: In fact, we could have used purified κ chains as the original immunogen, possibly increasing the proportion of useful clones recovered. Two considerations might have led us to immunize instead as described, i.e. using the intact κIgG3. First, the time and expense required for κ chain purification are not necessary, since we can readily identify the desired clones even among a large number of irrelevant ones. Second, we might be interested in simultaneously screening the 500 growing clones for anti-γ3(G3)-specific Ab as well, which could be used in other clinically or scientifically relevant assays.]

The final result consists of one or more stable cell lines, each producing a monoclonal antibody specific for human kappa chains, with specificity for either isotype or allotype. These Abs (or the cells producing them) can be shared by laboratories all over the world with the assurance that each lab is using Ab of precisely the same specificity. The Ab produced years later from the same cell line will be identical to the original (so long as rare mutants are avoided), and one batch will have precisely the same specificity as any other batch.

APPLICATIONS

Monoclonal Abs have found widespread use in many areas of clinical and basic relevance, including assays for many drugs and hormones, reagents for blood-typing and (to a more limited extent) HLA-typing, and for defining and diagnosing the presence of different serotypes of pathogenic viruses and bacteria. We’ve already mentioned (in Chapter 13, "CELL SURFACE MARKERS") the importance of those monoclonal antibodies defining several important markers, e.g. CD3, CD4 and CD8, as well as many others. Such antibodies can be used in the laboratory determination of lymphocyte subsets (CD4/CD8), for the depletion of T-cells from potential bone marrow grafts (using anti-CD3, for example), and for immunosuppressive therapy (e.g. anti-CD3).