CHAPTER 12

Mouse immunoglobulin allotypes: description and special methodology

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Identification of immunoglobulins
   classes ........................................ 12.1
   Identification of allotypes ............... 12.1
   Terminology .................................. 12.2
   Demonstration of allelism .................. 12.3
   Demonstration of genetic linkage ......... 12.4
   Allelic exclusion ............................. 12.4
   Light chain variation ....................... 12.4

Allopolypeptide and specification of the Ig loci .................................. 12.5
   Allotypic specificities ....................... 12.6
   Rules for defining specificities ........... 12.6
   Recognition of allotypes by electro-
   phoretic mobility differences ............. 12.8
   Specialized laboratory methods .......... 12.8

This short introduction is intended to orient the reader to terminology, classification and some immunogenetic considerations in the study of mouse immunoglobulins. It is followed by more detailed sections on mouse immunoglobulin classes and genetics as well as a section on some of the special 'mini' methods developed for studying these immunoglobulins.

Identification of immunoglobulin classes

The immunoglobulin molecule is made up of a four-chain unit, containing two 'identical heavy (H) chains and two identical light (L) chains [1]. The structure of the H-chains determines the class of the molecule.

Classes were originally identified by physical characteristics such as size and electrophoretic mobility; however, all known classes now may be recognized by class specific antisera raised in another species, usually goat or rabbit. These antisera, which contain antibody specific for a particular H-chain, generally must be absorbed to remove contaminating anti-light chain antibody (which would react with all classes) and also antibody which reacts with other H-chains.

There are eight known H-chain classes in the mouse, each associated with particular biological activities (see Table 12.1) and most are represented by at least one myeloma protein [2-5]. All mouse strains tested so far have been shown to have all of the known immunoglobulin classes.

The loci determining at least six (and most likely all) H-chain classes are clustered in a single chromosome region (the Ig region). Alleles at each of these loci determine minor differences in H-chain structure within each class. The immunoglobulins determined by these alleles are called allotypes [6, 7].

The H-chain loci are extremely closely linked (genetically) to one another and more loosely linked to another chromosome region called the V-region, which contains loci controlling other aspects of immunoglobulin structure (idiotypes). The V-region also contains loci controlling immune responses to certain antigens [8].

Identification of allotypes

Allotypes within a given class of immunoglobulins are products of alternate forms of the gene, i.e. alleles, at the locus determining the structure of the H-chain of that class of immunoglobulins. Typically,
### Table 12.1. Mouse immunoglobulin classes

<table>
<thead>
<tr>
<th>Class*</th>
<th>Locus</th>
<th>Number of alleles known in inbred strains</th>
<th>Number of specificities described</th>
<th>Some biologic activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG2a (γG)</td>
<td>Ig-1</td>
<td>8</td>
<td>11</td>
<td>Fixes complement, mediates cell lysis, fixes to tissues of other species and mediates local anaphylaxis</td>
</tr>
<tr>
<td>IgA (γA)</td>
<td>Ig-2</td>
<td>5</td>
<td>4</td>
<td>Does not fix complement, secreted into milk, tears, intestinal lumen, nasal secretions</td>
</tr>
<tr>
<td>IgG2b (γH)</td>
<td>Ig-3</td>
<td>6</td>
<td>7</td>
<td>Fixes complement, mediates cell lysis, passes placenta</td>
</tr>
<tr>
<td>IgG1 (γF)</td>
<td>Ig-4</td>
<td>2</td>
<td>2</td>
<td>Does not fix complement, fixes to tissues of same species, mediates local anaphylaxis, passes placenta</td>
</tr>
<tr>
<td>IgG3</td>
<td></td>
<td></td>
<td></td>
<td>Does not fix complement, does not fix to skin, passes placenta</td>
</tr>
<tr>
<td>IgD</td>
<td>Ig-5</td>
<td>2</td>
<td>2</td>
<td>Present on lymphocyte membranes, not found in circulation; function unknown; present on most B cells in adult mice</td>
</tr>
<tr>
<td>IgM (γM)</td>
<td>Ig-6</td>
<td>2</td>
<td>2</td>
<td>Fixes complement, mediates cell lysis (more efficient than IgG2a and IgG2b); present on most B cells in adult mice</td>
</tr>
<tr>
<td>IgE</td>
<td></td>
<td></td>
<td></td>
<td>Binds to most cells, responsible for allergic reactions</td>
</tr>
</tbody>
</table>

* Potter-Lieberman class notation given in parentheses [9].

Allotypes are recognized by antisera raised by immunizing one mouse strain with immunoglobulin from a second strain; however, other means may be used. For example, electrophoretic mobility of the Fc fragment of the H-chain was used to first identify alleles at the Ig-4 (IgG1) locus, although antigenic differences have been identified now for the allotypes in that class as well. Using antisera, allotypes for a given class may be identified either by the presence of a unique antigenic determinant (specificity) or as a unique combination of antigenic determinants each of which is also found in other combinations in other allotypes.

Antiallotype antisera often contain several antibody populations. These may react with allotypic determinants on different classes of immunoglobulins or with different determinants on a single class. Heterologous sera have been shown to detect allotypic differences. Such sera are not easily obtained, probably because allotypes represent such small differences between members of an immunoglobulin class that they are not as effective immunogens as isotypic (species) determinants in heterospecific immunization. When populations of antibody reacting with allotypic determinants are found in heterologous antisera, they frequently represent only a small fraction of the total anti-Ig (anti-immunoglobulin) response. Therefore, these sera must be extensively absorbed before they can be useful as reagents for detecting allotypes. Nonetheless, for certain purposes (e.g. fluorescent staining or indirect plaque-forming cell detection) heterologous anti-allotype reagents have proved extremely useful and well worth the trouble to prepare.

**Terminology**

For historical reasons, two bases for notation of mouse immunoglobulins are in use, a practice which often creates confusion for those beginning to work or read in the field. When speaking of antibodies or immunoglobulins as proteins, the class name, for example IgM or IgG2a, is most often used.* When discussing genetic (allelically-determined) structural differences between immunoglobulins or antibodies within a given class, the genetic or allotypic notation is used. For example, Ig-1 is the locus for IgG2a, IgG2a in the BALB/c strain is called Ig-1a and IgG2a in C57BL/10 strain is called Ig-1b. Thus, although logically it would suffice to call those

* In the older literature, γ is often used in place of Ig in class name, i.e. γM for IgM, etc.
immunoglobulins determined at the Ig-1 locus, Ig-1 globulins, they are in any nongeneric context, and often in a genetic context as well, referred to as IgG2a globulins, making it necessary for the reader to learn both class and allotypic terminology and to shift easily from one to the other if he is to make his way facilely through the mouse immunoglobulin literature.*

To make matters even worse, the individual immunoglobulin H-chain, whose structure determines both the class and the allotype of the immunoglobulin molecule of which it is a part, and is, in fact, the direct product of a particular Ig allele at a particular Ig locus, is named in Greek symbols in correspondence with the class terminology, e.g. the products of alleles at the Ig-2 locus are $\gamma$A H-chains and are all called $\alpha$-chains, with no specification of the allotype of the chain.

Attempts to simplify this notational jungle have met with little or no success as yet. Alternate systems have been suggested, but no agreement on nomenclature for mouse immunoglobulins, especially for allotypes, has been reached.

**Definition of H-chain loci**

There are certain problems in the demonstration that H-chains of the different classes are coded for by separate loci. These problems stem from the difficulty in studying a large enough number of mammalian progeny to detect crossing-over between closely linked genetic loci. Although upwards of 2000 progeny of appropriate crosses have been examined in detail, no direct evidence of a cross-over between Ig loci has been found. Thus, by the original definition of a locus as a segment of the chromosome defining a particular characteristic and separable by crossing-over from segments defining other characteristics, all of the Ig loci would be lumped as one.

The current definition of a locus in mammalian systems, however, is not based on a demonstration of crossing-over. In analogy with the bacterial cistron, a locus is defined as a place on the chromosome at which there exists a DNA sequence (gene) coding for a polypeptide chain. Although deletions and duplications can occur, in general a haploid chromosome set in a species carries one locus (hence one allele) for each of the proteins produced by the species. Therefore, if two polypeptide chains produced by a species differ structurally from one another, but the loci are linked, two or more alleles in a single haploid set, they may be presumed to be determined by separate loci even when no cross-over has been observed between the loci.

By these criteria, H-chain structure in each immunoglobulin class is determined at a separate locus. First, immunoglobulin H-chains in the different classes have been shown clearly to differ structurally from each other. Serologic studies have shown that each H-chain class carries unique antigenic markers. These differences have been confirmed by peptide mapping studies and in some cases by sequence analysis. Second, genetic studies presented in the next sections show that the loci controlling H-chain structure are all inherited in a single haploid set.

**Demonstration of allelism**

Alleles are defined as alternate forms of the gene (DNA sequence) at a given locus. Thus, by definition, alleles are not inherited in the same haploid set. Alleles are identified by their products (or lack thereof) on the basis of structural, functional, serological or physical criteria. Two or more alleles may exist for any given locus. Conventionally, a locus is not named until at least two alleles have been identified, i.e. until a polymorphism exists for a trait.*

Six loci have been named for the six mouse immunoglobulin classes in which polymorphic variations, i.e. allotypes, have been described. For each locus, the minimal criteria for allelism were met by genetic testing as follows. Two inbred mouse strains, each with a different H-chain allotype for a given class, were crossed to obtain heterozygotes. Both allotypes were found in the heterozygotes. Heterozygotes were then crossed, either back to one of the parental strains, or to a strain carrying a third allotype, or to themselves. In all cases, the expected ratios for segregating co-dominant alleles were found, indicating that a single haploid set always

* Another notion is used by Potter, Lieberman and some other workers [9]. Although it represents a logical attempt to combine the protein and gene notation, it is not generally used because it does not accord with the World Health Organization internationally accepted nomenclature for human immunoglobulins [10].

* Even more cautiously, but perhaps reasonably for immunoglobulins whose complete genetic control is to say the least still quite arcane, the reader should store the reservation that some allotype loci may be regulatory ones.
12.4 Immunogenetics

carried either the allele donated by the mother of the heterozygotes or the allele donated by the father never both, never neither [7]. These studies demonstrating allelism also show that a single haploid set carries each of the named H-chain loci.

Demonstration of genetic linkage

In crosses where the parental strains had allotypic differences at two or more H-chain loci, the genetic testing showed that the loci in a given haploid set were always inherited together indicating that the structural genes determining at least four of the mouse H-chain classes are clustered quite closely on the chromosome. This close genetic linkage could have considerable biological importance in the differentiation of immunoglobulin-producing cells.

Thus far, the testing of a large number of progeny (over 2000) has yielded no direct evidence of crossing-over between H-chain loci. Nonetheless, the existence of a number of cross-reacting allotypes at each locus are probably signs of past cross-overs. It is likely that continued progeny testing will eventually directly demonstrate crossing-over in the mouse Ig region since in humans, where the immunoglobulin genes are also closely linked, at least one family has been found where crossing-over has occurred [11].

Efforts to relate the Ig region to other mapped genetic markers in the mouse have thus far been unsuccessful, although crosses with markers in all of the known linkage groups in the mouse have been studied. Since male heterozygotes express the H-alleles, Ig cannot be sex linked.

Within the last few years a number of otherwise unmapped loci have been shown to be genetically linked to the Ig region. These include loci which control immunological responsiveness (e.g., response to dextran) and loci which control variable region structure (idiotypes). Several cross-overs have been observed between these markers and the Ig region [8, 12]. Another locus, controlling pre-albumin structure, has also been shown to be linked to the Ig region [13]. Its position with respect to the Ig region and the segment of chromosome controlling variable region markers has not yet been established. Thus, the Ig (H-chain) loci and the variable region loci form a linkage group which as yet has not been connected with any known linkage group in the mouse.

Cytogenetic attempts to locate the Ig loci have also thus far been unsuccessful [14]. 'Tobacco' mice, which have seven metacentric chromosomes resulting from end-to-end fusion of the 14 out of 20 of the mouse acrocentric chromosomes were crossed with normal mice and progeny backcrossed to obtain strains which contain one of the metacentric chromosomes on an otherwise normal chromosomal background. Although strains carrying each of the metacentric chromosomes were obtained, none of these carried the allelotype of the metacentric origin strain. Whether the Ig region was lost during backcrossing or whether it remains on one of the non-metacentric chromosomes in the tobacco mouse thus remains undetermined.

Allelic exclusion

One of the fascinating peculiarities of the genetic expression of the Ig alleles is that while the heterozygote produces immunoglobulins of both allotypes, individual immunoglobulin-producing cells in the heterozygote produce either one allotype or the other. The mechanism by which one of the two immunoglobulin alleles is excluded, or, put in the positive sense, the mechanism by which only one allele is turned on in a given cell, is not understood.

Recent studies suggest that allelic exclusion should more appropriately be called haplotype exclusion. Individual B cells (precursors of antibody producing cells) have now been shown to simultaneously express two or more immunoglobulin classes (IgD, IgM and sometimes IgG). In an allotype heterozygote, the allotype marker on each of these immunoglobulins shows that in a given cell, the immunoglobulins expressed are controlled by loci on the same chromosome. In other words, either the paternal derived or maternally derived Ig chromosome region appears to be functional in a given cell. Thus, in each cell, one parental haplotype is expressed and the other excluded [6].

Light chain variation

Both kappa and lambda chains are present in all classes of mouse immunoglobulins but lambda is found in only 5 per cent or less of the immunoglobulins. These chains are structurally homologous to human kappa and lambda [15]. Neither serologically detected allotypes nor amino acid compositional indicators of constant region genetic differences have been found. However, a V-region genetic difference detected by peptide 'finger printing' has been found. The variant peptide is obtained in a yield of under
20 per cent, consistent with its representation on a single V-region subgroup [16].

**Alleles and specificities of the Ig loci**

A summary description of the six known immunoglobulin H-chain classes, including associated biological activities, is presented in Table 12.1. Both the notation we use and Porter & Lieberman's alternate notation are given. As the table shows, allelic differences are known for only four of the classes, IgG2a, IgA, IgGb, and IgGl, defining the loci Ig^{-} to Ig^{-} respectively. The known alleles and distribution of allotype specificities at each of the Ig loci are presented in Tables 12.2–5.

Each Ig^{-} allele in Table 12.2 is assigned to a type strain whose immunoglobulins, by definition, are the standard for comparison for that allele. Since the Ig loci are closely linked genetically and since the largest number of alleles have been described at the Ig^{-} locus, the type strain assigned to each Ig^{-} allele is also assigned as the type strain for the entire Ig chromosome region of which that allele is a part, and alleles at subsequently discovered loci in the Ig region are named consistently with the Ig^{-} allele. For example, the BALB/c strain, which is Ig^{-}a, is assigned the Ig^{c} chromosome region, and therefore, by definition, as new Ig loci are recognized, the allele carried by BALB/c is designated by a superscript 'a', i.e. Ig^{-}a is the allele at the Ig^{-} locus in BALB/c. It is important to note that the assignment of Ig^{-}a and Ig^{-}b to BALB/c is not in any way meant to imply that BALB/c IgG2a is structurally more closely related to BALB/c IgA than to IgA molecules determined by other alleles at the Ig^{-} locus.

**Table 12.2. The Ig^{-} Locus** [7]

<table>
<thead>
<tr>
<th>Type strain</th>
<th>Allele</th>
<th>Specificities</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>Ig^{-}a</td>
<td>1(G7)^+ 2(G8)</td>
</tr>
<tr>
<td>C57BL/10J</td>
<td>Ig^{-}a</td>
<td>- - 6 7 8</td>
</tr>
<tr>
<td>DRA/2J</td>
<td>Ig^{-}a</td>
<td>2(G8) 3 - 7</td>
</tr>
<tr>
<td>AKR/J</td>
<td>Ig^{-}a</td>
<td>1(G7) 2(G8) 5 7</td>
</tr>
<tr>
<td>A/J</td>
<td>Ig^{-}a</td>
<td>1(G7) 2(G8) 6 7 8</td>
</tr>
<tr>
<td>CEJ</td>
<td>Ig^{-}a</td>
<td>1(G7) 2(G8) - - - -</td>
</tr>
<tr>
<td>RIUJ</td>
<td>Ig^{-}a</td>
<td>2(G8) 3 - - - -</td>
</tr>
<tr>
<td>SEA/Gn</td>
<td>Ig^{-}a</td>
<td>1(G7) 2(G8) 6 7 10(G1) 12(G6)</td>
</tr>
</tbody>
</table>

* Ig^{-} determines IgG2a immunoglobulin H-chains.
* Porter-Lieberman determinant names given in parentheses [9].

Specificity 12 described only by Potter & Lieberman.

At Ig^{-} to Ig^{-}, although some alleles at each of the loci have been defined, allotypic differences have not, as yet, been recognized between immunoglobulins in all of the type strains. In these cases the recognized alleles are formally named as a composite of the alleles of the undifferentiated type strains, e.g. Ig^{-}a, although in general usage Ig^{-}b is used to describe the allele for IgA of BALB/c origin. This type of notation system was adopted, among other reasons, in the expectation that as the search for new antisera continues, some sera may be found which differentiate between the proteins produced by the different type strains; however, it is quite possible that two type strains which carry different Ig^{-} alleles could have identical Ig^{-} alleles. As yet, no example of the reverse case has been found, i.e. two strains with different alleles at Ig^{-}, Ig^{-} or Ig^{-} having the same Ig^{-}.

In Table 12.6, a catalogue of 70 inbred mouse strains listed according to Ig^{-} allele is presented. Since, in the many cases where testing of inbred strains has been possible, the other Ig alleles have been shown to conform to the Ig^{-} allotype, it is reasonable to assume that if an inbred strain is Ig^{-}a, it is Ig^{-}b etc.

A somewhat looser correlation among inbred strains exists for Ig^{-}a-chain loci and the loci which determine variable region structure. For example, although strains carrying the Ig^{c} chromosome region all appear to carry the Ig^{-} through Ig^{-} alleles, only some carry the entire chromosomal segment haplotype found in the Ig^{c} type strain (BALB/c).

Two major subgroups have been identified within the Ig^{c}-carrying strains: one group, including BALB/c, which carries an allotypic determinant detectable on the Fab fragment of IgG2a and another
group, including C3H, which does not [17]. The restriction of this determinant to the IgG2a immunoglobulins indicates that it is present on the portion of the H-chain found in the Fab fragment, most likely in CH1. (The Ig region H-chain markers, in contrast, all appear to be present on the Fc H-chain fragment, i.e. in CH2 or CH3.) Peptide mapping studies of IgG2a myeloma proteins from BALB/c and C3H confirm a structural difference between the CH1 domains (Fab) in these two strains, but show no difference between CH2 or CH3 domains (Fc) [18].

The subdivision of the Ig* H-chain allotype group of strains is consistent with evidence demonstrating that variable region markers carried by strains carrying the Fab allotypic determinant are different from variable region markers carried by strains in which the Fab determinant is absent [8]. Thus, at least two chromosomes carrying the Ig* region are maintained among inbred mouse strains. Continued identification of new variable region markers may, in time, allow an estimate of how numerous these different chromosomes are.

Allotypic specificities

Each allele at a given locus is actually defined by the unique combination of antigenic specificities found on the immunoglobulin it determines (Tables 12.2-5). These specificities represent the reactions of the immunoglobulins of a given class with a panel of anti-allotype antiserum, and therefore represent structural differences between the immunoglobulin H-chains determined by the alleles at a given locus.

The specificities for each locus are numbered according to the order of their discovery. Therefore, specificity 3 for the Ig-1 locus has no structural relation to specificity 3 for the Ig-2 locus. When specificities at more than one locus are being discussed, the more complete designation, Ig-1-3 or Ig-3-3 should be used to prevent confusion.

Rules for defining specificities

A detailed exposition of how all of the specificities in the Ig system were defined would be too long for this review. However, since this procedure has some general utility, we will present a few illustrative examples in addition to the following set of 'rules' for defining specificities.

1. A strain producing an alloantiserum has none of the specificities recognized by that antiserum; (2) the immunizing strain has all the specificities detectable by that antiserum regardless of the type of antigen used to detect the antibodies included in the antiserum; however, (3) the specificities detected in the immunizing strain by the antiserum made against it are not necessarily all the antigen specificities present in the immunizing strain, i.e. antibodies may not be present to all the specificities.

Several of the Ig specificities have been defined by the ability of anti-allotype sera to precipitate 125I-labelled immunoglobulins, either myeloma proteins or immunoglobulins isolated from normal mouse sera, since this method is often more sensitive than Ouchterlony testing. Most of the other specificities have been defined by studying the nature of the inhibition of precipitation of labelled immunoglobulins when unlabelled (test) immunoglobulins, often in whole serum, from various strains are introduced into the assay. (For methods, see p. 12.19).

In the inhibition assay, if the test (inhibitor) antigen has no specificities in common with the labelled antigen there is no inhibition of precipitation. If, on the other hand, the test antigen is identical to the labelled antigen in its reactions with the antiserum (has all the specificities detected in the

<table>
<thead>
<tr>
<th>Type strains</th>
<th>Alleles</th>
<th>Specificities</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/cJ, SEA/Gn</td>
<td>Ig-2a</td>
<td>2(A12)† 3(A13) 4(A14)</td>
</tr>
<tr>
<td>C3HBL/10J</td>
<td>Ig-2 †</td>
<td>—            —</td>
</tr>
<tr>
<td>DBA/2J, RIII/J</td>
<td>Ig-2a</td>
<td>1            —</td>
</tr>
<tr>
<td>AKR/J, AJ</td>
<td>Ig-2a</td>
<td>—            3(A13) —</td>
</tr>
<tr>
<td>CE/J</td>
<td>Ig-2†</td>
<td>—            —    4(A14)</td>
</tr>
</tbody>
</table>

* Ig-2 determines IgA immunoglobulin H-chains.
† Potter-Lieberman determinant names given in parentheses [9].
 Specificities 3 and 4 have been described only by Potter and Lieberman.
assay), there is complete inhibition of precipitation.
In the case where the test antigen is partially identical
to the labelled antigen (has some of the specificities
detected in the assay, i.e. cross-reacts), there is
partial (incomplete) inhibition of precipitation. When
more than one specificity is detected by an antiserum,
varying the combinations of labelled antigen and
inhibitor antigen is often useful in reducing the num-
ber of possible specificities detected in a given assay.

**Table 12.5. The Ig-4 Locus**

<table>
<thead>
<tr>
<th>Type</th>
<th>Strains</th>
<th>Alleles</th>
<th>Specificities</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>DBA/2J, SEA/Gn</td>
<td>Ig-4&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>AKR/J</td>
<td>A/J</td>
<td>Ig-4 &lt;sup&gt;9&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>C57BL/10J</td>
<td>A/J</td>
<td>Ig-4 &lt;sup&gt;9&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>111/J</td>
<td>C57BL/10J</td>
<td>Ig-4*</td>
<td>2</td>
</tr>
</tbody>
</table>

* The Ig-4 locus determines IgG1 immunoglobulin H chains.

Continuing with the ‘rules’ then: (4) the specifici-
ties detected by an antiserum in reactions with a
labelled antigen, from a strain other than the im-
munizing strain, are those specificities detected by
the antiserum which are present in both the im-
munizing and labelled antigen strain; (5) a test
serum which completely inhibits has all the specifici-
ties detected by the antiserum in that assay, while a
test serum which partially inhibits a reaction has
some but not all of the specificities detected in that
assay; (6) two strains, which each partially inhibit
in a given reaction, need not share any specificities
with each other, but each shares some specificities
with the labelled antigen strain and the immunizing
strain; and (7) the number of specificities is always a
minimum estimate of the number compatible with
the results.

In the definition of specificities, the following
notation is used [7]: An <sup>125</sup>I-labelled preparation of
γ-globulin is indicated with an asterisk following the
symbol of the strain from which it was prepared
(for example, C3H*), while normal sera used in
inhibition assays are listed by the strain symbols
(e.g. C3H). The symbols C3H*<sup>+</sup>-C57BL anti-C3H
refer to the use of a labelled C3H γ-globulin prepara-
tion with a C57BL anti-C3H antiserum in an
inhibition assay. The expression ‘C3H I’ means
C3H has specificity one. The expression ‘C57BL-I’
means C57BL does not have specificity one.

Several Ig-1 specificities are here defined:
(1) C3H*<sup>+</sup>-C57BL anti-C3H precipitation is not
completely inhibited by DBA. C3H therefore has at
least one specificity not present in DBA (C3H 1;
C57BL-1; DBA-1).
(2) DBA<sup>+</sup>-C57BL anti-C3H precipitation is com-
pletely inhibited by AKR. Therefore C3H, DBA,
and AKR share at least one specificity, 2 (C3H 2;
C57BL-2; DBA 2; AKR 2).
(3) AKR<sup>+</sup>-C57BL anti-C3H precipitation is not
completely inhibited by DBA. Therefore AKR and
C3H must share at least one specificity that is not
present in DBA (AKR 1).
(4) C3H<sup>+</sup>-C57BL anti-C3H precipitation is not
completely inhibited by AKR. C3H therefore has
at least one specificity not present in AKR (C3H 6;
C57BL-6; AKR-6).
(5) Since C57BL anti-C3H recognized specificity 6,
statement (2) proves that DBA does not have 6
(DBA-6).
(6) DBA<sup>+</sup>-C57BL anti-DBA precipitation is not
completely inhibited by C3H or AKR. Therefore
DBA has at least one specificity not present in
either of these two strains (C3H-3; C57BL-3; DBA 3; AKR-3).
(7) C3H anti-C57BL precipitates C57BL*, but not C3H*, DBA*, AKR*, or AJ*. C57BL therefore has at least one specificity not present in the latter four strains (C3H-4; C57BL4; DBA-4; AKR-4; AJ-4).
(8) C3H* C57BL anti-C3H precipitation is completely inhibited by A/J. Therefore A/J has all specificities previously assigned to C3H (A/J 1, 2 and 6).

Recognition of allotypes by electrophoretic mobility differences
As mentioned earlier, although it has been the custom with immunoglobulins to resort to serology for characterization, allotypic differences need not be recognized only by serologic methods. For a long time, no methods were found which elicited alloantisera reacting with IgG1 (Ig-4) globulins. Faced with this problem, we turned to another classical criterion for demonstrating polymorphism at loci which determine protein structure electrophoretic mobility, and were able to define two alleles at the Ig-4 locus, one in BALB/c and the other in C57BL/6 [18].

For these studies rabbit antiserum for the IgG1 class was raised and rendered specific by absorption with immunoglobulins of the other classes. This antiserum was used to develop immunodiffusion patterns following electrophoretic separation in agar gels of IgG1 Fe fragments obtained by papain digestion.
All of the type strains and several strains from each allele group were then tested for electrophoretic mobility differences in IgG1. Only two mobility types were seen. Thus, the Ig-4 locus was defined and two alleles, Ig-4a and Ig-4b, designated.
Recent innovations in techniques of immunization have now made it possible to elicit good alloantisera reacting with IgG1 globulins. Nonetheless, only the two originally defined alleles at the Ig-4 locus have been recognized by these sera (see Table 12.6).

Recognition of allotypes by fluorescent staining with anti-IgG1 antisera
Since IgD and IgM are present on nearly all adult B cells, allotypes in these classes are identifiable by immunofluorescent staining of B cell or spleen cell populations [5]. Using fluorescence-activated cell sorter (FACS) analysis, we have shown that anti-bodies to IgM (Ig-6) or IgD (Ig-5) allotypes bind specifically to these allotypes on B cell membranes [20]. (Antiallotype sera were incubated with the cells and the antibody bound then measured by a specific fluorescent 'second step' reagent.) Presence of anti-Ig-5 and anti-Ig-6 antibody in the sera was confirmed by immunoprecipitation and SDS polyacrylamide gel electrophoresis (SDS-PAGE) studies [20].

IgG allotypes on B cells are also identifiable by immunofluorescent staining and FACS; however, because there are so few IgG-bearing cells in spleen, FACS separation and functional testing is required to unequivocally demonstrate the allotype presence of B cell membranes [21].

Specialized laboratory methods
The methods used for the study of mouse immunoglobulin allotypes are essentially the same as those used in other immunogenetic studies and described elsewhere in this volume. We will present here only those special modifications necessitated generally by the comparatively small volumes of reagents and test sera available from mice.

Production of antisera
Rabbit antisera
Rabbit antisera are made against mouse whole sera, partially purified normal immunoglobulins, and purified myeloma proteins and fragments (papain digestion products, i.e. Fe and Fab pieces) of immunoglobulins. Two injections, each of approximately 100 µg of purified protein (50 µg in the case of the papain fragments) or 100 µl of whole serum, evoke antisera which give strong precipitin arcs in immunoelectrophoresis.
The first injection of the antigen emulsified in complete Freund's adjuvant made with oil to water, 3:1 is given in the foot pads and in several subcutaneous sites using about 0.1 ml/site. After approximately 4 weeks, a second injection of the antigen, without adjuvants, is given intramuscularly. Starting at 1 week after the second injection and continuing weekly thereafter for several weeks, 30-60 ml of blood are drawn from the heart.
Preparation of antisera specific for a given immunoglobulin class often requires absorption of the antisera as well as careful isolation of the immunizing protein or fragment. Methods for absorption are presented in a later section.
<table>
<thead>
<tr>
<th>Ig-1*</th>
<th>Ig-1*</th>
<th>Ig-1*</th>
<th>Ig-1*</th>
<th>Ig-1*</th>
<th>Ig-1*</th>
<th>Ig-1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c*</td>
<td>C57/J</td>
<td>PL/J</td>
<td>C57BL/10J*</td>
<td>DBA/2J</td>
<td>AKR/J</td>
<td>C57BL/6J*</td>
</tr>
<tr>
<td>BALB/C</td>
<td>F/ Ao</td>
<td>POLY1/Ao</td>
<td>BAB/PoHz</td>
<td>DBA/2J</td>
<td>AL/N</td>
<td>NZB/B1</td>
</tr>
<tr>
<td>BUB/Bn</td>
<td>H-2a/Go</td>
<td>POLY2/Ao</td>
<td>B10.D2newHz</td>
<td>DBA/2J</td>
<td>AL/N</td>
<td>NZB/B1</td>
</tr>
<tr>
<td>CBA/J</td>
<td>J/K/Bi</td>
<td>PRUNT/Ao</td>
<td>B10.D2oldHz</td>
<td>DBA/2J</td>
<td>AL/N</td>
<td>NZB/B1</td>
</tr>
<tr>
<td>CH/J</td>
<td>MA/H</td>
<td>ST/J</td>
<td>C57BL/H</td>
<td>SM/J</td>
<td>RF/J</td>
<td>C57BL/H</td>
</tr>
<tr>
<td>C3H/He</td>
<td>MA/MyJ</td>
<td>STR/N</td>
<td>C57Bl/He</td>
<td>STA/Jc</td>
<td>SWR/J</td>
<td>C57Bl/He</td>
</tr>
<tr>
<td>C3H/SW/He</td>
<td>NZT/B1</td>
<td>T6/H</td>
<td>C57BL/Ka</td>
<td>WB/Re</td>
<td>WB/Re</td>
<td>C57BL/Ka</td>
</tr>
<tr>
<td>C57BL/cdJ</td>
<td>PBR/Ao</td>
<td>129/Brga</td>
<td>C57BL/cd</td>
<td>WB/Re</td>
<td>WB/Re</td>
<td>C57BL/cd</td>
</tr>
<tr>
<td>C57L/J</td>
<td>PBR/Ao</td>
<td>129/Brga</td>
<td>C57BL/cd</td>
<td>WB/Re</td>
<td>WB/Re</td>
<td>C57BL/cd</td>
</tr>
<tr>
<td>SJL/J</td>
<td>PBR/Ao</td>
<td>129/Brga</td>
<td>C57BL/cd</td>
<td>WB/Re</td>
<td>WB/Re</td>
<td>C57BL/cd</td>
</tr>
</tbody>
</table>

* Type strain for each allele.
† C3H and C3H-SW form a congenic trio C3H = H2*, Igα; C3H-SW = H2*, Igα; and C3H-B = H2?, Igα.
‡ BAB is congenic with BALB/c: BALB/c = Igα; BAB = Igα. BAB are derived by further backcrossing from BALB/c-2, received from Dr M. Potter at the 13th backcross generation. Congenic strains are almost identical genetically except at the indicated loci.
Mouse antiallotype sera: IgG and IgA
Although normal serum or normal γ-globulin can be used as antigen to stimulate anti-allotype antibody, far better results have been obtained by using, as the antigen, sera or immunoglobulins from animals which contain antibodies directed to some tissue or protein component in the recipient strain. For example, whereas BALB/c mice immunized with normal (C57BL/6) serum failed to produce any detectable anti Ig-1b, immunization of the same strain with C57BL/6 anti DBA/2-spleen-antisemur (which has a high anti H-2d titre) was quite successful (BALB/c and DBA/2 are both H-2d). Similarly, an antisemur to a complement-component antigen, Hve (22), was also effective in stimulating anti-allotype serum. In many cases the first antigen injection was in complete Freund's adjuvant but boosts were aequous.

In our hands this method of immunization is particularly effective for raising antibody reactive with Ig-1 (IgG2a) allotypes, but only marginally useful for raising antibody to allotypes on other classes. With the exception of the immunization of C57BL/6 with DBA/2, where antisemurs often react well with Ig-2 (IgA) as well as Ig-1, most immunizations by this method result in low titres in occasional animals for all antigens other than the Ig-1 (IgG2a) allotypes.

More effective immunization for other classes has been obtained by challenging the animal with immunoglobulins in the form of an externally created antigen-antibody complex. Recently, we have used a method established by Dresser, Taylor & Wortis (personal communication) in which Bordetella pertussis vaccine is used to immunize mice from one strain, these mice are bled, and their sera incubated in vitro with B. pertussis to allow complex formation. The whole mixture is then injected into a second mouse strain of a different allotype to generate an antiallotype response. This method has proved excellent for obtaining antibody reactive with Ig-4 (IgG1) allotypes as well as antibodies reactive with Ig-1 (IgG2a) allotypes. To a lesser extent, it has been useful for producing antibody to Ig-3 (IgG2b) allotypes. Strangely, we found no antibody produced reactive with Ig-2 (IgA) allotypes.

The inability to obtain antibody to Ig-2 with this method is most likely due to a poor IgA response to the B. pertussis, resulting in very little IgA being available for immunization in the antigen-antibody complex. By administering the B. pertussis par ora (putting the killed organisms in the drinking water) Dr Tohru Masuda, in our laboratory, has succeeded in producing an anti-pertussis serum demonstrably rich in IgA antibody which, when combined with B. pertussis and administered as described above, elicits excellent antibodies to Ig-2 allotypes in some cases with little or no contamination with antibodies to allotypes of other classes.

Not only, however, is it important to choose an appropriate protocol for preparing the immunogen to elicit antibody to a particular allotype, but the choice of recipient mouse strain can considerably influence the success of the antiallotype immunization. As many investigators involved in preparation of antiallotype sera, notably Dr Rose Lieberman and Dr Michael Potter, have pointed out, it is not uncommon for two different mouse strains carrying the same allotype to respond quite differently when presented with the same antigen-antibody complex in an identical protocol, suggesting that there may be genetic factors controlling the immune response to allotypic antigens.

Mouse antiallotype antisera: IgM and IgD
Recently, Goding, Warr and Warner observed that some C57BL/6 (Ig*, H-2d) alloantiseras prepared against CBA (Ig*, H-2k) spleen cells contained antibody reactive with IgD allotypic determinants (5). This immunization procedure has now been shown to be effective for producing antibody to both IgM (Ig-6) and IgD (Ig-5) allotypes in several strain combinations (6). Four weekly injections of 10⁷ spleen cells are generally sufficient to obtain the anti-allotype antibodies, but effective immunization appears to require donor and recipient to differ for H-2 as well as Ig haplotype. Thus, antisera prepared in this manner contain antibody reactive with a variety of cell surface determinants controlled by loci (e.g. H-2, Ia) in addition to antibody reactive with IgD and IgM.

Antibody to IgM (Ig-6) allotypes is easily isolated from the antisera by affinity chromatography using serum or myeloma IgM coupled to Sepharose. No method, however, has as yet been established for isolating antibody to IgD (Ig-5) from the antibodies reactive with other cell surface constituents. IgD is found on B cell membranes. It is not present in mouse serum. No IgD myelomas have been found in mouse. Therefore, no source of IgD for preparing an affinity chromatography column is currently available.

Removal of the contaminating antibody to cell
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Protocol No.</th>
<th>Dose and timing</th>
<th>Bleed</th>
<th>Dose and timing</th>
<th>Bleed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. pertussis</em> killed</td>
<td>I</td>
<td>Day 1: Total of $2 \times 10^9$ bacteria in 0.2 ml saline injected i.p. and in 4 places s.c. Days 3 and 5: same but in 4 places s.c. Days 21 and 37: same as day 1</td>
<td>Pool bleed</td>
<td>Days 1, 3, 5, 22, 26, 50, 57: $10^9$ bacteria + 20 mlitre antiserum from Step 1 in 0.2 ml saline per mouse. Mix at room temperature and inject i.p. Boost as on day 1</td>
<td>Day 64 and weekly. Bleed individually and test, or pool bleed depending on circumstances. Boost when titres go down. 10 days after boost, bleed and test.</td>
</tr>
<tr>
<td><em>B. pertussis</em> heat killed</td>
<td>II</td>
<td>Mice supplied with drinking water containing $2 \times 10^8$ bacteria/500 ml for 30 days (5 mice per cage)</td>
<td>Day 30: bleed. Check titre by bacterial agglutination. Check class of antibody. Pool bleed weekly.</td>
<td>Days 1, 3, 5, 21, 37 and monthly booster. Incubate serum from Step 1 with number of bacteria just sufficient to completely absorb anti-pertussis activity, 3 hours at 37°C and overnight in cold. Wash 3X with cold saline. Resuspend in saline to $2 \times 10^4$/ml. Mix with equal volume of CFA. Inject 0.2 ml</td>
<td>Day 45 and weekly, test individually</td>
</tr>
<tr>
<td>H-2</td>
<td>III</td>
<td>1/5 spleen/animal in isotonic MEM injected i.p. Boost with same monthly bleed</td>
<td>Bleed 3 weeks after injection, then weekly</td>
<td>Day 1: 20 mlitre antiserum from Step 1 + 20 mlitre CFA injected i.p. and 4 places s.c. Days 21, 28 and monthly booster: 10 mlitres in 0.2 ml saline i.p.</td>
<td>Day 35 bleed and test. Pool bleed all positive animals weekly</td>
</tr>
<tr>
<td>IV1A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Days 1, 8, 15, 22: $10^7$ spleen cells from strain differing for Ig allotype and H-2; boost monthly thereafter</td>
<td>Day 29 and weekly thereafter</td>
</tr>
</tbody>
</table>

* Protocol established by Dr Tohru Masuda, this laboratory.
† Complete Freund's adjuvant.
‡ Protocol established by Goding, Warr and Warner [3].
§ Incubate antiserum plus *B. pertussis* wash. Test conjugates in radioimmune assay. Use sera whose complexes show high levels of IgA and low levels of other immunoglobulins.
¶ Kindly supplied by Lederle Laboratories.
<table>
<thead>
<tr>
<th>Allotype serum</th>
<th>Antiallotype producing strain</th>
<th>Allotype donor strain</th>
<th>Protocol No.*</th>
<th>Antigen for alllotype donor</th>
<th>Reaction with</th>
<th>Ig-1</th>
<th>Ig-2</th>
<th>Ig-3</th>
<th>Ig-4</th>
<th>Ig-5</th>
<th>Ig-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>b anti-a</td>
<td>(LF/1 or C57BL/10)</td>
<td>BALB or C3H.SW</td>
<td>I</td>
<td>B. pertussis</td>
<td>+ + + †</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b anti-a</td>
<td>SJL/J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e anti-a</td>
<td>NZB</td>
<td>BALB</td>
<td>II</td>
<td>B. pertussis</td>
<td>+ + + o.w.</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>d anti-a</td>
<td>AKR</td>
<td></td>
<td></td>
<td></td>
<td>- + + o.w.</td>
<td>+</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>b anti-a</td>
<td>C57BL/10</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>b anti-a</td>
<td>C57BL/10</td>
<td>BALB</td>
<td>III</td>
<td>H-2</td>
<td>+ + + o.w.</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b anti-a</td>
<td>LP/J</td>
<td></td>
<td></td>
<td></td>
<td>+ + + o.w.</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a anti-b</td>
<td>BALB/c</td>
<td>(C57/BL/10 or LP/J)</td>
<td>I</td>
<td>B. pertussis</td>
<td>+ + + o.w.</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a anti-b</td>
<td>C3H.SW</td>
<td>BALB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a anti-b</td>
<td>C57BL/10</td>
<td>DBA/2</td>
<td>III</td>
<td>H-2</td>
<td>+ + + o.w.</td>
<td>+</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>d anti-c</td>
<td>C3H</td>
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<tr>
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<td>LP</td>
<td>AKR</td>
<td>III</td>
<td>H-2</td>
<td>+ +</td>
<td>+</td>
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<tr>
<td>c anti-d</td>
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<td>DBA/2</td>
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<td></td>
</tr>
<tr>
<td>a anti-e</td>
<td>NZB</td>
<td>A/J</td>
<td>III</td>
<td>H-2</td>
<td>+ + o.w.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b anti-e</td>
<td>C57BL/10</td>
<td>BALB/c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>a anti-b</td>
<td>SJL</td>
<td>BAB/20</td>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b anti-a</td>
<td>SJL</td>
<td>BALB/c</td>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* See Table 12.7.
† Antibody activity was surveyed either by Ouchterlony analysis or **121** precipitation. Those reactions scored + or + + are often difficult to detect in Ouchterlony tests.
‡ The SJL congenic strains are H-2'; the BALB/c congenic strains are H-2'.
o.w.: occasional animals producing weak antibody reactions.
Blank spaces indicate no testing data because of unavailability of isolated antigens, but may be presumed negative or weak positive since no unaccountable lines appeared in Ouchterlony testing against normal sera with these antisera.
No antibody to Ig-5 or Ig-6 allotypes has been found with protocols I-III.
surface determinants by in vivo or in vitro absorption with tissue from congenic mice carrying the donor surface antigens but not the donor IgD allotype should in theory be possible. In practice, however, such absorption is not feasible because the anti IgD titre is low compared to the titre of contaminating antibodies. Thus, until a satisfactory method is developed for isolating the anti IgD antibody population, the use of these sera is essentially restricted to use under conditions where the contaminating antibodies do not interfere with detection of IgD. This means that the reagents can be used only with IgD-bearing cells which do not carry surface determinants detected by the contaminating antibody.

The strategy adopted to overcome this problem is to prepare the antisera by immunizing one member of an allotype congenic pair with spleen cells from an unrelated strain carrying the same allotype as the other congenic partner. The antibody is then used with cells from the latter’s congenic partner. Thus, using the congenic pair SJL (IgD) and SJA (IgD), SJL mice are immunized with BALB/c (Igα) spleen cells (which have the same allotypes as SJA) and the antibody tested on SJ/A cells. In this way, the SJL antibody produced against BALB/c cell surface antigens other than those determined in the Ig chromosomal region is irrelevant because the test strain (SJA), carries the same surface determinants as the antibody producer (SJL) except for Ig region determinants such as IgD. This same strategy can be used for antibody to IgM allotypic determinants if isolation of the antibody is impractical.

From the foregoing, it is clear that the preparation of allotype reagents is at present an art bordering on a science. As a guideline for the novice venturing into this field, some of the experience in our laboratory is summarized in Tables 12.7 and 12.8. These observations are not the result of exhaustive testing and should not be considered as definitive. Minor changes in dosage or timing do not appear to be critical. A great deal of additional variable information on techniques of immunization may be found in the literature, especially in the work of Drs Lieberman and Potter [9] e.g. one of Lieberman’s immunization techniques is described below in the section entitled ‘Haemagglutination inhibition using myeloma protein coupled indicator cells’.

Absorption of antisera: isolation of specific antibody from multispecific antisera using Sepharose conjugated immunoadsorbents

Recently it has become common to absorb antisera with antigens rendered insoluble by covalent binding to an insoluble support such as Sepharose. Absorption by this method is excellent, ensuring that the absorbed sera do not contain either the soluble antigen-antibody complexes or residual soluble antigen frequently found when sera are absorbed with soluble antigen. In addition, the antibody removed by the insoluble absorbing antigen may be recovered by elution, relatively free of contamination with irrelevant immunoglobulins. We have found the Sepharose-cyanogen bromide method [23] to be particularly useful for preparing mouse antigen or antibody immunoadsorbents.

The data in Table 12.9 illustrate the isolation of two allotypotype antibodies using antigen-Sepharose column adsorptions and elutions. In the first step, passage through a Sepharose Ig-1a column removed virtually all of the anti Ig-1a antibody from a complex antisera containing anti Ig-1a and anti Ig-4a while nearly all of the anti Ig-4a activity was recovered at the column front. The bound anti Ig-4a activity was then eluted with 0.1 M acetic acid. Finally a small amount of contaminating (probably non-specifically trapped) anti Ig-4a activity found in the eluate was removed by passing the eluate through a Sepharose Ig-4a column. Recoveries, calculated in antigen binding units, were excellent, running from at least 50 per cent to as good as 90 per cent. The resolved components were each specific for the appropriate antigen within the limits of the assay.

For the separation described above, the antisera used was an IgG anti IgA, prepared by immunizing LP mice with BALB/c anti-pertussis conjugated to pertussis organisms p. 10.11. The serum was loaded onto a Sepharose-Ig-1a column* PBS (0.01 mol/l phosphate, pH 7.1, i = 0.15 mol/l NaCl) was then passed through the column and fractions collected until no more 280 nm absorbing material came off.† The initial fractions collected accounted for 90 per cent of the anti Ig-4a activity in the original serum, while the anti Ig-1a activity recovered (0.2 per cent of original anti Ig-1a) bordered on the limits of detectability. Thus, in one pass the column specifically

* Before use, the column was washed with 0.1 mol/l acetic acid to remove any extraneous protein, then PBS to return to neutrality. Then, to block non-specific adsorption, 2 ml of LP normal serum was run through, followed by PBS washing until no more 280 nm absorbing material appeared in the effluent.
† Recent experience indicates that Clq helps to stabilize the antigen-antibody complex on the column. Therefore 0.05 mol/l Tris is probably a better buffer for washing (p. 12.19).
**Table 12.9. Recoveries of anti Ig-1a and anti Ig-4a activity.**

<table>
<thead>
<tr>
<th>Fraction Description</th>
<th>Anti Ig-1a</th>
<th>Anti Ig-4a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units/ml*</td>
<td>Total units</td>
</tr>
<tr>
<td>Original serum Ig* anti Ig* . . . (‘A’)</td>
<td>12.5</td>
<td>130</td>
</tr>
<tr>
<td>Passed ‘A’ through Ig-1a Sepharose†</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Column eluted with 0.1 M acetic acid</td>
<td>6.3</td>
<td>66</td>
</tr>
<tr>
<td>Activity bound to Ig-1a Sepharose . . . (‘B’)</td>
<td>3.2</td>
<td>67</td>
</tr>
<tr>
<td>Passed ‘B’ through Ig-4a Sepharose‡</td>
<td>0.7</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* 1 unit of antibody = that volume able to precipitate two-thirds of maximum precipitable ^125I antigen precipitated by the original serum $\times 10^{-5}$.
† 5 ml column bed, with total of 10 mg bound RPC-5 (Ig-1a myeloma protein).
‡ 0.5 ml column bed, with total of 1 mg bound S-8 (Ig-4a myeloma protein).
removed (adsorbed) essentially all the anti Ig-1a activity from the serum.

To recover the bound anti Ig-1a, 0.1 mol/l acetic acid was passed through the column. A protein peak moved at the acid front. 2.5 ml fractions were collected into tubes containing 2.5 ml 2X PBS in 3 per cent BSA, which immediately brought the pH of the eluted material near neutrality. The pooled material collected under the protein peak accounted for 51 per cent of the original anti Ig-1a activity. In addition, a small amount (5 per cent of original) of anti Ig-4a activity appeared which was either trapped non-specifically or able to react with Ig-1a as well.

To remove this small amount of contaminating anti Ig-4a activity from the anti Ig-1a pool, the pool was concentrated and then passed through a smaller Sepharose Ig-4a column. As above, fractions were collected, the column washed and the bound material eluted. All of the anti Ig-1a (51 per cent) passed through the column, while no detectable anti Ig-4a passed through, yielding a good supply of antibody specific for Ig-1a.

The material eluted from the Ig-4a Sepharose column contained the anti Ig-4a activity which either had specifically bound to the Ig-1a in the first column or was trapped non-specifically in that column, plus about 2 per cent of the anti Ig-1a which in this case was either specifically bound to the Ig-4a protein or non-specifically trapped. Inhibition tests with the anti Ig-4a and anti Ig-1a activity in the eluate indicated the presence of two distinct antibody populations each specific for its own antigen. Thus, about 2 per cent of the antibody passed through these Sepharose-immunoglobulin columns is non-specifically trapped.

Assays for detection of anti-class and antiallotype antibodies and measurement of immunoglobulin class and allotype levels

Several types of assays are described here for the detection of antibodies to class and allotypic determinants on mouse immunoglobulins. By standardizing the antibody concentration, each of these assays can be used for quantitative estimation of immunoglobulin levels; however, each assay has characteristics which make it more suitable for particular purposes. For example, Ouchterlony analysis is rapid, semi-quantitative and relatively insensitive, while the radioimmune assay is more laborious but also considerably more sensitive and accurate. Perhaps the most versatile assay of those presented is the solid-phase radioimmune assay, which can be used to measure allotypic and class contributions to an overall immune response in addition to being suitable for measuring total antibody response to a variety of antigens (including allotypes) and for measuring allotype and class levels in sera.

Ouchterlony analysis (double diffusion)
Miniaturized hexagonal patterns, with holes separated 5 mm from their centres are cut with a flattened 18 gauge hypodermic needle from a 6 ml bed of agar (1 per cent ionagar in 0.05 mol/l Barbital, pH 8.2) on a 2 in x 3 in microscope slide. After development of the precipitation lines (3-6 hours at room temperature) slides are immersed briefly in water to replace the air in the wells with liquid, a piece of Whatman #1 filter paper is slid over the agar to make firm contact, and the covered slide is removed from the water, blotted and dried in a warm (not hot) air-stream. When slides are dry, the paper comes off easily, leaving an agar film on the glass with the precipitate firmly imbedded in the film. Excess soluble protein is removed by soaking the slide overnight in 0.5 per cent NaHCO₃ and the precipitation lines permanently stained with 1 per cent Buffalo black in water-methanol-glacial acetic acid (5:5:2) (1 min in stain solution and approximately 5 min in 5 per cent acetic acid to destain). If patterns are carefully cut so that the peripheral wells are equidistant from the centre, and if the wells are quantitatively loaded, Ouchterlony analysis can be used as a reliable semi-quantitative estimate of Ig levels.

Immunoelectrophoresis (IEP)
Slides for IEP are prepared as for Ouchterlony plates except that the slide is cleaned with ethanol and then dipped in 0.1 per cent agar in water and allowed to dry before the agar bed is laid onto the slide. This pretreatment prevents slippage of the agar when troughs are cut.

Electrophoresis is carried out for 1 hour at 5 V/cm, then the precut troughs are cleared of agar, filled with serum, the slide allowed to develop (3-6 hours at room temperature), and stained as above.

Passive haemagglutination
Erythrocytes coated with either non-agglutinating ('incomplete') or sub-agglutinating levels of antibody are agglutinated by antiallotype antisera of the appropriate specificity, using either mouse anti-sheep erythrocyte antibody and sheep erythrocytes
or antibody raised in one mouse strain to the histocompatibility-2 (H-2) antigen of a second. RBC are coated by incubation with antibody at a non-agglutinating dilution and then washed several times to remove the accompanying non-antibody immunoglobulin which, because it too carries the allotype, would otherwise inhibit the reaction. After washing, coated cells are incubated with dilutions of anti-allotype antibody in microagglutination trays and read in standard fashion.

While this method works quite well, we find we seldom use it, partly because the washing is cumbersome if many samples of anti-SRBC are to be tested and partly because the agglutination could be due to reaction of the antiallotype serum with any or all classes of antibody represented in the anti-SRBC, or even due to reaction with components of complement or other factors which adhere to the erythrocytes.

**Haemagglutination inhibition using myeloma protein coupled indicator cells**

This method, modified from the techniques of Dr. Rose Lieberman, [24] is quite simple and is nearly as sensitive as radioimmune assays. For some uses, in particular for screening large numbers of backcross mice for allotype segregation where only a qualitative plus or minus result for each mouse is needed, this method is ideal for speed and convenience. It is less useful for careful quantitation of immunoglobulin concentrations.

In this assay, sheep red blood cells coupled with a mouse myeloma protein are agglutinated by anti-allotype sera. This agglutination can be inhibited by the addition of competing soluble immunoglobulin of the same allotype. Thus, agglutination in a test means the absence of inhibition and the lack of the particular allotype in the added sample. A button or negative result is due to the inhibition of agglutination caused by allotype positive material in the sample.

A purified mouse myeloma protein is covalently coupled to sheep red cells with glutaraldehyde by the method of Evans et al. [25]. 0.5 ml of packed sheep red cells (approximately 2.5 ml of sheep blood preserved in Alsever's solution) is washed three times in isotonic saline and the red cells resuspended to 8 ml in saline. Three mg of purified myeloma protein is added to the cells in 1 ml of saline or PBS. Then the cells are continually stirred while 1-3 ml of saline containing 0.1 ml of 25 percent glutaraldehyde is added over a 15 min period. A peristaltic pump provides a conveniently slow and steady addition of the glutaraldehyde. The cells are mixed for another 45 min and are then washed three times in saline and resuspended in 100 ml of 0.1 percent BSA in PBS/1E (0.01 m phosphate buffered, 0.15 mol/l saline, mmol sodium azide, mmol EDTA, pH 7.2). These cell preparations are stable at 4°C for a year or more. The cells are tested without antibody to see if they settle cleanly in negative buttons. The BSA concentration may have to be raised to 1 percent to give clear buttons.

When appropriate myeloma proteins are available antiallotype sera are raised by the immunization procedure of Lieberman [24] because of its speed and the high titre of antisera produced. 0.5 ml of a 0.3 mg/ml solution of a myeloma protein is emulsified with 0.5 ml of complete Freund's adjuvant (CFA) and injected into the hind foot pads and subcutaneously in four axial and inguinal sites. This is repeated 3 to 4 days later in incomplete Freund's adjuvant (IFA). Two more injections of the protein alone are given intraperitoneally at 3 to 4 day intervals. The mice are bled a week after the fourth injection. If needed weekly boosts are given (0.5 ml, 0.15 mg, Lp.). Positive mice are bled twice weekly. Large amounts of antibody-containing ascites fluid can be obtained after 4 or more weekly i.p. injections of 0.2 ml CFA [26]. The success of this type of immunization schedule is highly dependent on the mouse strain being immunized as this response is under strong Ir gene control. Lieberman [27] lists many strain combinations; to these can be added the findings that good responses to BALB/c IgG2b (lg3') occur in A/He mice, to NZB IgG2b (lg3') in BALB/c and to NZB IgA (lg2') in C58 but not BALB/c. It must also be realized that this immunization results in a high level of anti-idiotypic in the response. For this reason different myeloma proteins of the same class and allotype must be used for the immunization and for coupling to the sheep red cells so that agglutination will be due solely to antiallotype activity.

The haemagglutination assay is done in polystyrene Microtiter V bottom plates using 0.1 percent BSA in PBS to as the antigen diluent. First the antiallotype serum is titrated alone to determine its endpoint. A dilution of antiallotype corresponding to 1-2 haemagglutinating units (i.e. the end point or twice that concentration) is chosen for the inhibition
Mouse immunoglobulin allotypes 12.17

assay. This is often a dilution of one part of antiserum in several thousand of 0.1 per cent BSA. Test sera or other immunoglobulin preparations may then be titrated in the diluted antibody. The endpoint of inhibition is proportional to allotype concentration which can be estimated from the inhibition given by purified protein standards. However, to determine the presence or absence of a segregating allotype in hundreds of backcross mice, the assay is used as a simple spot test. One μl samples of each of the test sera are added to 50 μl drops of the appropriate antiallotype dilution in Microtiter wells. Twenty-five μl drops of the 0.5 per cent v/v indicator cells are then added, and the well contents mixed by agitating the plate. The plates are covered and set aside at room temperature for 3 hours to overnight and then scored. Positive agglutination means the serum does not contain the tested allotype. Inhibition of agglutination indicates the presence of the allotype. Ambiguous settling patterns ('dirty buttons') can usually be resolved by tilting the plate vertically for several minutes and examining the 'run down' pattern. Equivocal results can also be checked by retesting with 5 μl of test serum.

Immunofluorescent staining
Before staining of spleen cell suspensions or T-depleted spleen cell suspensions (B cells), erythrocytes are lysed by incubating the cells for 2 min at 4°C in balanced salt solution in which the NaCl is replaced with an equimolar concentration of NH₄Cl. Cells are stained by two-step staining. Spleen cells (10 x 10⁶) are centrifuged in conical tubes and re-suspended in 0.1 ml of anti-mouse antisera. After 30 min incubation at 4°C the cells are underlayered with heat-inactivated fetal calf serum (FCS) and centrifuged. Cells are then washed once with Dulbecco's phosphate buffered saline (PBS) with 5 per cent FCS.

Cells are resuspended in 0.1 ml fluorescein-conjugated goat anti-rabbit IgG ('goat anti-rabbit IgG) or rabbit anti-goat IgG ('rabbit anti-goat Ig'). After 30 min incubation at 4°C the cells are underlayered with FCS and washed with Dulbecco's PBS with 5 per cent FCS.

Radioimmune tube immunoprecipitation assay: detection of antiallotype antibody
For the last several years, the workhorse assay in our laboratory for the detection of antiallotype antibody has been the precipitation of isolated immunoglobulins isotopeally traced labelled with 125I. This same assay, with an added step in which competitive unlabelled immunoglobulins are introduced to 'inhibit' precipitation of the labelled antigen has proved quite sensitive and reliable for the quantitative estimation of immunoglobulin levels in serum or culture fluids, as well as for the recognition and characterization of allotypic determinants on various immunoglobulins.

Isolation of immunoglobulins for use as labelled antigens. Because myeloma tumours produce a large amount of a single immunoglobulin, frequently with the concurrent lowering of all other serum immunoglobulins, sera or ascites fluid from myeloma-bearing mice provide ideal starting material for the isolation of a highly homogeneous immunoglobulin with little contamination from other immunoglobulins. Therefore, whenever possible, i.e. when ever there is a passagable myeloma tumour producing a protein carrying the required antigen, myeloma proteins are isolated for use as labelled antigens. In cases where no myeloma protein is available, a fraction of the normal serum immunoglobulins enriched for the particular class required is used.

Proteins are usually isolated using standard methods of column chromatography frequently with separation on DEAE-cellulose. We find it occasionally useful to separate small amounts of protein by electrophoresis in agar on a 2 in x 3 in (IEP) microscope slide. To extract the protein, the agar is cut into slices, placed in centrifuge tubes with 0.5 ml of buffer and spun at 50 000 rev/min for 30 min, which breaks the agar emulsion and frees the protein into the supernatant. This method, however, sometimes introduces contaminants which interfere somewhat with 125I labelling.

Induction with 125I. Indination of approximately 100 μg portions of γ-globulin is performed by the chloramine-T method [28] (see Chapter 14) with an average labelling of less than 1 atom 125I/molecule. Passage through anion exchange resin columns followed by dialysis against saline is used to remove unbound iodide. Approximately 5–30 per cent of the added iodine becomes covalently bound to the protein, and is therefore TCA precipitable. With some preparations, 10–15 per cent of the radioactivity was not easily removed by dialysis but was nevertheless not precipitable by TCA. In these cases, precipitation of the protein by 50 per cent saturated ammonium sulphate (0–10° C) was an effective means of
separating the protein from the non-precipitable radioactivity.

Measurement of antiallotype antibody. In addition to providing a quantitative measure of antiallotypic activity, precipitation of $^{125}$I labelled antigen appears to be consistently far more sensitive for antiallotype detection than immunodiffusion. Many sera that do not show any precipitation in agar precipitate the labelled antigen, even with antisera diluted of 1:1000. This is especially true for antibody to Ig-4 allotypes in our hands. Therefore, in searching for new antisera, as well as in titrating antisera and testing for contaminating antibody in absorptions, sera are routinely tested by this method.

For the assay, 50 μl of labelled antigen in 3 per cent BSA-tris (i.e. 0.05 mol/l tris buffer, final pH 7.6, containing 3 per cent bovine serum albumin (BSA), diluted to contain approximately $2 \times 10^3$ cpm/50 μl, is placed in a 6 × 50 mm culture tube. To this is added, with rapid mixing, 50 μl of the antisera serially diluted in S-Dil, i.e. 9 parts of 3 per cent BSA plus 1 part normal rabbit serum. The reason for using a diluent containing normal rabbit serum will be discussed in the next section. The tubes are incubated at 37°C for 3 hours, chilled to 4°C for at least 30 min, and then centrifuged in the cold at 10000 g for 10 min. 50 μl of the supernatant is carefully removed and placed in a disposable counting tube containing approximately 0.5 ml of normal saline. Samples are counted in a well-type crystal, scintillation counter.

To facilitate rapid pipetting of reagents and accurately sampling in the large number of tubes we routinely do per assay, we have designed a screw driven syringe holder for a 10 ml syringe which will deliver 50 μl of reagent from the syringe reservoir for each click on the operating wheel or, with saline in the syringe and a special tip on the delivery hose, will withdraw 50 μl from the 100 μl volume in the assay tube and then expel that 50 μl and 150 μl saline wash into the counting tube. Delivery with the machine is at least as accurate as with micropipettes, and, since the machine does not fatigue, is perhaps somewhat more accurate than micropipetting for large assays.

Requirement for C1q in precipitation of $^{125}$I-labelled antigens. During the development of the $^{125}$I-labelled antigen precipitation assay, we found that demonstration of maximal antibody titres with many antisera required the presence of a non-specific serum factor from normal rabbit serum. As a routine precaution, we therefore always included 5 per cent normal rabbit serum in the final assay mixture. Recently, consistent with findings reported by McKenzie et al [29], we found that the rabbit serum could be replaced by purified human C1q, a subunit of C1, the first component of haemolytic complement [30], kindly supplied to us by Dr M. A. Calcott in Dr Muller-Eberhard's laboratory. This is demonstrated as follows.

Without exogenously added C1q a typical antiallotypic antisera reaches two-thirds of maximum precipitation at 0.3 μl antisera/assay tube. With 5 per cent normal rabbit serum in the incubation mixture, the same endpoint is reached at 0.05 μl/tube. Depletion of C1q from the antisera by inactivation at 56°C for 30 min reduces the titre in the absence of exogenously added C1q such that two-thirds maximum is not even approached at 5 μl/tube, but the titre is restored to maximum again by the presence of 5 per cent normal rabbit serum. Complement inactivated (56°C for 30 min) rabbit serum will not restore the titre. Finally, 0.3 μg isolated human C1q restores the titre as well as the normal rabbit serum.

The C1q appears to react selectively with antigen-antibody complexes, since large amounts of free immunoglobulin or antibody will not inhibit precipitation while antibody-antigen complexes such as anti-DNP DNP-HSA will sequester the C1q and prevent precipitation of the complex if C1q is limiting.

The dependence on C1q for precipitation may mean that antibody which does not bind complement will not be detected in this kind of assay. Some circumstantial evidence points to the existence of such antibody in some sera [31], but we have not explored this question at any length.

The C1q in mouse, human, rabbit, goat and guinea-pig sera seem about equally effective in precipitating labelled complexes, however, without a measure of C1q levels in these sera, no definite statement can be made. Isolated human C1, kindly supplied by Dr Dean Lincott, was only marginally active until treated with EDTA to separate the C1q from the other C1 components. The whole serum samples also increased in activity after EDTA treatment suggesting that only C1q works to facilitate precipitation.

The facilitation also demonstrates a salt concentration sensitivity. At a NaCl concentration equiva-
lent to that in normal serum (0-15 mol/l). No precipitation occurs at all. Somewhere below 0.1 mol/l, precipitation begins to occur and is maximal at about 0.07 mol/l. Assays are routinely run therefore at 0.05 mol/l Tris with no added sodium chloride.

We have bound partially purified C1q to Sepharose using the Cyanogen–Bromide method [23] to make a C1q-Sepharose column (see insoluble immunoadsorbants, p. 12.13). The bound material retains preformed antigen–antibody complexes when these are passed through the column at low salt concentration and releases the complex still intact at 0.15 mol/l NaCl. Elution of the complexes from the column regenerates the column, and it once again binds complexes when the salt concentration is reduced.

Quantitation of immunoglobulins by radiimmune (tube) assay

Inhibition of antibody precipitation of labelled antigen

For quantitative estimation of immunoglobulins, either in whole sera or isolated preparations, we have slightly modified a method originally described to us by Dr John Fahey in which unlabelled antigen is introduced at varying dilutions in a reaction mixture containing a standard amount of antiserum at a limiting dilution and 125I-labelled antigen. The unlabelled antigen competes for the antibody and thus "inhibits" the precipitation of the labelled antigen. Comparison with a standard curve for inhibition by a solution containing a known amount of unlabelled antigen allows accurate quantitation of the unknown.

The inhibition assay is performed as follows (slightly modified from [32]):

As in direct precipitation, 50 µl of labelled antigen diluted in 3 per cent BSA-tris such that 50 µl contain approximately 2 × 10^5 cpm, is added first. Next 5 µl of unlabelled test or standard antigen at varying dilutions in 3 per cent BSA-tris is added and the tubes mixed. Last, 50 µl of antiserum at a dilution in S-dil chosen such that the amount added is sufficient to precipitate about two-thirds of the maximum number of counts precipitable by that antiserum is added (Fig. 12.1). Since rapid mixing is essential, antiserum is generally added to two tubes at a time and those are then immediately mixed with a Vortex Junior mixer (Scientific Industries, Springfield, Mass.). Incubation and sampling is as described above in the section on precipitation of labelled antigen. All tests are done in duplicate.

![Image](image-url)

**Fig. 12.1.** Precipitation of 125I-labelled antigen by antiallootype serum. P: fraction of counts precipitated. Each tube contains ∼0.013 µg of 125I-labelled IgG2a myeloma protein, GPC-8, in 50 µl 3 per cent BSA-tris plus 1 µg anti IgG (LP anti BALB/c-peritussis) diluted in 50 µl S-dil. Arrow indicates amount of antiserum (0.25 µl) chosen for use in quantitative inhibition of precipitation assay presented in Fig. 12.2. Better linearity in the inhibition assay would be obtained choosing ∼0.18 µl antiserum.

Two sets of controls are included with the assay and generally repeated for each group of fifty tubes. One set contains 5 µl of 3 per cent BSA-Tris instead of inhibiting antigen and 50 µl of S-dil instead of antiserum to establish the number of counts expected in the absence of precipitation of the labelled antigen (no-precipitation control). This figure is used as the denominator to calculate the percentage of labelled antigen precipitated in assay tubes. The second control set contains 5 µl of 3 per cent BSA-tris instead of inhibiting antigen and establishes therefore the amount of labelled antigen precipitated by the antiserum in the absence of inhibitor (maximum precipitation control).

A series of dilutions of a known inhibitor protein or normal serum pool carrying the alloctype under test is always included in each assay, to provide data for a standard curve by comparison with which the allotype levels in the unknowns are determined. For convenience in interpolating values when reading from this curve, the reciprocal of the percentage of labelled antigen precipitated is plotted as a function of inhibitor concentration. This simple manoeuvre yields a standard curve which is linear over a wide range of concentrations.

There are straightforward reasons for expecting a
linear relationship between amount of inhibitor added and the reciprocal of percentage of radioactivity precipitated. These may be understood as follows. In the assay, the concentrations of antibody and labelled antigen are held constant. C1q concentration (also constant) is in excess. The concentration of inhibitor protein is varied from that which gives no detectable inhibition to one at which inhibition is apparently complete. Over most of this range, the total amount of antigen precipitated by the antibody is constant, and the unlabelled competitive (inhibitor) antigen simply competes for place in the precipitate, thereby decreasing the specific radioactivity of the total precipitated antigen. This decrease in specific activity is thus due only to dilution and is inversely proportional to the concentration of unlabelled antigen.

Put into mathematical terms, if for each assay tube:

\[ P: \text{fraction of radioactivity precipitated,} \]
\[ Ab: \text{\mu g of antigen precipitated (bound) by the amount of antibody used,} \]
\[ Ag: \text{\mu g of labelled antigen used,} \]
\[ Ag^*: \text{number of counts/min in Ag, and} \]
\[ u: \text{varying amount (in} \mu \text{g) of unlabelled (inhibitor) antigen added} \]

then

\[ \frac{Ag^*}{Ag} \text{spec radioactivity of the labelled antigen in preparation and} \]
\[ \frac{Ag^*}{u + Ag} \text{spec radioactivity of total antigen in tube when varying amounts of unlabelled antigen are added.} \]

When no inhibitor is present,

\[ P = \frac{Ab}{Ag} \frac{Ag^*}{Ag} = \frac{Ab}{Ag} \text{max fraction of counts precipitated in the assay.} \]

When varying amounts of inhibitor \( u \) are present,\n
\[ P = \frac{Ab}{Ag^*} \frac{Ag^*}{u + Ag} = \frac{Ab}{Ag} \]

and, taking the reciprocal

\[ \frac{1}{P} = \left( \frac{1}{Ab} \right) + \frac{Ag}{Ab} \]

This is in the slope intercept form of the equation for a straight line when \( 1/P \) is plotted against \( u \). The inverse of the ordinate intercept of this line is equal to \( Ab/Ag \), the maximum fraction of labelled antigen which is precipitable by the amount of antibody

![Graph](a)

![Graph](b)

**Fig. 12.2.** Inhibition of precipitation of \(^{125}I\)-labelled antigen by homologous unlabelled antigen. \( P \): fraction of counts precipitated. Each tube contains \( \sim 0.013 \mu \)g of labelled IgG2a myeloma protein. GPC-8, in 50 \( \mu l \) 3 per cent BSA-tris, 0.25 \( \mu l \) IgG anti IgA (LP anti BALB/c-permutus) in 50 \( \mu l \) 2-dil and 3 \( \mu l \) unlabelled GPC-8 diluted in 3 per cent BSA-tris.

used. The slope, \( 1/Ab \), decreases as the absolute amount of antigen precipitated increases, hence as the amount of antibody used increases.

Data showing a typical reciprocal plot (linear) standard curve for an inhibition assay are presented in Fig. 12.2a. For comparison, the same data with percentage of labelled antigen plotted directly as a function of amount of inhibitor are presented in Fig. 12.2b. Although the data in both figures are presented in terms of \( \mu g \) of inhibitor added, it is, of course, equally possible to use a standard of unknown inhibitor concentration, e.g. normal serum, and express values for unknowns as \( \mu l \) of standard/\( \mu l \) unknown.

The inhibition curve presented here (Fig. 12.2a)
was chosen to show the most common departure from linearity observed in this system: a small initial lag before linearity is established. The 'lag' may be avoided by dropping the antibody concentration or raising the amount of antigen in the labelled preparation; however, it is frequently more convenient just to ignore the early part of the curve and read values from the linear region only. In this case, the true y intercept, i.e. the maximum fraction of labelled antigen precipitable at the antigen and antibody concentrations used in the assay, may be determined by extrapolation of the linear portion of the curve.

The observed 'lag' is probably due to the total antigen concentration being below saturation at the antibody concentration chosen, since theoretically linearity only obtains when $A_B$ (the amount of antigen precipitated in the assay) is constant. This interpretation is borne out by the demonstration that, for a given amount of labelled antigen, the length of the 'lag' increases with increasing antibody concentration and decreases with increasing antigen concentration.

Sensitivity of the assay varies according to the concentration of antibody and labelled antigen. Generally, the minimum amount of immunoglobulin carrying a particular allotypic antigen detectable is in the region of 0.02 mg/ml serum, although if need be the lower limit can be decreased by as much as an order of magnitude. Determinations may be carried out without interference even in the presence of a 20 000-fold excess of a non-cross-reacting immunoglobulin. Thus, as little as 10 $\mu$g of allotype-carrying immunoglobulin may be detected in a mouse with roughly 30 mg of total immunoglobulin.

Two precautions are necessary to prevent serious errors in the quantitative determinations. It is important to:

1. avoid the introduction of a large amount of extraneous antigen-antibody complexes, which will non-specifically consume $C_{1q}$ and therefore prevent complete precipitation of the labelled complex (see $C_{1q}$ p. 12.18), and

2. avoid situations where the standard or the unknown differs from the labelled antigen and does not carry all of the antigenic specificities detected by the antiserum in the assay. This can occur with cross-reacting antigens (such as allotypes) if the antiserum used reacts with two specificities, both of which are present on labelled antigen molecules but only one of which is present on the inhibitor. In such a case the inhibitor cannot inhibit precipitation completely; therefore, the reciprocal plot for the inhibitor is not linear, and quantitative comparisons become hazardous. If the standard and the labelled antigen are identical but the 'unknown' is different, values for the unknown read from the standard curve are invalid.

Linearity for a cross-reacting standard may be obtained by subtracting the fraction of radioactivity precipitation not inhibitable from the total fraction precipitated before converting to the reciprocal. It is, however, more satisfactory to choose appropriate combinations of antiserum and labelled antigen or to absorb the antiserum first, so that such manipulations are unnecessary.

Radioimmune assay: solid-phase (Plate) assays

These assays are particularly useful for the measurement of antibody responses to haptenics, protein or other antigens which easily adhere to plastic surfaces [32]. In brief, wells in polystyrene plates are coated with antigen, and then antisera are incubated in the coated wells. Next, radiolabelled anti-immunoglobulin antibody is incubated in the well. The number of counts bound provides a measure of the amount of specific antibody which has bound to the antigen adhering to the plate. By using specific radiolabelled anticlass or antiallotype reagents, the class and allotype representation in the bound antibody are individually measurable in this assay.

Radioiodination of antibody: affinity chromatography method. Specifically purified, radioiodinated antibody is required for many solid phase radioimmuno-binding assays. Generally, antibody is isolated first by affinity chromatography and then labelled with $^{125}$I. We have recently found, however, that preparation of labelled reagent is more efficient when the labelling step is interpolated between the adsorption and elution steps of the affinity chromatography purification, i.e. when the antibody is labelled while bound to the Sepharose-bound antigen [34]. This method, suitably miniaturized for use with mouse reagents, is presented here.

The column used to hold the immunoadsorbant consists of a Beckman microfuge tube (400 $\mu$l capacity) with its bottom tip cut off and plugged with glass wool. Sephadex G-25 is packed over the glass wool to a bed volume of 100 $\mu$l. Sepharose-myeloma protein containing about 50 $\mu$g of protein (10 $\mu$l bed volume) is layered onto the Sephadex. For maximal specificity of labelled reagents, the volume of Sepharose-myeloma protein should be limiting with
respect to the amount of antibody used. The Sephadex G-25 is used to create a manageable bed volume and to achieve a faster separation of eluted protein from the acidic solution used for elution. Immediately before use, the column is washed with 1-2 ml of 0.2 mol/l glycine-HCl, pH 2.3, and neutralized with phosphate buffered saline (PBS), pH 7.5, is layered onto the column. The Sepharose and the top of the Sephadex bed are mixed in this ‘cocktail’ for 15-30 sec, then sodium metabisulfite (100 µg in 25 µl of 0.05 mol/l phosphate buffer, pH 7.5) is added to the slurry and the column washed immediately with 3-4 ml of PBS.

The labelled antibody is eluted with 0.2 mol/l glycine-HCl containing 1 per cent bovine serum albumin (BSA), pH 2.3 (14 drops/min). Two-drop fractions (approximately 60 µl/drop) are collected into 120 µl of 0.3 mol/l borate buffer, containing 1 percent BSA and 0.2 per cent sodium azide, pH 8.6. The percentage of protein-bound 

\[ ^{14} \text{C} \]

in each fraction is determined by precipitation with trichloroacetic acid in the presence of 1 per cent carrier BSA. In some instances the eluted radioiodinated antibody is further purified by binding and elution from a second immunosorbent column.

Measurement of allotype and class representation in antisera (plate assay). Antibody to the hapten dinitrophenyl (DNP) is used here as an example. A solution of 1 per cent BSA, 0.005 mol/l EDTA, 0.1 per cent NaNO₃, PBS (pH 7.6) is used for washing and dilution. All steps are performed at room temperature. Flexible polystyrene U-bottom microtitre plates are coated with DNP by incubating 50 µl of DNP-BSA (2 mg/ml) per well for an hour. Test sera or standard anti-DNP sera at various dilutions are added to the coated wells (20 µl/well) and incubated for 3 hours. The plate is then washed twice and radioiodinated antiallotype antibody (20 µl/well, approximately \( 3 \times 10^{6} \) cpm) is added and incubated for another 3 hours. Finally, the plates are washed twice and the wells separated and counted in a well-type gamma counter.

To conveniently separate the wells, the adhesive plastic sheet usually used to seal microtitre plates is applied to the bottom of the plate. The top of the plate is then sliced away with a hot wire (mounted horizontally, 10 mm high, on a lattice table) leaving the liberated wells adhering to the bottom sheet in their original position and ready for easy transfer to tubes for counting. This assay can be used to measure the total anti-DNP antibody content in an antiserum or, by appropriate choice of labelled reagent, the individual contribution of each allotype and class to the response. Similar measurement can be made for other protein antigens or haptens so long as these can be bound to the polystyrene plates.

Quantitative determination of allotype levels. Immunoglobulin allotype levels are determined by an antibody consumption type assay. Fifteen µl of radioiodinated antiallotype antibody (approximately \( 2.5 \times 10^{6} \) cpm) are mixed with 15 µl of a known myeloma protein (standard) or a test serum and incubated for 4 hours at room temperature. Aliquots of 20 µl are then transferred into microtitre wells previously coated with a myeloma protein of the same allotype for 90 min at room temperature (0-1 to 1 mg/ml). The plates are washed twice with diluent and the microtitre wells separated and counted.

For maximal specificity in this assay, the amount of myeloma protein coated on the wells should be adjusted so as to be just sufficient to obtain maximal binding of the amount of labelled antibody used.

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