LAH 4/15

Chapter 27
Mouse Immunoglobulin Allotypes

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The subject of immunoglobulin allotypes can be viewed in two ways; historically and functionally. Historically, the study of murine immunoglobulin allotypes provided the first glimpse into the complex organization of what is now known as the immunoglobulin heavy chain constant region (Igh-C) locus. Since the 1980s, molecular biological analyses have confirmed these pioneering studies and provided an exquisitely detailed picture of the structure and organization of the Igh-C locus at the level of nucleotide sequence. From a functional standpoint, today, immunoglobulin allotypes are primarily used as powerful markers to follow populations of B cells and their secreted immunoglobulins in a variety of adoptive transfer experiments. The purpose of this chapter is to provide investigators with the necessary information for an understanding of murine immunoglobulin allotypes and allotypic specificities and their uses in current immunological studies.

Identification of immunoglobulin isotypes

The immunoglobulin molecule is a four-chain unit containing two identical heavy (H) chains and two identical light (L) chains [1]. Each chain can be divided into a variable region and a constant region. The variable regions of the H and L chains can jointly define the structure of the antibody combining site. The constant regions of the H chains define the *isotype* (class/subclass) of the molecule and determine many of its biological properties. Isotypes were originally identified by physical characteristics such as size and electrophoretic mobility; however, now all known mouse Ig isotypes can be easily distinguished with isotype-specific antisera or monoclonal antibodies.

There are eight known H-chain isotypes in the mouse, each associated with particular biological activities (Table 27.1). Seven of these isotypes are routinely present in sera from virtually all mice. The eighth (IgD) is difficult to detect in sera but is expressed on the majority of mature B lymphocytes found in spleen, lymph node, and peripheral blood. Clonally derived immunoglobulin-secreting cell lines (myeloma tumors and hybridomas), which can produce large amount of each of the known isotypes (including IgD), are available.

The genetic mechanisms involved in the generation of immunoglobulin heavy chains are highly complex. Each H chain is encoded by four non-contiguous segments of DNA: V_H (variable region), D_H (diversity), J_H (joining) and C_H (constant region) (See chap 16). The constant region genes, which determine H-chain isotype structure, are tightly clustered in a single 200-kb region of chromosome 12 designated as the Igh-C complex [2]. This region is very closely linked to the chromosome region containing J_H segment genes and somewhat more loosely linked to the chromosome region that codes for the specificity-defining structures of the H chain (V_H and D_H). Thus the DNA that codes for an individual Ig H chain is a compound gene created by chromosomal rearrangements that juxtapose constant region and variable region H chain genes.

Immunoglobulin allotypes—a brief history

The segment of DNA that codes for a single polypeptide chain (or in the case of immunoglobulin, part of the chain) is defined as the genetic *locus* for the protein. When multiple forms of the same protein (differing slightly in amino acid sequence) are found within the population, the locus coding for that protein is said to be *polymorphic* and the alternate forms are defined as the *alleles* of that locus. For immunoglobulins encoded within the Igh-C locus these alleles are referred to as *allotypes*. Some of the Igh-C loci are highly polymorphic, with as many as nine alleles identified, although others are considerably less polymorphic. However, at least two alleles have been detected at all of the Igh-C loci.

Allotypes were first identified in the mouse in the early 1960s [4, 5], several years after the identification of genetic polymorphisms in rabbit [6] and human [7] Ig. The availability of numerous inbred strains of mice, each with a genetically fixed set of allotypes, greatly facilitated the study of these polymorphisms. As with the Ig isotypes, mouse allotypes have been identified by physical properties such as electrophoretic mobility or by their reactivity with heterologous antibodies raised in goats, rabbits, or rats. (In fact, because heterologous anti-mouse Ig isotype antisera are often raised against myeloma/hybridoma proteins they can contain considerable anti-allotypic activity and therefore show a strong reactivity bias to allotypic determinants of the immunizing protein [8]. We found a commercial anti-mouse IgG2a sera that had 10- to 20-fold higher reactivity with IgG_{2a} from BALB/c compared to C57BL. A phone call to the company confirmed that the antiserum had been raised against a BALB/c myeloma protein. Investigators should be careful when using such heterologous antisera as second-step reagents to reveal monoclonal first steps in ELISA or FACS assays.) Typically, however, they are detected by their distinctive reactivity patterns with *allo-antisera* raised by immunizing one mouse strain with immunoglobulin from a second strain. Thus, as a rule, individual allotypes (*alleles*) are defined by the presence of unique combinations of allo-antigenic determinants (*allotypic specificities*), as identified by allo-antisera or monoclonal antibodies.

By 1964, the idea of a chromosome region containing several closely linked loci coding for the various Ig isotypes in the mouse [9, 10] and in humans [11] had gained considerable acceptance. By the late 1960s, the chromosome region containing the mouse Ig H-chain genes was well established with polymorphisms identified for four of the eight Ig loci and a long series of alleles at two of the four polymorphic loci [12, 13]. Studies on Ig allotypes were further facilitated by the development of strains of mice congenic for the Igh-C loci [14]. These strains are described in detail later.

During the next decade, genetic polymorphisms were identified

Table 27.1. Mouse immunoglobulin isotypes

Isotype	Locus	Number of alleles known in inbred strains	Number of allotypic determinants identified	Some biologic activities
IgG2a (γ2a)	Igh-1	12	17	Fixes complement; mediates cell lysis; fixes to tissues of other species; mediates ADCC, mediates local anaphylaxis; passes placenta
IgA (α)	Igh-2	6	8	Does not fix complement; secreted into milk, tears, intestinal lumen, and nasal secretions
IgG2b (γ2b)	Igh-3	6	12	Fixes complement; mediates cell lysis, mediates ADCC (less efficiently than IgG2a); passes placenta
IgG (γ1)	Igh-4	3	3	Does not fix complement; fixes to tissues of same species; mediates local anaphylaxis; passes placenta
IgD (δ)	Igh-5	3	5	Trace amounts in circulation; present on most B cells in adult mice; no known function
IgM (μ)	Igh-6	4	6	Fixes complement, mediates cell lysis (more efficiently than IgG2a and IgG2b); present on most B cells in adult mice
IgE (ε)	Igh-7	2	2	Binds to mast cells; involved in allergic reactions
IgG3 (γ3)	Igh-8	None[a]	3[a]	Does not fix complement; does not fix to skin; passes placenta

[a] Although no allotypic differences have been observed in inbred strains, three monoclonal antibodies have detected differences between the IgG3 of wild strains of mice and inbred strains [3].

for IgD [15], IgM [16], and IgE [17] and a large number of Ig H-chain allotypic determinants and alleles were identified. The overall concept of the region, however, remained essentially static until the late 1970s when evidence from molecular studies divided the Ig chromosome into separate V, D, J, and C regions containing genes that could rearrange during lymphoid development to constitute a single H chain gene capable of being expressed in an antibody-producing cell. The current view of the Igh-C region as containing the loci that code for the H chain constant region structure emerged at this point.

Since their discovery, immunoglobulin allotypes have been of interest as members of a highly polymorphic, genetically complex system. In addition, as markers of specific cell lineages and genotypes, they have proved extremely useful for dissecting cell lineages and regulatory mechanisms within the immune system. For example, the ability to distinguish allotypes was the key to the demonstration that bone-marrow derived (B) cells produce antibodies, although thymus-influenced Thy-1$^+$ cells participate indirectly (as helper cells) in antibody production [18]. Furthermore, the suppression of allotype production in adoptive transfer assays provided some of the earliest evidence demonstrating the existence of suppressor T cells [19]. Today, the availability of a variety of pairs of Igh-C congenic strains and of monoclonal anti-allotypic antibodies has been critical to studies of B cell differentiation and development [20, 21].

Alleles, specificities, and haplotypes of the IgH-C loci

Terminology of Ig heavy chains

For historical reasons, immunoglobulins are referred to by two different systems of notation depending on the context in which they are being discussed (a practice that often creates confusion for those beginning to work or read in the field). In general, isotype names such as IgM or IgG2a are used when speaking of antibodies or immunoglobulins as proteins or as a functional molecules in biological assays such as complement fixation. Allotype names (such as Igh-6 or Igh-1), in contrast, are usually used in discussions where genetic (allelically determined) differences between immunoglobulins or antibodies are important, such as in genetic linkage studies or in studies where the production of individual allotypes is independently regulated. Thus, although each isotype name is synonymous with the name of the locus that encodes it (such as IgG2a and Igh-1), routine practice maintains both notations as current. To make matters worse, isolated immunoglobulin H chains (without associated L chains) are designated by yet another system: Greek letters are used to identify each H chain (in correspondence with its isotype name where possible). Allotypes are ignored in this notation system. The IgA H chains encoded at the Igh-2 locus, for example, are referred to as α chains regardless of which Igh-2 allele encodes them (see Table 27.1).

In the early studies of allotypic determinants there were two different systems for designating the Igh-C loci and their allotypic determinants; however, a standard system for the genetic nomenclature for the Ig loci was adopted in 1979 [22]. In this system, the Ig H-chain loci are designated Igh and numbered according to the order in which they were discovered to be polymorphic. Allotypic specificities were first discovered for IgG2a; therefore, the locus coding for IgG2a is designated Igh-1. The genetic designation of each of the Igh-C loci is given in Table 27.1. For each locus the allotypic specificities or determinants are designated by individual numbers following the locus. Thus the 17 known allotypic determinants of the IgG2a (Igh-2) locus are designated Igh-2.1 through

Table 27.2. The Igh-1 locus

Allele	Type strain	Specificities															
a	BALB/c	1	2	–	–	–	6	7	8	10	–	12	13	14	15	–	17
b	C57BL/10J	–	–	–	4	–	–	7	–	–	–	–	–	–	–	–	–
c	DBA/2J	–	2	3	–	–	–	7	–	–	–	–	–	–	–	–	–
d	AKR/J	1	2	–	–	5	–	7	–	–	–	12	13	14	–	16	17
e	A/J	1	2	–	–	5	6	7	8	–	–	12	13	14	–	16	17
f	CE/J	1	2	–	–	–	–	–	8	–	11	–	13	–	–	16	17
g	RIII/J	–	2	3	–	–	–	–	–	–	–	–	13	14	15	–	17
h	SEA/Gn	1	2	–	–	–	6	7	–	10	–	12	13	14	15	–	17
j[a]	CBA/H	1	2	–	–	–	6	7	8	10	–	12	13	14	15	–	17

[a] Shown to be different from BALB/c by its positive reaction with a conventional antiserum; detected specificity was on Fab [44].

Igh-2.17 (Table 27.2). Inasmuch as this nomenclature is well-established, references to the earlier designations have been dropped from this chapter. For descriptions of the previous systems of Ig allotype nomenclature and designation of the specificities, the reader is referred to earlier reviews of mouse Ig genetics [23, 24].

Table 27.3. The Igh-5 locus

Allele	Type strain	Specificities[a]				
a	BALB/c	1	–	–	4	–
b	C57BL/10	–	2	3	–	–
e	A/J	1	–	3	–	5

Alleles and allotypic specificities

Alleles are alternate forms of a gene (DNA sequence) at a given locus. By definition, they cannot be inherited in the same haploid set and must segregate in the progeny derived from a heterozygous parent. Thus they are classically demonstrated by back-cross and intercross studies that reveal the pattern of inheritance of the trait under study. Alleles, which when referring to immunoglobulins are called allotypes, have been identified for all of the Igh-C loci. For each locus the alleles are designated by a letter following the locus name. Thus, the IgG2a allotype of BALB/c is Igh-1a although the allotype of C57BL/6 is Igh-1b. The convention for designating newly identified alleles is discussed later.

Except for the IgG3 alleles, the genetic criteria for allelism of the genes coding for these allotypes have been met by back-cross analyses; that is, two inbred mouse strains, each with a different H-chain allotype for a given isotype, were crossed to obtain heterozygotes that produces both allotypes. The heterozygotes were then back-crossed to one of the parental strains, to a strain carrying a third allotype, or to themselves. In all cases, the expected ratios for segregating codominant alleles were found, indicating that a single haploid set always carried either the allele donated by the mother of the heterozygotes of the allele donated by the father (never both; never neither [13]).

Although each allele is definitively characterized by its nucleotide or protein sequence, practically they are identified serologically using allo-antisera or monoclonal antibodies that detect differences in the protein sequence between a given Ig isotype obtained from two inbred strains of mice. The unique pattern of reactivity of an antiserum or monoclonal antibody with a given isotype from a panel of inbred strains of mice defines an *allotypic specificity*. For example, specificity 3 of the Igh-5 locus is defined by a monoclonal antibody that only reacts with IgD from C57BL/10 and A/J but not BALB/c mice (Table 27.3). The specificities for each Igh-C locus are numbered according to the order of their discovery. Therefore, specificity 3 for the Igh-5 locus has no structural relation to specificity 3 for the Igh-1 locus. When specificities at more than one locus are being discussed, the more complete designation, Igh-5.3 (specificity 3 of the Igh-5 locus) or Igh-1.3 is used to prevent confusion.

With the advent of monoclonal antibodies it was recognized that a single allotypic specificity may be composed of multiple distinct alloantigenic determinants, referred to as *allotopes*, located on different parts of the Ig molecule. For example, a minimum of eight antibodies with a reactivity pattern characteristic of the allotypic specificity Igh-1.4 (found on C57BL IgG2a immunoglobulins) have been identified. Cross-blocking studies in solid-phase radioimmunoassays (RIAs) using these antibodies have demonstrated that they detect physically distinct determinants scattered along the Igh-1b molecule [25, 26]. Multiple allotopes have also been identified for specificities Igh-1.14, Igh-1.17 [26] and Igh-5.3 [27].

A given allotypic specificity can be unique to one allele (such as specificity 4 of the Igh-1 locus that is only present on the Igh-1b allele, see Table 27.2) or shared by multiple alleles (such as specificity 12 which is present on the Igh-1a, 1d, 1e, 1g, 1h, and 1j alleles). For a given Igh-C locus each allele is defined by the presence of a unique combination of allotypic specificities. Thus, the Igh-5a allele (IgD of BALB/c) is defined by the presence of the Igh-5.1 and 5.4 specificities and the absence of specificities Igh-5.2, 5.3, and 5.5 (see Table 27.3).

The Igh-C complex and Igh-C haplotypes

Definition and nomenclature. In crosses where the parental strains had allotypic differences at two or more H-chain loci, backcross and intercross testing showed that the loci in a given haploid set were always inherited together. These early studies demonstrated that the structural genes encoding at least four of the mouse H-chain isotypes (those for which anti-allotype antisera were available at the time) are quite closely linked on the chromosome [13]. Later genetic studies demonstrated that the remaining three polymorphic loci were also tightly linked to the same gene complex now designated Igh-C. Upward of 8000 progeny of appropriate crosses have been examined in detail, yet no direct evidence of a cross-over between Igh-C loci has been

Table 27.4. Distribution of alleles in the Igh-C haplotypes

Haplotype	Prototype strain	Immunoglobulin locus and heavy chain isotype[a]						
		Igh-6 IgM	Igh-5 IgD	Igh-4 IgG1	Igh-3 IgG2b	Igh-1 IgG2a	Igh-7 IgE	Igh-2 IgA
a	BALB/c	a	a	a	a	a	a	a
b	C57BL/10J	b	b	b	b	b	b	b
c	DBA/2	a	a	a	a	c	?	c
d	AKR/J	n (d)[c]	a	d	d	d	a	d
e	A/J	e	e	a	e	e	a	d
f	CE/J	a	a	a	f	f	?	f
g	RIII/J	a	a	a	g	g	?	g
h	SEA/J	a	a	a	a	h	?	a
j	CBA/H	a	a	a	a	j	a	a
k	KH-1[d]	?	b	a	a	k	?	c
l	KH-2[d]	?	b	a	a	l	?	c
m	Ky[d]	?	b	b	b	m	?	b
n	NZB	n (d)	a	d	e	e	?	d
o	AL/N	e	e	a	d	d	?	b
p	SWR/J	?	?	?	f	c	?	b

[a] The loci are listed in the order that they occur on chromosome 12.
[b] The strain has not been typed for all specificities at this locus.
[c] This allele was first identified in NZB mice and thus given the designation Igh-6n. However, recent studies indicate that this allele is common to both AKR and NZB and should likely be designated Igh-6d. (see The Igh-6 (IgM) locus).
[d] Haplotypes identified in wild mice.

found [22]. As noted earlier, DNA sequence analysis now places all of the H-chain loci within a chromosome region that is 200-kbp long. The order (5′ to 3′) of the individual gene loci within the Igh-C region is Igh-6 (μ), Igh-5 (δ), Igh-8 ($\gamma 3$), Igh-4 ($\gamma 1$), Igh-3 ($\gamma 2b$), Igh-1 ($\gamma 2a$), Igh-7 (ϵ), and Igh-2 (α).

This close genetic linkage means that in any mating all of the Igh-C alleles from each parent are transmitted to the progeny as two haploid sets. Such a linked set of alleles, inherited as a group, is referred to as a *haplotype*. In the case of the Ig heavy chain genes, each Igh-C haplotype is defined by a unique combination of alleles at each of the seven loci. Among inbred strains there are currently 12 known combinations of Igh-C alleles or haplotypes (Table 27.4). Each haplotype is designated by a superscript letter (usually corresponding to the allele of the highly polymorphic Igh-1 locus). Although multiple strains may carry the same haplotype, for each haplotype one strain is designated the prototypical or Type Strain.

If allotypic differences are identified between two strains previously considered to be identical, the type strain maintains its haplotypic designation and the second strain is given a new haplotype designation. For example, BALB/c and CBA were long consider to have the same Igh-C^a haplotype because they shared identical allotypic specificities at each of the Ig loci. However, a sera was identified that was positive on IgG2a of CBA but not BALB/c. This difference defined a new allele of Igh-1 (Igh-1j) and therefore CBA (and all others strains with this allele) were given the new haplotype designation, Igh-C^j. However, the alleles of IgM, IgD, etc., are still indistinguishable from BALB/c and remain designated Igh-6a, Igh-5a, etc. (See Table 27.4.)

Relationship between haplotypes. The 12 inbred haplotypes can be broadly classified as *independent haplotypes* (such as Igh-C^a or Igh-C^d) in which some or all of the loci have unique alleles or as *recombinant haplotypes* (Igh-C$^{n,\ o,\ and\ p}$) in which all of the alleles are indistinguishable from two or more independent haplotypes. The two most diverse (and extensively studied) haplotypes are the Igh-C^a haplotype of BALB/c and the Igh-C^b haplotype of C57BL/10. At every loci the alleles of these two haplotypes show extensive differences. For example, the alleles of IgG2a, Igh-1a and Igh-1b, share only five of 17 allotypic specificities. This divergance is not unexpected given the disparate origins of these two strains [14]. By definition the Ig alleles of BALB/c are designated "a"; Igh-1a, Igh-2a, etc., whereas the alleles of C57BL/10 are designated "b."

The rest of the independent haplotypes appear to be, to a varying extent, related to the Igh-C^a haplotype, in every case sharing at least one allotype in common. From analysis of the alleles and allotypic specificities these haplotypes can be grouped into families. Igh-C$^{h\ and\ j}$ are most closely related to Igh-C^a, differing at only a few specificities at the Igh-1 locus. Similarly, the Igh-C$^{c\ and\ g}$ haplotypes that differ from the Igh-Ca haplotypes at two and three loci, respectively, show similarity at the Igh-1 and Igh-2 loci. The final family consists of the Igh-C$^{d\ and\ e}$ haplotypes that are closely related to each other and differ from the Igh-C^a haplotype at five of the seven loci.

Although no cross-overs between H-chain loci has been seen in more than 8000 progeny of F1 matings, the haplotypes of two strains of inbred mice (NZB and AL/N) resemble natural recombinants [27–29] of the Igh-C^d and Igh-C^e haplotypes (Table 27.4). Interestingly, the recombination in both of these strains appears to have occurred between the Igh-4 and Igh-3 loci. This may indicate a recombinatorial "hot spot" similar to that identified in the H-2 gene complex [30]. Furthermore, gene sequencing data [31] and serological analysis [32] suggest the past occurrence of recombination or gene conversion events in wild mice, especially between the IgG_{2a} and IgG_{2b} loci. These rare events were probably facilitated by the high level of homology between exons encoding the constant region domains of different allotypes and even different isotypes [32].

Allelic (haplotypic) exclusion. The phenomenon originally called allelic exclusion and now referred to as haplotype exclusion is one of the fascinating peculiarities of the genetic mechanisms controlling Igh-C gene expression. Similar to heterozygotes for most codominant genes, allotype heterozygotes typically produce about

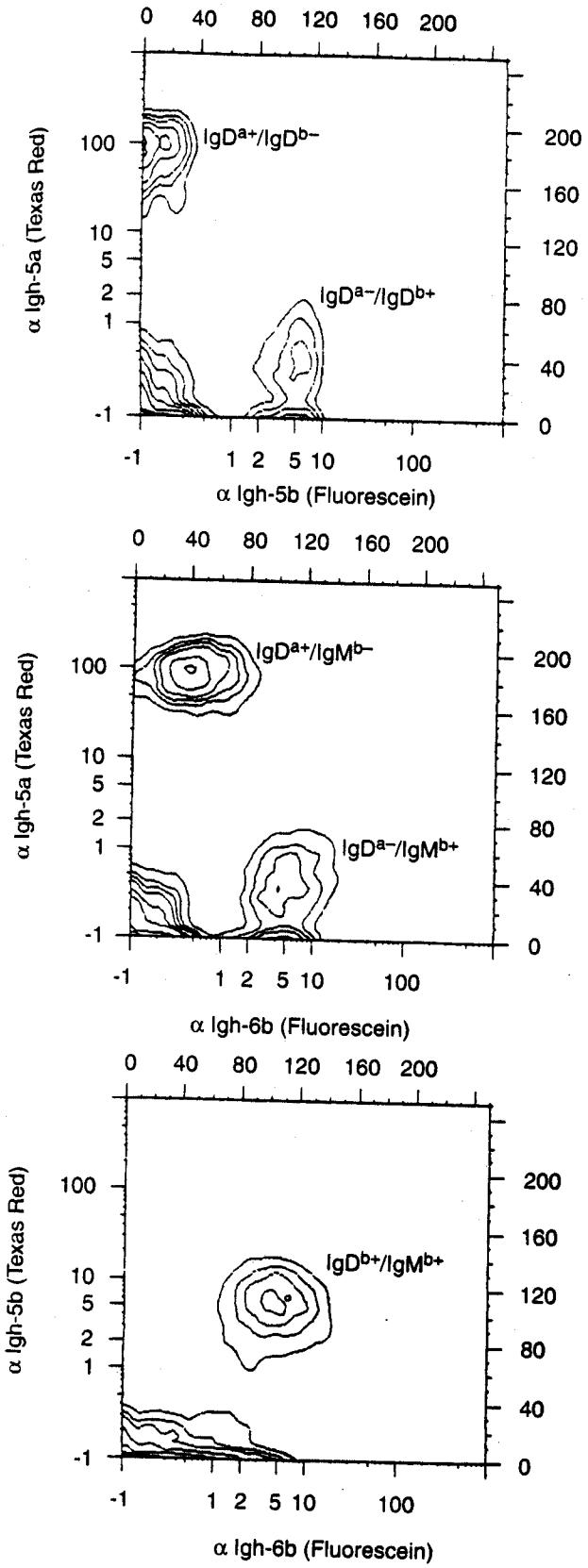

Fig. 27.1. *(BALB/c x C57BL/6)$_{F1}$ [Igh-C$^{a/b}$] spleen cells were stained with: (a) fluorescein-conjugated anti-IgD^{b} and biotin-conjugated anti-IgD^{a}; (b) fluorescein-anti-IgM^{b} and biotin-anti-IgD^{a}; (c) fluorescein-anti-IgM^{b} and biotin-anti-IgD^{b}.*

equal amounts of each parental allotype. Surprisingly, Pernis et al [33] found that the expected codominant production of allotype observed in the serum was not seen at the cellular level, that is, individual immunoglobulin-producing cells in allotype heterozygotes produced either the parental allotype or the maternal allotype but not both. This exclusion of one of the parental alleles in each cell expressing a given locus was called *allelic exclusion*. The allelic exclusion of the two IgD alleles in a (BALB/c x C57BL/10)$_{F1}$ {Igh-C$^{a/b}$} heterozygote is shown in Figure 27.1a. Spleen cells that are stained with a monoclonal antibody specific for the IgD^{a} allele do not bind a monoclonal specific for the IgD^{b} allele and vice versa.

Individual cells, however, are not restricted to producing the products of a single H-chain locus. Surface Ig positive splenic and lymph node cells typically express both IgM and IgD isotypes. In an Igh-C heterozygote, analysis of the allotype markers on these immunoglobulin isotypes graphically demonstrates that the immunoglobulin expressed in a given cell are controlled by alleles located on the same chromosome; that is, in an animal heterozygous for the eight-Igh-C loci, cells expressing the maternal allele of the IgM will express only the maternal allele of IgD [34]. Thus as shown in Figure 27.1b and 27.1c, in a (BALB/c x C57BL)F$_1$ {Igh-C$^{a/b}$} heterozygote, the spleen cells that react with a monoclonal antibody specific for the IgM^{b} allele do not bind a monoclonal antibody specific for IgD^{a} (Fig. 27.1b) but do bind one specific for the IgD^{b} allele (Fig. 27.1c). Furthermore, sorted cells producing IgD and IgM encoded by a given parental chromosome give rise to IgG-producing cells that express alleles of the same parental haplotype [35]. In other words, either the parentally derived or maternally derived Igh-C chromosome region appears to be functional in a given cell. Thus allelic exclusion should more appropriately be called *haplotype exclusion*.

In each case Texas red-avidin was used as a second step for the biotin-conjugated monoclonal antibody. The following monoclonal antibodies were used: AMS 9.1 (anti-IgD^{a}); AF6 to 122.2 (anti-IgD^{b}) and AF6 to 78.25 (anti-IgM^{b}) [27].

Functionally, we now know that haplotype exclusion is a result of the fact that in each cell only one allele undergoes a functional VDJ rearrangement allowing for the production of Ig mRNA and protein. However, the molecular mechanism(s) or signals that prevent both alleles from functionally rearranging both alleles are still not completely understood. However, studies of transgenic mice with targeted deletions or expressing functionally rearranged immunoglobulin genes have provided insights into the basic molecular events required for the process of allelic exclusion to occur. There appears to be an absolute requirement for the membrane form of the μ heavy chain because expression of transgenic μ lacking a membrane exon does not inhibit the rearrangement of endogenous μ [36]. The fact that targeted deletion of either the μ membrane exon [37] or $\lambda 5$ [38] severely disrupts B cell development suggests that expression of a membrane form a μ, in conjunction with $\lambda 5$ and V_{preB} is necessary for both allelic exclusion and ongoing B cell differentiation. Although expression of the membrane-form of IgM is necessary for allelic exclusion, it is not sufficient. In most of the membrane μ transgenic mice analyzed, a large but variable population of cells express endogenous μ, alone or in conjunction with transgenic μ [39–41]. It is not clear whether this is due to a "natural leakiness" in the allelic exclusion induced by just the μ transgene or to the

Table 27.5. Monoclonal antibodies recognizing Igh-C[a] and Igh-C[b] allotypic specificities

Primary reactivity (specificity)	Clone name	Isotype of antibody	Isotype recognized	Reactive alleles	Non-reactive alleles	Sources[a]	Reference
Igh-1a (1.13)	Ig(1a) 8.3	IgG2a	IgG2a	a, d, e, f, g, h, j, n, o	b, c, p	A, P	[25, 43]
Igh-1b (1.4)	Igh(1b) 5.7	IgG3	IgG2a	b	a, c, d, e, f, g, h, j, n, o, p	H, P	[25, 43]
Igh-2a (2.9)	HY-16	IgG2a	IgA	a, f, g, j	b, c, d, e	H	from Michael Potter
Igh-2b (2.10)	HIS-M2	IgG1	IgA	b, d, e, f	a, c, d, g	K, H	Frans Kroese pers. commun.
Igh-3a/1a (1.17/3.10)	21–48 (16.3)	IgG2a	IgG2a/2b	3a, d, e, f, g 1a, d, e, f, g, h, j	3b, 1c, 1p	H, P	[26, 43]
Igh-3b (3.9)	412-72 (24.1)	IgG1	IgG2b	b	a, d, e, f, g	H, P	[26, 43]
Igh-4a (4.1)	Ig(4a) 10.9	IgG2a	IgG1	a, d	b	A, P	[25, 43]
Igh-4b (4.2)	412-79 (22.9)	IgG2b	IgG1	b	a, d	H, P	[25, 43]
Igh-5a (5.4)	AMS 9	IgG2b	IgD	a	b, e	A, P	[27]
Igh-5b (5.3)	AF6-122	IgG1	IgD	b, e	a	A	[27]
Igh-5b (5.2)	217–170	IgG1	IgD	b	a, e	A, P	A. Stall, unpubl. data
Igh-5e (5.5)	AF4-73	IgG2b	IgD	e	a, b	A, P	[27]
Igh-6a (6.5)	331.12	Rat IgG2b	IgM	a, b, n	e	A	[56, 57]
Igh-6a (6.1)	DS-1	IgG1	IgM	a	b, e, n	A, P	[29]
Igh-6b (6.4)	AF6-78	IgG1	IgM	b, n	a, e	A, P	[27]
Igh-7a (7.1)	UH297	Rat IgG2c	IgE	a	b	O, H, P	[62]
Igh-7b (7.2)	JKS-6	Rat IgG2a	IgE	b	a	O, H, P	[63]

[a] Sources: Cell lines: A, American Type Culture Collection, Rockville, MD; H, Dr. L. A. Herzenberg, Stanford University, Stanford, CA; K, Dr. F. Kroese, University of Groningen, Groningen, Netherlands; O, Dr. K. Okumura, Juntendo University, Tokyo, Japan. Commercial antibodies: P, Pharmingen, San Diego, CA.

Table 27.6. Igh-1 allotypes in inbred mouse strains[a]

Igh-1a			Igh-1b		Igh-1c	Igh-1d	Igh-1e	Igh-1f	Igh-1g	Igh-1h	Igh-1j
BALB/cJ[b]	C58/J		C57BL/10J[b]	LP/J	DBA/2J[b]	AKR/J[b]	A/J[b]	CE/J[b]	RIII/J[b]	SEA/Gn[b]	CBA/H
BALB/cGa	F/Ao	POLY1/Ao	BAB/PoHz	SJL/J	DBA/1J		A/HeN	DE/J	DA/Hu	BDP/J	CBA/N
BUB/Bn	H-2G/Go	POLY2/Ao	B10.D2(new)	SM/J	I/Ao			N/Ao	FZ/Di	BSL/Di	C3H/H
CHI/Ao	JK/BI	PRUNT/Ao	B10.D2(old)	STA/Je	JB/Di			NH/N	STB/Je	P/J	C3H/SJ
C57BR/cdJ	MA/H	SJA	C57BL/H	WB/Re	RF/J					SEC/Gn	PL/J
C57L/J	MA/MyJ	ST/J	CWB/Hz	WC/Re							
	NZY/B1	STR/N	C57BL/Ka	WH/Rd							
	PBR/Ao	T6/H	C57BL/6J	WK/Re							
		129/RrGa	H-2H/Go	58N/Sn							
			H-21/Go	101/R1							

[a] Each Igh-1 allele is assigned to a type strain whose immunoglobulins, by definition, are the standard for comparison for that allele. Because the Igh loci are closely linked genetically, and because the largest number of alleles have been described as the Igh-1 locus, the type strain assigned to each Igh-1 allele is also assigned as the type strain for the entire Igh chromosome region of which that allele is a part. Alleles at subsequently discovered Ig loci are then named consistently with the Igh-1 allele, *i.e.* Igh-1 allotypes usually indicate the Ig haplotype of the strain.
[b] Type strain for each allele.

fact that this is not a completely accurate model of allelic exclusion.

Alleles and specificities of the Igh-C loci

The following sections detail the alleles and distribution of allotype specificities for each of the Igh-C loci identified in inbred mice. Where known, monoclonal antibodies recognizing the various allotypic determinants are noted and their use is described. Table 27.5 provides a list of readily available monoclonal antibodies specific for the Igh-C[a] or Igh-C[b] alleles for each allotypes. Sources for commercial reagent and/or cell lines are given. With the availability of these reagents it is easy for any investigator to perform allotype specific ELISA or FACS assays. Table 27.6 catalogs the Igh-1 allele of some seventy strains of inbred mice. As noted earlier, in most cases the Igh-C haplotype of a given strain is equivalent to the Igh-1 allele. However the alleles have not been characterized for every loci in all of the strains and investigators should confirm for themselves the alleles for their strains of interest.

The Igh-1 (IgG2a) locus

The Igh-1 locus is the most extensively characterized and the most polymorphic of all the immunoglobulin loci (Table 27.7).

Table 27.7. The Igh-1 locus

Allele	Type strain	Specificities															
a	BALB/C	1	2	–	–	–	6	7	8	10	–	12	13	14	15	–	17
b	C57BL/10J	–	–	–	4	–	–	7	–	–	–	–	–	–	–	–	–
c	DBA/2J	–	2	3	–	–	–	7	–	–	–	–	–	–	–	–	–
d	AKR/J	1	2	–	–	5	–	7	–	–	–	12	13	14	–	16	17
e	A/J	1	2	–	–	5	6	7	8	–	–	12	13	14	–	16	17
f	CE/J	1	2	–	–	–	–	–	8	–	11	–	13	–	–	16	17
g	RIII/J	–	2	3	–	–	–	–	–	–	–	–	13	14	15	–	17
h	SEA/Gn	1	2	–	–	–	6	7	–	10	–	12	13	14	15	–	17
j[a]	CBA/H	1	2	–	–	–	6	7	8	10	–	12	13	14	15	–	17

[a] Shown to be different from BALB/c by its positive reaction with a conventional antiserum; detected specificity was on Fab [44].

Table 27.8. The Igh-2 locus

Allele	Type strain	Specificities									
a	BALB/c	–	2	3	4	–	–	–	–	9	–
b	C57BL/10	–	–	–	–	5	–	–	8	–	10
c	DBA/2	1	–	–	–	–	6	7	8	–	–
d	AKR/J	–	–	3	–	–	6	–	–	–	10
f	CE/J	–	–	–	4	–	–	–	–	9	10
g	RIII/J	1	–	–	–	–	6	–	–	9	–

Table 27.9. The Igh-3 locus

Allele	Type strain	Specificities									
a	BALB/c	1	2	–	4	7	8	–	10	–	12
b	C57BL/10	–	–	–	4	7	8	9	–	11	12
d	AKR/J	1	–	3	–	7	8	–	10	11	12
e	A/J	1	–	3	–	7	–	–	10	11	–
f	CE/J	1	2	3	4	–	–	–	10	–	–
g	RIII/J	1	2	–	4?	–	–	–	10	–	12

Table 27.10. The Igh-4 locus

Allele	Type strain	Specificities		
a	BALB/c	1	–	3
b	C57BL/10	–	2	3
d	AKR/J	1	–	–

Table 27.11. The Igh-7 locus

Allele	Type strain	Specificities	
a	BALB/c	1	–
b	C57BL/10	–	2

Seventeen allotypic determinants define nine alleles. The specificities are described by Herzenberg et al [24], Potter and Lieberman [42] and Huang et al [43]. Specificity 1.17 is distinct from specificity 1.13 as the former is expressed on both Igh-1 and Igh-3 heavy chains, although the latter is found only on Igh-1 heavy chains [43]. Monoclonal antibodies detecting specificities 1.2, 1.4, 1.13, 1.14, 1.15, 1.16, and 1.17 have been described [43].

Comparison of DNA sequence of the Igh-1 locus from BALB/c and C57BL/6, the two most divergent alleles, showed that they differed by 10% at the nucleotide level and 15% for the deduced protein sequences [45]. These differences were not evenly distributed: most differences were in the hinge region and the CH3 domain. Using a panel of hybrid IgG2b-IgG2a immunoglobulins, a number of the allotypic determinants present on the Igh-1a (IgG2a) heavy chain have been localized to the CH2 and CH3 domains [46]. However, comparison of the amino acid sequences of the IgG2a H chain constant regions with genetically or physically distinct Igh-1a determinants failed to reveal straightforward correlation between individual allotopes and specific amino acid substitutions [47]. In fact, more allotypic determinants unique to Igh-1a molecules were found than would be predicted from a simple analysis of the sequence of Igh-1a, Igh-1b, Igh-3a, and Igh-3b proteins. Thus the tertiary structure of the immunoglobulin molecule likely plays a major role in the presentation of allotypic determinants [26] [also see The Igh-6 (IgM) Locus].

Some of the anti-allotypic monoclonal antibodies react with allotopes associated with the exon expressed in the secreted form of IgG2a [48]. These antibodies can be used in ELISA assays to measure serum immunoglobulin but do not recognize the membrane form of the molecule and cannot be used for cell surface immunofluorescence.

The close relationship between Igh-1 (IgG2a) and Igh-3 (IgG2b) is seen with respect to both sequence analysis and allotypic specificities. Sequence comparison of the Igh-1b and Igh-3b alleles of C57BL suggests that some of the allotypic specificities of Igh-1b have resulted from gene conversion of the Igh-1b allele by the Igh-3b allele [45]. This is supported by the fact that a mAb has been identified that reacts with an allotypic specificity shared by Igh-1a and and Igh-3a [43].

The Igh-2 (IgA) locus

Ten allotypic specificities have been identified that define the six known alleles of the IgA locus (Table 27.8). Specificities 2.1 to 2.8 were characterized using allo-antisera and are described by Herzenberg et al [24], Lieberman [23], Potter and Lieberman [42], and Tada et al [42]. Specificities 2.9 and 2.10 are defined by the monoclonal antibodies HY-16 and HIS-M2, respectively. Both mAbs can be used for both cell surface immunofluorescence and immunohistochemistry (Frans Kroese, University of Groningen, personal communication).

The Igh-3 (IgG2b) locus (Table 27.9)

Specificity 3.1 differs from specificity 3.10 because it is found only on IgG2b molecules. The monoclonal antibody detecting

Table 27.12. The Igh-6 locus

Allele	Type strain	Specificities					
a	BALB/c	1	2	–	–	5	–
b	C57BL/10	–	–	3	4	5	6
e	A/J	–	?	–	4	–	–
n (d)	NZB/B1J	–	2	–	4	5	6

specificity 3.10 also detects an allotypic marker of IgG2a molecules [43] (see The Igh-1 (IgG2a) locus. The specificities are described by Herzenberg et al [24], Potter and Lieberman [42], and Huang et al [43]. Monoclonal antibodies detecting specificities 3.4, 3.9, 3.10, 3.11, and 3.12 have been described [42].

The Igh-4 (IgG1) locus (Table 27.10)

Specificities are as described by Parsons et al [28]. Monoclonal antibodies detecting specificities 4.1 and 4.2 have been described [43]. The mAb recognizing the Igh-4a allele has been used successfully in ELISA, FACS, and immunohistochemistry assays. The anti-Igh-4b mAb has a significantly lower affinity and should be used with care [43].

The Igh-5 (IgD) locus

Five allotypic specificities have been identified that define three alleles of IgD [27] (see Table 27.3). Although originally characterized by allo-antisera [49, 50], monoclonal antibodies recognizing each of the known allotopes of IgD are available [27] (Table 27.5). Researchers using these antibodies should be aware that, for unknown reasons, mAbs that react with Igh-5b generally have a lower avidity/staining than anti-Igh-5a or -5e antibodies. Thus by flow cytometric analysis, B cells from BALB/c stained with anti-Igh-5.4 will appear to express more IgD than those from C57BL stained with anti-Igh-5.3. In fact, when analyzed with an anti-IgD isotypic antibody, both strains express equivalent levels of IgD (A. M. Stall, personal observation).

The relative location of the specificities on the δ chain has been determined by loss of mAb binding after limited trypsin digestion [27]. On the Igh-5a allele the 5.1 allotope is located on the Fab fragment and the 5.4 allotope is on the Fc fragment. Similarly, on the Igh-5e allele the 5.5 allotope is on the Fab although the 5.3 allotope in on the Fc. The location of Igh-5.2 is unknown.

The Igh-6 (IgM) locus (Table 27.12)

Studies using allo-antisera originally identified four allotypic specificities (6.1 to 4) which defined the a, b, and e alleles of IgM [16, 51]. The b and n alleles were originally distinguished based on allo-antisera recognizing specificities 6.2 and 6.3. Because these antisera were not tested on the Igh-C^d haplotype the allele for this haplotype could not be assigned. Similarly none of the anti-allotypic monoclonal antibodies can distinguish the Igh-C b, d, or n alleles. However, as noted earlier, recent studies suggest that the Igh-C^n haplotype is a the result of a recombinant between the Igh-C^d and Igh-C^e haplotypes. This is supported by the finding that AKR and NZB have essentially identical V region haplotypes (R. Riblet, personal communication). Thus it appears likely that AKR has the Igh-6n and not the Igh-6b allele of IgM. If formally proved this allele would be redesignated Igh-6d.

Within the last 10 years monoclonal antibodies that can distinguish the 6a, 6b, and 6e alleles have been produced. These antibodies are probably the most commonly used today because they can be used in adoptive transfer studies to identify both secreted antibodies and the cells that produce them [20, 21, 52]. The monoclonal antibodies, DS-1 [29] and RS-3 [53] have identical specificities recognizing the Igh-6.1 allotope. MB86 recognizes the Igh-6.4 specificity [54]; however, some care is required when using this antibody because it does not appear to recognize the allotope on IgM molecules containing the $\lambda 2$ light chain [55]. The Igh-6b allele can also be identified using the mAb, AF6-78 [27], which recognizes the Igh-6.6 specificity and is not affected by differences in light chain. As with the anti-Igh-5b mAbs, the anti-Igh-6b mAbs do not stain as brightly as the anti-Igh-6a mAbs. Two rat monoclonal antibodies, 331.12 [56, 57] and BET-1 [58], recognize the Igh-6.5 specificity although differing in their relative reactivity. Both antibodies are negative for the 6e allele; however, 331.12 reacts equally well with the 6a, 6b, and 6n alleles while BET-1 reacts strongly with the 6a allele and weakly with the 6b and 6n(d) alleles. In the past BET-1, based on this differential reactivity, has been used to distinguish Igh-6a and 6b alleles. Given the present availability of anti-Igh-6a monoclonal antibodies, the use of BET-1 as an anti-allotypic is not recommended.

The ability of small differences in amino acid sequence to elicit large differences in allotypic specificities is highlighted in the case of IgM. Although there are five distinct allotopes that distinguish the Igh-6a and 6b alleles, comparison of the nucleotide sequences of the μ genes from BALB/c and C57BL/6 reveals only a single amino acid difference in the CH1 domain [59]. As expected, all of the anti-Igh-6 allotypic antibodies tested bind to the CH1 domain [57]. Furthermore, Gause et al have shown that a single point mutation in CH1 of the Igh-6b allele can result in the loss of reactivity with anti-Igh-6b reagents and the acquisition of reactivity with anti-Igh-6a reagents. Thus a single amino acid difference can result in multiple antigenic allotypic epitopes.

Interestingly, in the case of IgM, all of the known allotopes are dependent on the expression of a light chain. Velardi et al have shown that none of the anti-IgM allotypic antibodies will react with isolated free μ heavy chain or with pre-B cells that express μ heavy chain in the absence of light chain [57]. Moreover, as noted earlier, the reactivity of the mAb, MB86, depends on the isotype of the light chain. To date all of the known anti-IgM allotype monoclonal antibodies react with $\mu + \kappa$ light chains, however, the reactivities of the various anti-IgM allotypic antibodies have not been tested on all possible combinations of μ and λ light chains. When studying non-conventional strains, investigators are advised to test the monoclonal antibodies on IgM antibodies associated with each of the light chain isotypes.

The Igh-7 (IgE) locus (see Table 27.11)

Two alleles of IgE were originally identified by Borges et al [17] using conventional alloantisera. DNA sequences comparison of IgE from BALB/c and B10.A revealed that there were 12 single-base changes that resulted in seven amino acid substitutions in CH3 (two) and CH4 (five) [60]. Numerous attempts to generate anti-allotypic monoclonal antibodies by conventional allo-immunizations have proved unsuccessful; however, recently Rat monoclonal antibodies specific for Igh-7.1 [61, 62] and Igh-7.2 [63] have been produced. These antibodies show strong allotypic specificity and can be used for ELISA and immunofluorescence assays and

Table 27.13. Igh-C congenic strains

Strain	Inbred background	Source of Igh-C locus	Number of backcrosses	References	Source[a]
C.B-20[b]	BALB/c	C57BL/Ka	20	[22, 29]	1
C.B-17[b]	BALB/c	C57BL/Ka	17		2
BAB/25[b]	BALB/c	C57BL/Ka	25	[16, 29]	3
C.D2-11	BALB/c	DBA/2	11		5
C.A-21	BALB/c	A/J	21		2
C.AL-20	BALB/c	AL/N	20	[20, 29]	1
B.C-20	C57BL/Ka	BALB/c	20	[22]	1
SJA/9	SJL	BALB/c	9		4
SJA/20	SJL	BALB/c	20	[16]	3
CBA.Igh[b]	CBA/Tufts	C57BL/6	14	[61]	6

[a] Sources: 1, The Jackson Laboratory, Bar Harbor, ME; 2, Dr. R. Riblet, Molecular Biology Institute, La Jolla, CA; 3, Dr. L. A. Herzenberg, Stanford University, Stanford, CA; 4, Dr. K. Okumura, Juntendo University, Tokyo; 5, Dr. M. Potter, NIH, Bethesda, MD; 6, Dr. H. Wortis, Tufts University, Boston, MA.

[b] See text for the relationship of these strains.

for allotype-specific inhibition of passive cutaneous anaphylactic (PCA) reactions [63].

The Igh-8 (IgG3) locus

Despite numerous attempts by a number of investigators, no allelic differences in IgG3 have been identified in inbred strains of mice. Inasmuch as nucleotide sequences of the γ3 gene are not available for BALB/c and C57BL, it is not clear whether this is due to a lack of polymorphic differences or an inability to generate appropriate allo-antisera. Huang et al generated xenogeneic rat anti-mouse IgG3 monoclonal antibodies that recognized at least three different epitopes on BALB/c IgG3 molecules [3]. Although these antibodies detected no polymorphism among 40 inbred strains tested, genetic polymorphism of IgG3 isotype were found among wild mice and form the basis for designating this locus as Igh-8.

Igh-C congenic strains of mice

The usefulness of monoclonal anti-allotypic antibodies as tools in studies of B cell development has been greatly facilitated by the ready availablity of Igh-C congenic strains of mice. Two inbred strains that are genetically identical except for a single chromosomal segment (including the gene locus of interest) are referred to as *congenic* strains. Congenic mice were first developed by Snell [64] to study histocompatibility genes. The strains have proved to be a major tool in the dissection of the role of the MHC complex in the immune system. Similarly, the development of Igh-C congenic strains has greatly facilitated the study of the genetic polymorphisms of immunoglobulins and the role that Igh-C linked genes play in the regulation of the immune system [65]. Today, Igh-C congenic strains are major tools for adoptive transfer experiments in the study of B cell ontogeny and development [20, 21, 66–68].

Congenic strains are developed by the recursive back-crossing of mice heterozygous for the gene locus of interest to the background strain. As an example, C.B-20 mice are considered to be identical to BALB/c except for the segment containing the Igh-C^b loci derived from C57BL/Ka. BALB/c were crossed with C57BL/Ka to produce F_1 progeny that were then back-crossed to the parental BALB/c. The progeny of the back-crossed were typed for the Igh-C^b allele, and the positive animals (Igh-$C^{a/b}$) were again back-crossed to BALB/c. This process is repeated for many generations, after which the Igh-$C^{b/b}$ homozygotes are selected and maintained by brother-sister matings. A minimum of nine back-cross generations are needed for the strains to be considered congenic. After 20 back-crosses, the probability is >99% that the two strains will be homozygous for loci of more than than 20 map units from the locus of interest–in this case, Igh-1 [69].

Two sets of nomenclature have been used for Igh-C congenic strains. In the standard genetic system the strain is designated by the background strain followed by a period, the gene locus introduced and the number of backcross generations. In this system the C.B-20 strain would be designated BALB/c.Igh-1^b/N20; where the Igh-1b locus of C57BL was backcrossed 20 generations onto a BALB/c background. In the second system used primarily for Igh-C congenic lines, the strain is designated by an abbreviation for the background strain (such as C for BALB/*c*), followed by an abbreviation for the donor strain (B for C57*B*L), followed by the number of backcross generations. Thus a strain designated BALB/c.Igh-1^b/N20 in the first system would be designated C.B-20. A number of Igh-C congenic strains that have been developed are listed in Table 27.13.

In general Igh-C congenic mice are identical for both the immunoglobulin constant region genes *and* the variable region genes (Igh-V). However, in contrast to the Igh-C loci, a number of recombinational cross-overs have been observed within the Igh-V loci. This was first observed in the development of the BALB/c. Igh-Cb congenic strains (C.B-20, C.B-17, and BAB/25). Dr. Micheal Potter had backcrossed the Igh-1b locus of C57BL/Ka onto BALB/c for 13 generations. At that point mice were sent to the Fox Chase Cancer Center and Stanford University. The Fox Chase line was backcrossed four more generations and then inbred. This line became C.B-17. The Stanford line was backcross once, inbred, and originally designated BAB/14. (It has since been backcrossed to the 25th generation and is now designated BAB/25). The original Potter line was backcrossed to the 20th generation and is designated C.B-20. Given their common heritage it was assumed that these strain were essentially equivalent and allotypic analysis comfirms that all strains carry all of the Igh-C^b loci. However, analysis of anti-Dextran antibodies showed that BAB/14 (and now BAB/25) expresses idiotypic markers of both BALB/c and C57BL [23, 70]. Thus BAB/25 like BALB/c (but not C57BL) can respond to α(133,136) dextran. The cross-over appears to have occurred within the V_H7182 loci of the Igh-V region, giving BAB/25 most of the V_H genes from BALB/c (R. Riblet, personal communication). Both C.B-17 and C.B-20 appear to have the Igh-V loci of C57BL.

Other differences of unknown origin can appear in congenic stains. The SJA/9 line was generated by backcrossing the Igh-1a locus of BALB/c onto SJL for nine generations before inbreeding. The line also continued to be backcrossed to the 20th generation generating the SJA/20 line. Analysis of the levels of natural and antigen-induced serum IgE found that although SJL and SJA/20 mice have similar low levels of IgE, SJA/9 mice produce little or no serum IgE [71, 72]. Thus, although congenic strains can be powerful tools for the study of immune regulation and B cell development, investigators cannot always assume that two Igh-C congenic strains are immunologically identical except for their allotypic markers.

Table 27.14. Allotype immunization protocols

Step 1: Immunization of allotype donor				Step 2: Immunization of recipient for production of anti-allotype serum	
Antigen	Protocol	Dose and timing	Bleed	Dose and timing	Bleed
B. pertussis heat killed[a]	I	D 1: total of 2×10^9 bacteria in 0.2 ml saline injected i.p. and in four places s.c. D 3 and 5: same but in four places s.c. D 21 and 37: same as d 1	Pool bleed D 25 D 32 D 45	Days 1, 3, 5, 22, 26, 50, 57: 10^9 bacteria + 20 ml antiserum from step 1 in 0.2 ml saline per mouse. Mix at room temperature and inject i.p. Boost as on d 1	D 64 and weekly. Bleed individually and test, or pool bleed depending on circumstances. Boost when titers go down. Ten days after boost, bleed and test.
B. pertussis heat killed	II[b]	Mice supplied with drinking water containing 2×10^{10} bacteria/500 ml for 30 d (5 mice per cage)	D 30; bleed. Check titer by bacterial agglutination. Check isotype of antibody[c]. Pool blood weekly	D 1, 3, 5, 21, 37, and monthly booster. Incubate serum for step 1 with number of bacteria just sufficient to completely absorb anti-*pertussis* activity, 3 h at 37°C and overnight in cold. Wash three times with cold saline. Resuspend in saline to 2×10^{10}/ml. Mix with equal volume of CFA[d]. Inject 0.2 ml	D 45 and weekly, test individually
H-2	III	1/5 spleen/animal in isotonic MEM injected i.p. Boost with same monthly	Bleed 3 wk after injection, then weekly	D 1: 20 ml antiserum from step 1 + 20 ml CFA[d] injected i.p. and four places s.c. D 21, 28, and monthly booster: 10 ml in 0.2 ml saline i.p.	D 35 bleed and test. Pool bleed all positive animals weekly
–	IV[e]	–	–	D 1, 8, 15, 22: 10^7 spleen cells from strain differing for Ig allotype and H-2; boost monthly thereafter	Fuse for hybridomas 3 d after last immunization
–	V	–	–	D 1, 75 μg in CFA s.c. and footpad; d 4, 7 in IFA s.c. and footpad followed by 9 weekly boosts; rest 4–8 and boost with 75 μg i.v.	Fuse for hybridomas 3 d after last immunization

[a] From Lederle Laboratories.
[b] Protocol established by Dr. Tohru Masuda.
[c] Incubate antiserum plus *B. pertussis*; wash. Test conjugates in radioimmunoassay. Use sera whose complexes show high level of IgA and low level of other immunoglobulins.
[d] Complete Freund's adjuvant.
[e] Protocol established by Goding, Warr and Warner [15], modified by Stall and Loken [27].

Generation and characterization of allotypic specificities

The preparation of anti-allotype reagents is an art bordering on a science. As a guideline for the novice venturing into this field, a summary of the experiences of investigators in this field is shown in Tables 27.14 and 27.15. These observations are not the result of exhaustive testing and should not be considered definitive. Minor changes in dosage or timing do not appear to be critical. A great deal of additional information on techniques of immunization may be found in the literature, especially in the work of Potter and Lieberman [42]. Fortunately, over the past 15 years monoclonal antibodies have been generated to many allotypic specificities. In particular monoclonal antibodies to all of the alleles of the Igh-Ca and Igh-Cb haplotypes are readily available (see Table 27.5).

Allogeneic anti-allotypic antibodies

For the generation of allogeneic (mouse anti-mouse) anti-allotypic antibodies it is not only important to choose an appropriate protocol for preparing the immunogen to elicit antibody to a particular allotype, but also the choice of recipient mouse strain can considerably influence the success of the immunization. As many investigators involved in the preparation of anti-allotype sera (notably Drs. R. Lieberman and M. Potter) have noted, 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. Thus there may be key genetic factors controlling the immune response to allotypic antigens [73].

Mouse anti-mouse immunoglobulin immunizations: IgG, IgA, and IgM. Although the protocols discussed below were developed for making conventional antisera, the same considerations apply to immunizations for the generation of monoclonal antibodies. While normal serum or normal immunoglobulin can be used as antigen to stimulate anti-allotype antibody, far better results have been obtains by immunizing with antisera or antibodies directed

Table 27.15. Allotype immunization results[a]

Anti-allotype serum	Anti-allotype-producing strain	Allotype donor strain	Protocol no.[b]	Antigen for allotype donor	Reaction with					
					Ig-1	Ig-2	Ig-3	Ig-4	Ig-5	Ig-6
b anti-a	(LP/J or C57B1/10)	(BALB or CSW)	I	*B. pertussis*	+++[c]	–	+	++		
b anti-a	SJL/J	(BALB or CSW)			+++	o.w.	+	+++		
n anti-a	NZB	BALB	II	*B. pertussis*	++	++	+++	–		
d anti-a	AKR	BALB			–	++	o.w.	–		
b anti-a	C57BL/10	BALB			+++	++	–	++		
b anti-a	C57BL/10	BALB	III	H-2	++	o.w.	o.w.	–		
b anti-a	LP/J	BALB			+++	o.w.	o.w.	–		
a anti-b	BALB/c	(C57/BL/10 or LP/J)	I	*B. pertussis*	+++		o.w.	+		
a anti-b	C3H.SW	C57BL/10	III	H-2	+++		o.w.	++		
a anti-b	BALB	C57BL/10			+++	–	–	–		
b anti-c	C57BL/10	DBA/2	III	H-2	++	++				
a anti-c	C3H	DBA/2			+/–	–				
d anti-c	AKR	DBA/2			+/–	–				
b anti-d	LP	AKR	III	H-2	++					
b anti-d	C57BL/10	AKR			++					
c anti-d	DBA/2	AKR			++					
a anti-n	BALB/c	NZB	III	H-2	++		o.w.			
b anti-e	C57BL/10	A/J			+++		o.w.			
a anti-b	SJA	BAB/20	IV	spleen cells					+++	+++
b anti-a	SJL	BALB/c	IV						+++	+++
b anti-e	C57BL/10	A/J	IV						+++	
a anti-b	BALB/c	C57BL/10	IV						+++	+++

[a] Blank spaces indicate no testing data because of unavailability of isolated antigens, but may be presumed negative or weak positive because no unaccountable lines appeared in Ouchterlony testing against normal sera with these antisera. No antibodies to IgM or IgD have been seen with protocols I to III.

[b] Incubate antiserum plus *B. pertussis*; wash. Test conjugates in radioimmunoassay. Use sera whose complexes show high level of IgA and low level of other immunoglobulins.

[c] Antibody activity was surveyed by Ouchterlony analysis or ^{125}I precipitation. Those reactions scored + or ++ are often difficult to detect in Ouchterlony tests. o.w., occasional animals producing weak antibody reactions.

to some tissue or protein component in the recipient strain (see Table 27.15; protocol III). The resulting antigen:antibody complexes render the weakly antigenic allotypic determinants much more immunogenic. For example, whereas BALB/c mice immunized with normal C57BL/6 serum failed to produce any detectable anti-Igh-1b antibodies, immunization of the same strain with C57BL/6 anti-DBA/2 spleen antiserum (which reacts with cell surface determinants on BALB/c cells) was quite successful (BALB/c and DBA/2 are both H-2^d). Although this method of immunization is particularly effective for raising antibody reactive with Igh-1 (IgG2a) allotypes, it results only in low antibody titers for all other allotypes in an occasional animal. However, immunization of C57BL/6 with DBA/2 Ig frequently generates antisera that react well with Igh-2 (IgA) in addition to Igh-1.

More effective immunization for allotypic determinants of other isotypes has been obtained by challenging the animals with immunoglobulins in the form of an externally created antigen-antibody complex. In this method established by Dresser, Taylor, and Wortis, *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 anti-allotype response (see Table 27.15; protocol I). This method has proved excellent for obtaining antibody reactive with Igh-4 (IgG1) allotypes as well as Igh-1 allotypes. To a lesser extent, it has been useful for producing antibody to Igh-3 (IgG2b) allotypes.

Unless the *B. pertussis* is administered orally, no antibody reactive with Igh-2 (IgA) allotypes is produced by this procedure. By administering the *B. pertussis* orally, an anti-*pertussis* serum demonstrably rich in IgA antibody is generated. When combined with *B. pertussis* and administered as described above, this serum elicits excellent antibodies to Igh-2 allotypes, in some cases with little or no contamination with antibodies to allotypes of other isotypes (see Table 27.1; protocol II).

Monoclonal anti-Igh-6 allotypes have been generated using purified IgM in a Freund's adjuvant emulsion as the immunogen [29] (Table 27.14; protocol V). In many cases anti-Igh-6 antibodies may not appear until two to three months into the immunization protocol. Appearance can be followed by ELISA. In general, it is not worth trying to generate monoclonal antibodies unless reasonable anti-allotypic titers are detectable in the serum of an animal.

When monoclonal antibodies are used as an immunogen, anti-idiotypic antibodies are often generated in addition to any anti-allotypic antibodies. For this reason, when screening for anti-allotypic antibodies in the serum or after the generation of hybridomas, always use a panel of (monoclonal) antibodies that is different from the immunogen.

Mouse anti-mouse spleen cell immunization: IgM and IgD. Antibodies directed against allotypic specificities of IgM (Igh-6 and IgD (Igh-5)) are effectively generated by immunizing mice with Igh-C and H-2 incompatible spleen cells. The difference in H-2 appears to be required to obtain a good response to IgM. Four weekly injections of 10^7 spleen cells, followed by a boost one month later, result in a vigorous anti-IgD response. Alloantibodies to IgM are more difficult to generate and may require two to

Table 27.16. Allotyping methods

Method	References
Electrophoretic analyses of Fc fragments	[12]
Ouchterlony analysis	[21] [43]
Passive hemagglutination	[74, 75]
Immunofluorescence staining	[27]
Radioimmune precipitation	[21, 47]
Solid-phase radioimmune competition and cobinding assay	[32, 76, 77]
ELISA	[78]
DNA restriction enzyme site polymorphisms	[31]

three subsequent monthly boosts (see Table 27.14; protocol IV) [16, 27].

Analyzing the alloantisera (or hybridomas) produced by this immunization scheme poses unique problems. Antibodies reactive with a variety of cell surface antigens (H-2, I-A, etc.) as well as IgD and IgM, are produced. Alloantibodies to IgM can be identified by conventional solid-phase RIA or ELISA assays. This is, however, not generally feasible for IgD and other methods must be used (see section on *immunofluorescence staining*).

Heterologous anti-allotypic antibodies

As noted earlier, heterologous (such as goat or rabbit) anti-mouse isotype antisera can contain substantial levels of anti-allotype-specific antibodies. Although these antisera can be made allotype specific with appropriate absorption, with the availablity of mAbs, the use of such antisera for allotyping is not recommended. The methods involved in the purification and absorption of allotype-specific heterologous antisera are discussed in earlier editions of this handbook [24].

Rat anti-mouse allotype immunization: IgM and IgE. The exceptions to the use of heterologous anti-allotypic antibodies are monoclonal rat anti-mouse allotype antibodies. As with any heterologous response, most of the antibodies produced will be directed against isotypic determinants. However, a small fraction will be allotype specific. By producing hybridomas, these allo-reactive antibodies can be isolated and carefully tested for their allotypic specificity. Anti-Igh-7 [62, 63] and anti-Igh-6 [29, 58] allotype mAbs have been produced using cells (protocol IV) [58] or purified Ig (protocol V) [29, 62, 63] as immunogens.

A potential problem with such mAbs is that they may only have an allotypic bias rather than true allotypic specificity. For example, the mAb, BET-1 [58], has a 10- to 20-fold higher affinity for the Igh-6a allele than for the Igh-6b or 6n alleles but does not react with the Igh-6e allele. When used at high concentrations, BET-1 will react with Igh-6b but not with Igh-6e. In contrast the mAb, 331.12 [56], reacts equally with the Igh-6a, 6b, and 6n but not 6e. In general, it is not recommended that mAbs such as BET-1, which only show a relative affinity difference, be used for the purpose of distinguishing allotypes.

Assays for detection of anti-isotype and anti-allotype antibodies, and measurement of immunoglobulin isotype and allotype levels

The basic methods that have been used to identify allotypic specificities (determinants) are presented in Table 27.16 and in many cases described elsewhere in this handbook. In essence, to identify new allotypic specificities by these classical methods, anti-allotype antisera or monoclonal anti-allotype antibodies raised in a particular inbred mouse strain are tested for reactivity with Ig (usually purified isotypes) from other mouse strains.

In general, new reagents (allo-antisera or monoclonal antibodies) whose reactivities mimic a previously defined pattern are treated as duplicates of the original reagent. This is true in an immunogenic sense because no new genetically distinct specificities can be defined with the second reagent. However, as noted earlier, there is no *a priori* reason to expect that the old and the new reagents detect the same allotpes, that is, a pair of such reagents could detect physically separate determinants that are inherited together in all animals available for testing. Although conventional allo-antisera do not lend themselves easily to the detection of such determinants, studies with monoclonal anti-allotype antibodies have revealed several sets of such "duplicate" antibodies. By definition, two monoclonal antibodies recognize different allotopes if they a) can be shown to bind to different proteolytic fragments of the heavy chain [25, 27] and/or b) each fail to block the binding of the other monoclonal antibody [26, 27].

Several types of assays can be used for the detection of isotype and allotypic determinants on mouse immunoglobulins or the antibodies to them. With the proper standardization, each of these assays can be used for quantitative estimation of immunoglobulin levels; however, each assay has characteristics that make it more suitable for particular purposes. For example, Ouchterlony or hemagglutination assays are rapid, semiquantitative and relatively insensitive, although ELISA and radioimmunoassays are somewhat more laborious but also considerably more sensitive and accurate. A list of the techniques available for allotyping, as well as references that fully describe their application, is given in Table 13. Only those special modifications necessitated by the comparatively small volumes of reagents and test sera available from mice are presented.

Double diffusion (Ouchterlony) analyses

Double immunodiffusion analyses rely on the presence of multiple reactive determinants on the antigen molecule, so that each antigen binds several antibodies. When the bivalent antibodies in turn bind a second antigen molecule, a network of antigen and antibodies is built up that eventually precipitates. Because monoclonal antibodies react with only one epitope per heavy chain, they rarely cause the formation of a sufficient lattice to effect precipitation. Thus, although especially useful with conventional antisera, this technique is not generally recommended for use with monoclonal anti-allotype antibodies; however, some monoclonal antibodies can be used. Investigators should test each mAb individually for its usefulness in their system. The use of anti-allotypic antibodies in this assay is no different from other antisera [24].

Hemagglutination techniques

These assays are conveniently performed in V-bottom 96-well microtiter plates. For passive hemagglutination, erythrocytes coated with either non-agglutinating ("incomplete") or subagglutinating levels of antibody are agglutinated by anti-allotype antisera of the appropriate specificity. Sheep erythrocytes can be coated with mouse anti-sheep erythrocyte antibody or mouse erythrocytes can be coated with antibody raised in one mouse strain to the H-2 antigens of the erythrocyte donor. Erythrocytes

are coated by incubation with antibody at a non-agglutinating dilution and then washed several times to remove the free immunoglobulin that, because it too carries the allotype, would otherwise inhibit the reaction. After washing, coated cells are incubated with dilutions of anti-allotype antibody and read in standard fashion. This technique requires that allotyping be performed on the appropriately immunized animals and that the isotype compositions of the test anti-erythrocyte antisera are appropriate for the anti-allotype reagents used.

An alternative approach is the hemagglutination inhibition assay. This method, modified from the techniques of Lieberman [79], is quite simple and sensitive. Because of its speed and convenience, it is particularly useful for screening large numbers of mice, but is less useful for careful quantification 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 indicates the lack of the particular allotype in the added sample, although inhibition of agglutination indicates allotype-positive material in the sample.

Immunofluorescence staining

Analysis of allotypic markers of cell surface immunoglobulins, notably IgM and IgD, is most conveniently performed using immunofluorescence staining. IgG allotypes on B cells are also identifiable by immunofluorescence staining and FACS; however, because of the low frequency of IgG-bearing cells in spleen, FACS separation and functional testing is required to unequivocally demonstrate the presence of allotypic determinants on B cell membranes [80].

Staining procedures using monoclonal anti-allotypic antibodies do not differ significantly from other immunofluorescence staining protocols. For indirect staining, as in other two-step assays, great care must be taken that the reactivity pattern of the second antibody is appropriate for the isotype of the monoclonal anti-allotypic antibody. For example, a rabbit anti-mouse IgG that reacts primarily with IgG2a and IgG2b will not be useful in assays using an IgG1 anti-allotype antibody. Also, when working with B cells, the second antibody must not recognize IgM or light chain determinants. Many heterologous anti-IgG reagents have low levels of anti-κ reactivity–even after absortion. For such reagents every lot and every vial should be tested for background staining on spleen cells of the appropriate mouse strain. Today, monoclonal rat anti-mouse isotypes are available and are preferable for use as second step reagents when necessary. However, when possible, staining with directly conjugated reagents is preferable to indirect staining in that backgrounds are often substantially lower in comparison to the amount of specific staining obtained.

ELISA and radioimmunoassays

Anti-allotypic antibodies are most commonly used in ELISA and radioimmune assays (RIA) for the detection of allotypic determinants, quantification of serum allotype and isotype levels, and measurement of allotype representation in antibody responses in immunoregulatory or mixed adoptive transfer studies. In this section general considerations for the use of anti-allotypic antibodies in RIA/ELISA assays are discussed. For more detailed methodological discussions of these types of assays, investigators should refer to other chapters in the handbook.

Measuring allotype and isotype representation in antibody responses

In measuring antibody responses to antigens, the use of monoclonal anti-allotypic antibodies as revealing antibodies in antigen-specific direct-binding assays is no different than using any anti-immunoglobulin reagent. Biotin- or direct alkaline phosphatase-conjugates are commercially available for many anti-allotype mAbs (see Table 27.13). If investigators need to produce their own reagents, biotin-conjugates are recommended because of their reproducibilty and ease of preparation. Allotype-specific responses to DNP-BSA [35], PC-BSA, and Dextran [81] have all been successfully measured using direct-binding assays. The only consideration in these assays is the relative affinity of a given anti-allotypic mAb. As noted in the sections on allotypic specificities, some mAbs (notably anti-Igh-4b and anti-Igh-6b) have a significantly lower affinity and must be used with care. In any case, standard curves must be run for *each* anit-allotypic mAb.

Measuring allotype and isotype levels in serum and culture fluids

The measurement of serum allotype levels is again similar to the general measurement of isotype level. The most common assay is a direct-binding sandwich assay in which an unlabeled monoclonal anti-allotypic antibody is used to coat the microtiter plate and a labeled conjugate of the same mAb is used to reveal the bound immunoglobulin (allotype). The advantage of this assay is that the same mAb is used, minimizing reagent preparation. The main disadvantage is that for IgG, because each Ig has only two allotopes (one on each heavy chain), the binding of one anti-allotypic mAb can sometimes block the binding of a second (or revealing) identical mAb. For this reason, each monoclonal anti-allotypic must be tested for its ability to be used in a sandwich assay. Successful results have been obtained in using DS-1 (anti-Igh-6a), AF6 to 78 (anti-Igh-6b), HY-16 (anti-Igh-2b), and Ig(1a) 8.3 (anti-Igh-1a).

An alternative approach is to perform an "asymmetrical" sandwich assay, in which the plate-coat mAb and the labeled revealing mAb recognize different allotopes on the same alleles [78]. Again the two mAbs must be tested in cobinding [43] or blocking [27, 43] experiments to make sure that there is no interaction in the binding of the two mAbs.

The limitations in using monoclonal anti-allotypic reagents for the detection of serum allotype levels can also be overcome by using a competition ELISA/RIA assay. In this form of the assay, purified monoclonal anti-allotype antibody is coated on the wells of a polyvinyl chloride U-bottom microtiter plate. Test sera at various dilutions are then added, with an enzyme-/radiolabeled myeloma or hybridoma protein carrying the relevant allotypic determinant. After one hour, the wells are rinsed and assayed for bound label that is either enzymatic activity or radioactivity.

If the allotypic determinant being assayed is present in the test serum, it will compete with the labeled protein for antibody sites on the antibody-coated well. Thus it will block the binding of the labeled material and the extent of blockage will reflect the relative concentration of the labeled and unlabeled protein. In allotype homozygous animals, the use of this assay with anti-allotype antibodies is equivalent to quantification of the immunoglobulin isotype on which the allotypic determinant is carried.

The amount of the allotype (or isotype) in the test sample is

determined by comparison of the inhibition with that obtained with serial dilutions of an antigen standard. This allows quantification of the allotope in the sample. Plotting the reciprocal of the number (or percentage) of counts bound against the amount of unlabeled protein yields a straight line standard curve for this assay, because the amount of label bound is directly proportional to the specific activity of the protein in the well (provided that the labeled and unlabeled proteins each carry the determinant detected by the bound antibody). Departures from linearity usually mean that the radio-labeled protein is sticking non-specifically to the well. (Ultra-centrifuging (in an Airfuge) reagents for these assays usually prevent this type of non-specific binding.) The theory underlying this method of plotting data has been discussed in detail earlier [24, 77].

Detection of allotype secretion by single cells

Using the enzyme-linked immunospot (ELISPOT) assay it is possible to detect and ennumerate the numbers of cells secreting immunoglobulin of a specific isotype or of a defined antigen specificity [82]. In essence, an ELISPOT assay is identical to an ELISA assay except that the antibody captured on the plate is secreted by single cells and that the enzyme substrate is added in agarose to localize the colored enzymatic products to the site of the cell. The author has successfully used anti-Igh-6a, Igh-6b, and Igh-2a mAbs in both allotype-specific sandwich assay and in antigen-specific direct binding assay [81]. All the considerations and constraints involving the use of anti-allotypic antibodies in ELISA assays apply equally to ELISPOT assays.

Acknowledgment

This chapter is derived in part from earlier versions coauthored by Drs. Leonore and Leonard Herzenberg. Their contributions to this chapter, to our understanding of murine immunoglobulin allotypes, and to the author's interest in the subject are gratefully acknowledged.

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