LAH 263

Chapter 97
Mouse immunoglobulin allotypes

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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 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, all known mouse Ig isotypes can now be distinguished with isotype-specific antisera raised in another species (usually goat or rabbit) and absorbed to remove contaminating antibodies reactive with other mouse H chains or with L chains. In addition, many of the mouse isotypes can be distinguished with isotype-specific monoclonal antibodies raised in the rat.

There are eight known H-chain isotypes in the mouse, each associated with particular biological activities (see Table 97.1). Seven of these isotypes are routinely present in sera from virtually all mice. The eighth (IgD) is difficult to detect in sera but is routinely present on the majority of the B lymphocytes found in spleen, lymph node and peripheral blood. Clonally derived immunoglobulin-secreting cell lines (myeloma tumours and hybridomas), which can produce large amounts 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) [2,3]. The constant region genes, which determine H-chain isotype structure, are tightly clustered in a single region (the Igh-C region) of chromosome 12 [4]. 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

The segment of DNA which codes for a single polypeptide 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. The immunoglobulins encoded by such alleles of an Igh-C locus are referred to as *allotypes*. Certain of the Igh-C loci are highly polymorphic, with as many as nine alleles identified, while 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 1962 [5,6], several years following the identification of genetic polymorphisms in rabbit [7] and human [8] Ig. The availability of numerous inbred strains of mice, each with a genetically fixed set of allotypes, greatly facilitated the study of these polymorphisms. Like the

Table 97.1. Mouse immunoglobulin isotypes

Isotype	Locus	Number of alleles known in inbred strains	Number of genetic specificities described	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
IgG1(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			Does not fix complement; does not fix to skin; passes placenta

Ig isotypes, mouse allotypes have been identified by physical properties such as electrophoretic mobility [9] or by their reactivity with antibodies raised in goats or rabbits. Typically, however, they are detected by their distinctive reactivity patterns with antibodies 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 alloantigenic determinants (*allotypic specificities*), as identified by alloantisera 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 [10,11] and in humans [12] 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 [9,13]. 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 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 evidence from molecular studies divided the Ig chromosome into separate C, V, D and J 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 H chain constant region structure emerged at this point.

Ever 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, while 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].

Terminology

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 which 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 functional molecules in biological assays such as complement fixation. Allotype names (e.g. Igh-6 or Igh-1), in contrast, are usually used in discussions where the genetic (allelically determined) differences between immunoglobulins or antibodies are important, e.g. 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 (e.g. 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. Thus the IgA H chains encoded at the Igh-2 locus, for example, are all called α chains regardless of which Igh-2 allele encodes them (Table 97.1).

Attempts to simplify this isotype/allotype notational jungle have met with little or no success; however, agreement has been reached on a standard system for the genetic nomenclature for the Ig loci [20]. 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 97.1. For descriptions of the previous systems of Ig allotype nomenclature, the reader is referred to earlier reviews of mouse Ig genetics [21,22].

Definition of H-chain loci

Upwards of 5000 progeny of appropriate crosses have been examined in detail, yet no direct evidence of a cross-over between Igh-C loci has been found [20]. Thus, by the classical 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, but rather 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 controlling them are inherited 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.

Immunoglobulin H chains of the different isotypes were shown clearly to differ structurally from each other, possessing unique antigenic markers and unique amino acid sequences. Also, genetic studies detailed below showed that the H-chain loci structures are all inherited as a single haploid set. Therefore each isotype was considered to be determined at a separate locus. The existence of individual loci for each of the Ig isotypes has been confirmed recently by molecular biological analyses, which show that the constant region of each H-chain isotype is encoded in the genome by a unique series of exons. The order (5′ to 3′) of the individual gene loci within the Igh-C region is Igh-6(μ), Igh-5(δ), Igh-8(γ3), Igh-4(γ1), Igh-3(γ2b), Igh-1(γ2a), Igh-7(ε), and Igh-2(α) [2].

Demonstration of alleles at Igh-C loci

As previously stated, 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.

Conventionally, a locus is not named until at least two alleles have been identified, i.e. until a polymorphism exists for a trait; however, as indicated above, once polymorphisms were demonstrated for several Igh-C loci, the existence of the remaining structurally distinct isotypes was taken as prima-facie evidence for the presence of Igh-C loci encoding those isotypes [23]. Thus, in its early days, the Ig chromosome region had several putative loci defined before allotypic markers were identified and classical genetic analyses were complete.

Allotypes have been identified for all Igh-C loci. The minimal criteria for allelism of the genes coding for these allotypes in each case (except for the recently discovered IgG3 alleles) have been met by genetic studies; i.e. two inbred mouse strains, each with a different H-chain allotype for a given isotype, were crossed to obtain heterozygotes which produced 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 co-dominant alleles were found, indicating that a single haploid set always carried either the allele donated by the mother of the heterozygotes or the allele donated by the father (never both; never neither) [13]. These studies demonstrating allelism also show that a single haploid set carries each of the identified H-chain loci. Such a unique set of Ig H-chain alleles, inherited as a group, defines an Igh-C *haplotype*.

Demonstration of genetic linkage between Igh-C loci

In crosses where the parental strains had allotypic differences at two or more H-chain loci, back-cross and intercross testing showed that the loci in a given haploid set were always inherited together. In early studies, this evidence 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. Later genetic studies demonstrated that the remaining three polymorphic loci were also tightly linked to the same gene complex now designated Igh-C. Most recently, molecular biological analyses have placed all of the H-chain loci within a chromosome region that is 200 kbp long [3]. This close genetic linkage has considerable biological importance in the differentiation of immunoglobulin-producing cells.

Although the testing of a large number of progeny

Table 97.2. Distribution of alleles of the Igh loci in the Igh haplotypes

		Immunoglobulin locus and heavy chain isotype*						
Haplotype	Prototype strain	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	b/n	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$	?	?	a	a	k	?	c
l	KH-2$	?	?	a	a	l	?	c
m	Ky$	?	?	b	b	m	?	b
n	NZB	n	a	d	e	e	?	d
o	AL/N	e/?	e	a	d	d	?	?
p	SWR/J	?	?	?	f	c	?	?

* The loci are listed in the order that they occur on chromosome 12 of the Balb/c strain.
? The strain has not been typed for all specificities at this locus.
$ Haplotypes derived from wild mice.

(over 5000) failed to yield direct evidence for crossing-over between H-chain loci [20,21,22], three strains of inbred mice (NZB, AL/N and AKR) resemble natural recombinants [24,25] (Table 97.2). Interestingly, the recombination in each of these three strains appear to have occurred between the Igh-5 and Igh-3 loci. This may indicate a recombinatorial 'hot spot' similar to that identified in the H-2 gene complex [26]. Furthermore, gene sequencing data [27] and serological analysis [28] suggest the past occurrence of recombination or gene conversion events in wild mice. 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 [27].

Loci that control variable region structure (idiotypes) have also been linked to the Igh-C region on chromosome 12. Several recombinations between Igh-V markers and the Ig region have been observed [29]. Genes encoding at least four T cell surface molecules have also been closely linked to the Ig chromosome region [30]. In addition, a locus (Pre) controlling pre-albumin structure has been shown to be linked to this region [31]. The order of these genes on chromosome 12 is Igh–V—Igh–C—(T cell antigens)—Pre [30].

Haplotype (allelic) 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. Like heterozygotes for most co-dominant genes, allotype heterozygotes typically produce about equal amounts of each parental allotype. Surprisingly, however, the co-dominant production of allotype observed in the serum is not seen at the cellular level, i.e. individual immunoglobulin-producing cells in allotype heterozygotes produce either the paternal allotype or the maternal allotype but not both. Thus the expression of one parental allele is excluded in each cell expressing a given locus. The allelic exclusion of the two IgD alleles in a (BALB/c × C57BL/10)F_1 {Igh-$C^{a/b}$} heterozygote is shown in Fig. 97.1a. 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 demonstrates that the immunoglobulins expressed in a given cell are

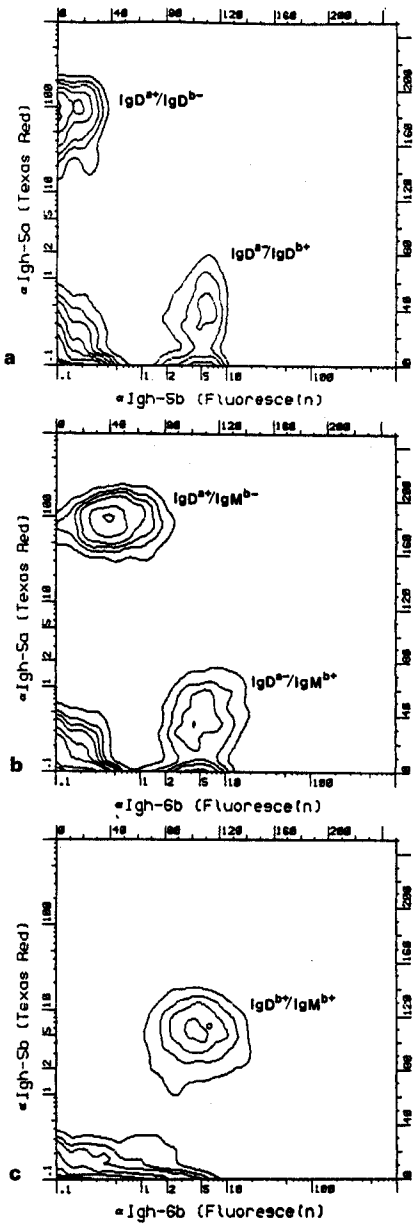

Fig. 97.1. (BALB/c × C57BL/6)$_{F1}$ [Igh-$C^{a/b}$] spleen cells were stained with:
(a) fluorescein-conjugated anti-IgD^b and biotin-conjugated anti-IgD^a;
(b) fluorescein–anti-IgM^b and biotin–anti-IgD^a;
(c) fluorescein–anti-IgM^b and biotin–anti-IgD^b.
In each case Texas red-avidin was used as a second step for the biotin-conjugated monoclonal antibody. The following monoclonal antibodies were used: AMS 9.1 (anti-IgD^a); AF6-122.2 (anti-IgD^b) and AF6-78.25 (anti-IgM^b) [24].

controlled by alleles located on the same chromosome; i.e. in an animal heterozygous for the Igh-C loci, cells expressing the maternal allele of IgM will express only the maternal allele of IgD [32]. This is demonstrated in Fig. 97.1b and c. In a (BALB/c × C57BL)F_1 {Igh-$C^{a/b}$} heterozygote, the spleen cells which react with a monoclonal antibody specific for the IgM^b allele do not bind a monoclonal antibody specific for IgD^a (Fig. 97.1b) but do bind one specific for the IgD^b allele (Fig. 97.1c).

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 [33]. In other words, either the paternally 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.

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 completely understood. The expression of an immunoglobulin H chain, however, requires at least two DNA rearrangements to productively align the V_H, D, and J_H segments to be expressed [34]. Furthermore, subsequent rearrangements apparently occur when cells 'switch' to production of different isotypes, e.g. from IgM and IgD to IgG. Since this switch associates a new Igh-C locus with the originally rearranged VDJ segment [3], mechanical considerations suggest that the evolution of haplotype exclusion is intimately linked with the evolution of the mechanisms responsible for isotype switching.

Alleles and specificities of the Igh-C loci

In inbred mice allelic differences are known for seven of the eight isotypes, with only IgG3 being monomorphic. Table 97.2 presents the designated type strains for inbred mice which carry each of the known Igh-C haplotypes and lists the alleles these strains possess at each locus. Each Igh-C haplotype is defined by a unique set of alleles at the seven polymorphic loci. Note that most of the haplotypes are defined by a unique allele at the highly polymorphic Igh-1 locus, but that some (NZB/BL and AL/N) are defined by a unique combination of alleles at each of the seven loci. Table 97.3 catalogues the Igh-1 haplotypes of some seventy inbred mouse strains.

Tables 97.4–97.10 show the alleles and distribution of allotype specificities at each of the Igh loci known in inbred mice. The alleles at each Igh-C locus are defined by the presence of a unique combination of allotypic specificities found on the Ig coded at that locus. Thus the 'a' allele of Igh-5 is defined by the presence of specificities Igh-5.1 and Igh-5.4 on IgD of the 'a' haplotype type strain: BALB/c (Table 97.8). Each allotypic specificity is identified by the unique pattern of reactivity of an antiserum or monoclonal antibody with a panel of inbred strains of mice. For example, specificity 3 of the Igh-1 locus is defined by an alloantiserum which only reacts with IgG2a from DBA/2J and RIII/J mice (Table 97.4). The specificities for each locus are numbered according to the order of their discovery. Therefore, specificity 3 for the Igh-1 locus has no structural relation to specificity 3 for the Igh-2 locus. When specificities at more than one locus are being discussed, the more complete designation,

Table 97.3. Igh-1 allotypes in inbred mouse strains†

Igh-1a			Igh-1b		Igh-1c	Igh-1d	Igh-1e	Igh-1f	Igh-1g	Igh-1h	Igh-1j
BALB/cJ*	C58/J		C57BL/10J*	LP/J	DBA/2J*	AKR/J*	A/J*	CE/J*	RIII/J*	SEA/Gn*	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)HZ	SM/J	I/Ao			N/Ao	FZ/Di	BSL/Di	C3H/Hz
CHI/Ao	JK/BI	PRUNT/Ao	B10.D2(old)Hz	STA/Je	JB/Di			NH/N	STB/Je	P/J	C3H/SW
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/BI	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/RI							

* Type strain for each allele.

† Each Igh-1 allele is assigned to a type strain whose immunoglobulins, by definition, are the standard for comparison for that allele. Since the Igh loci are closely linked genetically, and since the largest number of alleles have been described at 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.

Table 97.4. 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†	CBA/H	1	2	—	—	—	6	7	8	10	—	12	13	14	15	—	17

* Igh-1 determines IgG2a heavy chain constant regions.
† Shown to be different from BALB/c by its positive reaction with a conventional antiserum; detected specificity was on Fab [35].
§ Specificity Igh-1.1 has also been designated as specificity G1, 1.2 as G8, 1.10 as G1, and 1.12 as G6 [36]. The specificities are described by Herzenberg *et al.* [21], Potter & Lieberman [36] and Huang *et al.* [37]. Specificity 1.17 is distinct from specificity 1.13 as the former is expressed on both Igh-1 and Igh-3 heavy chains, while the latter is found only on Igh-1 heavy chains [37]. Monoclonal antibodies detecting specificities 1.2, 1.4, 1.13, 1.14, 1.15, 1.16 and 1.17 have been described [37].

Table 97.5. The Igh-2 locus*

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

* Igh-2 determines IgA heavy chain constant region.
† According to other nomenclature specificity 2.1 has been known as specificity A35, 2.2 as A12, 2.3 as A13, 2.4 as A14, 2.5 as A15, 2.6 as A17 [22]; 2.7 as 590, and 2.8 as 596 [38]. The specificities are described by Herzenberg *et al.* [21], Lieberman [22], Potter & Lieberman [36] and Tada *et al.* [38].

Table 97.6. 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

* The Igh-3 locus encodes IgG2b heavy chain constant regions.
† Specificity 3.2 has also been designated as specificity H11 and specificity 3.9 as H9 [36]. Specificity 3.1 differs from specificity 3.10 in that it is found only on IgG2b molecules. The monoclonal antibody detecting specificity 3.10 also detects an allotypic marker of IgG2a molecules [37]. The specificities are described by Herzenberg *et al.* [21], Potter & Lieberman [22], and Huang *et al.* [37]. Monoclonal antibodies detecting specificities 3.4, 3.9, 3.10, 3.11 and 3.12 have been described [37].

Igh-1.3 (specificity 3 of the Igh-1 locus) or Igh-3.3 is usually used to prevent confusion.

The genetic diversity demonstrated by the Igh loci described in these tables is extraordinarily extensive; however, it is minimal in comparison with the diversity of the alleles expressed in wild mice. Studies with polyclonal [41,42] and monoclonal anti-allotype reagents [28] have already revealed numerous new combinations of allotypic specificities in these mice even though a large sampling of animals from diverse geographical areas have yet to be explored.

Table 97.7. The Igh-4 locus*

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

* The Igh-4 locus encodes IgG1 heavy chain constant regions.
† Specificities are as described by Parsons *et al.* [24]. Monoclonal antibodies detecting specificities 4.1 and 4.2 have been described [37].

Table 97.8. The Igh-5 locus*

Allele	Type strain	Specificities†				
a	BALB/c	1	—	—	4	—
b	C57BL/10	—	2	3	—	—
e	A/J	1	—	3	—	5

* The Igh-5 locus encodes IgD heavy chain constant regions.
† The specificities are described by Stall & Loken [25]. Monoclonal antibodies to specificities 5.1, 5.3, 5.4 and 5.5 [25] and 5.3 [39] have been described.

Table 97.9. The Igh-6 locus*

Allele	Type strain	Specificities†					
a	BALB/c	1	2	—	—	5	—
b	C57BL/10	—	—	3	4	5^w	6
e	A/J	nd	nd	—	4	—	—
n	NZB/BlJ	—	2	—	4	5^w	6

* The Igh-6 locus encodes IgM heavy chain constant regions.
† Igh-6 specificities are described by Black *et al.* [16], Kung *et al.* [40] and Stall & Loken [25]. Igh-6.5 is recognized by the monoclonal antibody 331.12 [63]. Igh-6.6 is recognized by the monoclonal antibody AF6-78.25 [25].

Table 97.10. The Igh-7 locus*

Allele	Type strain	Specificity†	
a	BALB/c	1	—
b	C57BL/10	—	2

* The Igh-7 locus encodes IgE heavy chain constant regions.
† Igh-7 specificities by Borges *et al.* [17].

Identification and characterization of allotypic specificities

The basic methods for defining immunogenetic specificities (determinants) of the kinds used here to distinguish the Ig H-chain allotypes are presented in Table 97.11 and in many cases described elsewhere in this volume. 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 individual isotypes) from other mouse strains. The discovery of a unique reactivity distribution amongst the mouse strains tested with a given reagent defines a new specificity.

In general, new reagents whose reactivities mimic a previously defined pattern are treated as duplicates of the original reagent. This is true in an immunogenetic sense since no new genetically distinct specificities can be defined with the second reagent. However, there is no a priori reason to expect that the old and the new reagents detect the same physical structure, i.e. a pair of such reagents could detect physically separate determinants that are inherited together in all animals available for testing.

Table 97.11. Allotyping methods

Method	References
Electrophoretic analyses of Fc fragments	[9]
Ouchterlony analysis	[21] [43]
Passive haemagglutination	[44] [45] [46]
Immunofluorescence staining	[25]
Radioimmune precipitation	[21,47]
Solid-phase radioimmune competition assay	[28,48,49]
Solid-phase radioimmune co-binding assay	[28,49]
DNA restriction enzyme site polymorphisms	[27]

These considerations were basically moot prior to the monoclonal era since conventional antisera do not lend themselves easily to the detection of such determinants. However, studies with monoclonal anti-allotype antibodies have revealed several sets of such 'duplicate' antibodies. For example, a minimum of eight antibodies specific for the allotypic specificity Igh-1.4 (found on C57BL/10 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 [50,51]. Thus a single allotypic specificity may be composed of multiple distinct alloantigenic determinants located on different parts of the Ig molecule. These individual determinants are referred to as *allotopes*. Multiple allotopes have also been identified for specificities Igh-1.14, Igh-1.17 [51] and Igh-5.3 [25]. By definition, two monoclonal antibodies recognize different allotopes if they: (1) can be shown to bind to different proteolytic fragments of the heavy chain [25,50]; and/or (2) each fail to block the binding of the other monoclonal antibody [25,51].

Comparison of the amino acid sequences of the IgG2a H chain constant regions [52] with genetically or physically distinct Igh-1a determinants failed to reveal straightforward correlation between individual allotopes and specific amino acid substitutions. 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 was hypothesized to play a major role in the presentation of allotypic determinants [51].

Igh-C congenic strains of mice

Two inbred strains which 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 [53] to study histocompatibility genes. The strains have proved to be a major tool in the dissection of the H-2 complex. 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.

The 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 which were then back-crossed to the parental BALB/c. The progeny of the back-cross 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-$C^{a/b}$ progeny are intercrossed and 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 twenty back-crosses, the probability is >99% that the

Table 97.12. Igh-C congenic strains

Strain	Inbred background	Source of Igh-C locus	Number of back-crosses	References
C.B-20	BALB/c	C57BL/Ka	20	[22,29]
BAB/14	BALB/c	C57BL/Ka^{8*}	14	[16,29]
B.C-9	C57BL/Ka	BALB/c	9	[22]
C.AL-20	BALB/c	AL/N	20	[22,29]
SJA/20	SJL	BALB/c	20	[16]
C57BL/6.Igh^n	C57BL/6	NZB	14	[16]
CBA.Igh^b	CBA/Tufts	C57BL/6	14	[61]
CWB	C3H.SW	C57BL/6	20	[16,29]
BALB/c.Igh^b	BALB/c	C57BL/6	20	[**]
BALB/c.Igh^c	BALB/c	DBA/2	22	[**]
BALB/c.Igh^d	BALB/c	AKR	21	[**]

* BAB/14 possesses the Igh-C loci of C57BL/Ka. However, a cross-over appears to have occurred within the Igh-V region. Thus BAB/14 expresses idiotypic markers of both BALB/c and C57BL. See ref. 29.
** A. M. Stall, personal communication.

two strains will be homozygous for loci greater than 20 map units from the locus of interest—in this case, Igh-1 [54]. A number of Igh-C congenic strains which have been developed are listed in Table 97.12.

Specialized laboratory methods

Most of the methods for allotyping (identifying and distinguishing Ig alleles) rely on serological analyses. However, in the past, IgG1 molecules have been typed by electrophoretic analysis of Fc fragments [12]. More recently, it has become possible to analyse the genome directly for DNA sequence polymorphisms within the Igh-C structural genes or in regions closely associated with them [27]. A list of the techniques available for allotyping, as well as references which fully describe their application, is given in Table 97.11. Only those special modifications necessitated generally by the comparatively small volumes of reagents and test sera available from mice will be presented here.

Production of antisera

Rabbit antisera

Rabbit antisera can be made against mouse whole sera, partially purified normal immunoglobulins, purified myeloma or hybridoma proteins, or fragments of immunoglobulins (papain digestion products, i.e. Fc and Fab pieces). 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 is given in the footpads 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. Preparation of antisera specific for a given immunoglobulin isotype 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.

Mouse anti-allotype immunizations: IgG and IgA

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 obtained by immunizing with antisera or antibodies directed to some tissue or protein component in the recipient strain (Table 97.13; protocol III). 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-2d). Similarly, an antiserum to a complement-component antigen, Hc^1 [55], was quite effective in stimulating anti-allotype serum.

In the authors' hands, this method of immunization is particularly effective for raising antibody reactive with Igh-1 (IgG2a) allotypes but results only in low antibody titres in occasional animals for all other allotypes. Immunization of C57BL/6 with DBA/2 Ig, however, 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. The authors 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 anti-allotype response (Table 97.13; protocol I). This method has proved excellent for obtaining antibody reactive with Igh-4 (IgG1) allotypes as well as antibodies reactive with 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 (Table 97.13; protocol II).

Mouse anti-allotype immunization: IgM and IgD

Antibodies directed against allotypic specificities of IgM (Igh-6) and IgD (Igh-5) are most 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

Table 97.13. 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#	I	Day 1: total of 2×10^9 bacteria in 0.2 ml saline injected i.p. and in four places s.c. Days 3 and 5: same but in four places s.c. Days 21 and 37: same as day 1	Pool bleed Day 25 Day 32 Day 45	Days 1, 3, 5, 22, 26, 50, 57: 10^9 bacteria + 20 μl antiserum from Step 1 in 0.2 ml saline per mouse. Mix at room temperature and inject i.p. Boost as on day 1	Day 64 and weekly. Bleed individually and test, or pool bleed depending on circumstances. Boost when titres go down. Ten days after boost, bleed and test.
B. pertussis heat killed	II##	Mice supplied with drinking water containing 2×10^{10} bacteria/500 ml for 30 days (5 mice per cage)	Day 30; bleed. Check titre by bacterial agglutination. Check isotype of antibody*. Pool blood weekly	Days 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**. Inject 0.2 ml	Day 45 and weekly, test individually
H-2	III	1/5 spleen/animal in isotonic MEM injected i.p. Boost with same monthly	Bleed 3 weeks after injection, then weekly	Day 1: 20 μl antiserum from Step 1 + 20 μl CFA** injected i.p. and four places s.c. Days 21, 28 and monthly booster: 10 μl in 0.2 ml saline i.p.	Day 35 bleed and test. Pool bleed all positive animals weekly
—	IV^	—	—	Days 1, 8, 15, 22: 10^7 spleen cells from strain differing for Ig allotype and H-2; boost monthly thereafter	Day 29 and weekly thereafter

Kindly supplied by Lederle Laboratories.
Protocol established by Dr Tohru Masuda.
* Incubate antiserum plus *B. Pertussis*; wash. Test conjugates in radioimmunoassay. Use sera whose complexes show high levels of IgA and low levels of other immunoglobulins.
** Complete Freund's adjuvant.
^ Protocol established by Goding, Warr & Warner [15].

IgM. Four weekly injections of 10^7 spleen cells, followed by a boost 1 month later, result in a vigorous anti-IgD response. Alloantibodies to IgM are more difficult to generate and may require two to three subsequent monthly boosts (Table 97.13; protocol IV) [16,25].

Analysing the alloantisera (or hybridomas) produced by this immunization scheme poses unique problems. Antibodies reactive with a variety of cell surface antigens (H-2, Ia, 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 utilized (see section on immunofluorescent staining, p. 97.14).

Not only is it 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 preparation of anti-allotype sera (notably Drs Rose Lieberman and M. 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. Thus there may be key genetic factors controlling the immune response to allotypic antigens [56].

From the foregoing, it is clear that the preparation of anti-allotype reagents is an art bordering on a science. As a guideline for the novice venturing into this field, some of the experience in the authors' laboratory is summarized in Tables 97.13 and 97.14. These observations are not the result of exhaustive

Table 97.14. Allotype immunization results

Anti-allotype serum	Anti-allotype-producing strain	Allotype donor strain	Protocol no.*	Antigen for allotype donor	Reaction with					
					Ig-1	Ig-2	Ig-3	Ig-4	Ig-5	Ig-6
b anti-a	(LP/J or C57Bl/10)	(BALB or CSW)	I	*B. pertussis*	+++^	−	+	++		
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						+++	+++
b anti-a	SJL	BALB/c	IV						+++	+++
b anti-e	C57BL/10	A/J	IV						+++	
a anti-b	BALB/c	C57BL/10	IV						+++	+++

* See Igh-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.

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.

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 & Lieberman [36]. Fortunately, the potential for preparing monoclonal antibodies from animals immunized with the above protocols has now relieved much of the tedium associated with preparing anti-allotype reagents. Following each table detailing the specificities of the Igh-C loci (Tables 97.4–97.10), the specificities for which monoclonal antibodies are available are given.

Preparation of specific antibody

Monoclonal antibodies are by definition homogeneous with respect to specificity and are clearly preferable as reagents. Certain protocols, however, still dictate the use of antisera even though these 'conventional reagents' usually contain several contaminating activities. In general, it is wise to isolate the relevant antibodies from such sera rather than risk spurious reactions that are sometimes difficult to recognize as artifacts.

Antibodies to immunoglobulin determinants can be readily isolated by affinity purification, i.e. by absorption and elution on antigens rendered insoluble by covalent binding to an insoluble support such as Sepharose. Alternatively, contaminating antibodies can be removed by absorbing the undesired antibodies on insolubilized antigens. These solid-phase methods are clearly preferable to liquid-phase absorptions in that the resultant antisera do not contain either the soluble antigen–antibody complexes or residual soluble antigen.

Affinity-purified antibodies are prepared by first passing the antiserum over an antigenic protein covalently coupled to Sepharose. Then, after thorough washing, the bound antibody is eluted by dissociating the antigen–antibody complexes by altering the pH or by the addition of chaotropic ions (see Chapter 13 [43]). In general, a myeloma or hybridoma protein that carries the relevant determinants but differs from the immunogen is used as an absorbent for purification of anti-isotype and anti-allotype antibodies (in order to minimize contamination with anti-idiotype antibodies).

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

Several types of assays are described here 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 which make it more suitable for particular purposes. For example, Ouchterlony analysis is rapid, semi-quantitative and relatively insensitive, while the radioimmunoassay is somewhat more laborious but also considerably more sensitive and accurate. Perhaps the most versatile assay of those presented is the solid-phase radioimmunoassay, which can be used to measure allotypic and isotype contributions to an overall immune response, as well as the total antibody response to a variety of antigens (including allotypes). It can also be used to measure allotype and isotype levels in sera, and is particularly suitable for analysis using monoclonal anti-allotype antibodies.

Double diffusion 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 which eventually precipitates. Since monoclonal antibodies react with only one epitope per heavy chain, they rarely cause the formation of a sufficient lattice to effect precipitation. Thus, while especially useful with conventional antisera, these techniques are not recommended for use with monoclonal anti-allotype antibodies.

Slides for immunoelectrophoresis or Ouchterlony plates are cleaned with ethanol and then dipped in 0.1% agar in water and allowed to dry. This pre-treatment prevents slippage of the agar during subsequent manipulations. A bed of 1% agar in 50 mM-barbital buffer, pH 8.2, is then poured on to the slide (1 ml per sq. in). For Ouchterlony analyses, hexagonal patterns, with holes separated 5 mm from their centre wells, are cut with a flattened 18-gauge needle. If 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 immunoglobulin levels.

After development of the precipitation lines (3–6 h at room temperature) slides are immersed briefly in water to fill the wells with liquid, covered with a piece of Whatman #1 filter paper and dried in a warm (not hot) airstream. When slides are dry, the paper comes off easily, leaving on the glass an agar film with the precipitate firmly embedded. Soluble protein is removed by rinsing the slide overnight in 0.5% $NaHCO_3$, and the precipitation lines are permanently stained with 1% Buffalo black in water–methanol–

glacial acetic acid (5:5:2) (1 min in stain solution and approximately 5 min in 5% acetic acid to destain).

For immunoelectrophoresis, samples are loaded into wells separated by troughs cut into the agarose. After electrophoresis for 1 h at 5 V/cm, so that the sample is spread parallel to the length of the trough, the precut troughs are cleared of agar, and filled with antiserum. The slide is allowed to develop (3–6 h at room temperature), and is then rinsed and stained as above.

Haemagglutination techniques

These assays are conveniently performed in V-bottom 96-well microtitre plates. For passive haemagglutination, erythrocytes coated with either non-agglutinating ('incomplete') or sub-agglutinating 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 which, 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 haemagglutination inhibition assay. This method, modified from the techniques of Lieberman [44], 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, while 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 by immunofluorescence staining. IgG allotypes on B cells are also identifiable by immunofluorescence 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 [57]. It has also been demonstrated that membrane-bound Ig may not express the complete set of allotypic specificities found on the secreted molecule [58].

Staining procedures used do not differ significantly from other immunofluorescence staining protocols (see Chapter 13 in this handbook). For indirect staining, as in other two-step assays, care must be taken that the reactivity pattern of the second antibody is appropriate to the isotype representation in the anti-allotype antiserum. For example, a rabbit anti-mouse IgG which reacts primarily with IgG2a and IgG2b will not be useful in assays using an IgG3 anti-allotype antibody. Also, when working with B cells, the second antibody must not recognize IgM or L chain determinants. In general, staining with directly coupled reagents is preferable to indirect staining in that backgrounds are substantially lower in comparison to the amount of specific staining obtained.

Radioimmunoassays

For the last several years, the solid-phase radioimmunoassay (RIA) has been the work-horse of the authors' laboratory. In various incarnations, it has been used for: detection of allotypic determinants; quantification of serum allotype and isotype levels; identification, characterization and quantification of anti-allotype antibodies; and measurement of allotype representation in antibody responses in immunoregulatory studies. The individual RIA assays developed for these purposes derive from, and have almost totally replaced, the radioimmune tube assays that the authors developed earlier and described in detail in the previous edition of this handbook. In this section, the authors briefly outline their current RIA assays and refer the reader to other chapters in this volume for more detailed methodological discussions.

For these assays, immunoglobulins to be used as radio-labelled antigens or monoclonal antibodies are isolated from the ascites fluids produced by myeloma- or hybridoma-bearing mice. In cases where no myeloma or hybridoma proteins of the appropriate allotype are available, a fraction of normal serum immunoglobulins enriched for the particular isotype required is used. Proteins are usually isolated using standard methods of ammonium sulphate precipitation, ion-exchange chromatography and gel filtration [37,43].

Approximately 100 μg of purified immunoglobulin is iodinated with 0.4 mCi of ^{125}I by the iodogen [59] or

chloramine-T methods [60] to an average labelling of less than one atom ^{125}I/molecule. From 5 to 30% of the added iodine becomes covalently bound to the protein and is, therefore, TCA precipitable. Passage over a small G25 column is sufficient to remove unbound iodine.

If conventional antisera are used, these can either be affinity purified and labelled as above or the labelling step can be interpolated between the adsorption and elution of the affinity chromatography purification. In other words, the antibody is labelled with chloramine T while bound to the affinity matrix.

Measuring allotype and isotype levels in serum and culture fluids

To detect the presence and level of allotypic determinants in sera or culture fluids, the following procedure is used. Purified monoclonal anti-allotype antibody is coated on to the wells of a polyvinyl chloride U-bottom microtitre plate. Test sera at various dilutions are then added, along with a radio-labelled myeloma or hybridoma protein carrying the relevant determinant. After 1 h, the wells are rinsed, removed, and assayed for bound radioactivity [48].

If the allotypic determinant being assayed is present in the test serum, it will compete with the radio-labelled protein for antibody sites on the antibody-coated well. Thus it will block the binding of the radio-labelled material and the extent of blockage will reflect the relative concentrations of the radio-labelled and unlabelled 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 unlabelled protein yields a straight line standard curve for this assay, since the amount of radioactivity bound is directly proportional to the specific activity of the protein in the well (provided that the labelled and unlabelled proteins each carry the determinant detected by the bound antibody). Departures from linearity usually mean that the radio-labelled protein is sticking non-specifically to the well. (Airfuging 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 [21].

Measuring allotype and isotype representation in antibody responses

Much of the authors' work on immunoregulatory mechanisms is based on using solid-phase RIA to measure allotype and isotype representation in antibody responses to protein antigens and to haptens such as dinitrophenyl (DNP). Microtitre plates are coated with protein antigens or with DNP conjugated to bovine serum albumin (BSA). Usually the authors use both a minimally substituted and a highly substituted conjugate (e.g. DNP_5-BSA and DNP_{20}-BSA). Test sera or standard anti-DNP sera at various dilutions are added to the coated wells and incubated. The plate is then washed, and radio-iodinated anti-allotype antibody is added and incubated. Finally, the plates are washed twice and the wells separated and counted in a well-type gamma counter.

This assay, which usually requires considerably less than 10 μl of a test serum, measures the anti-protein or anti-DNP antibody content in an antiserum in terms of the contribution of the individual contribution of each allotype and isotype to the response. In addition, it provides a measure of the average affinity of the anti-DNP antibody in each of these allotype and isotype responses (based on the contrast in the amount of antibody bound to the minimally substituted and highly substituted hapten (DNP) conjugates) [33].

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