CHAPTER 13

Mouse immunoglobulin allotypes: description and special methodology

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

Identification of immunoglobulin classes

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

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

There are six known H-chain classes in the mouse, each associated with particular biological activities (see Table 13.1) and represented by at least one myeloma protein [2–4]. All mouse strains tested so far have been shown to have all of the known immunoglobulin classes, although genetic differences in the classes from one strain to another exist. The immunoglobulins arising from these genetic differences are called allotypes [5].

Identification of allotypes

Allotypes within a given class of immunoglobulins are products of alternate forms of the gene, i.e. alleles, at the locus determining the structure of the H-chain of that class of immunoglobulins. Typically, allotypes are recognized by antisera raised by immunizing one mouse strain with immunoglobulin from a second strain; however, other means may be used. For example, electrophoretic mobility of the Fc fragment of the H-chain was used to first identify alleles at the Ig-4 (γG1) locus, although antigenic differences have been identified now for the allotypes in that class as well. Using antisera, allotypes for a given class may be identified either by the presence of a unique antigenic determinant (specificity) or as a unique combination of antigenic determinants each of which is also found in other combinations in other allotypes.

Antiallotype antisera often contain several anti-

body populations. These may react with allotypic determinants on different classes of immunoglobulins or with different determinants on a single class.

Heterologous sera have been shown to detect allotypic differences; however, such sera are not easily obtained, probably because allotypes represent such small differences between members of an immunoglobulin class that they are not as effective immunogens as isotypic (species) determinants in heterospecific immunization.

Terminology

For historical reasons, two bases for notation of mouse immunoglobulins are in use, a practice which often creates confusion for those beginning to work or read in the field. When speaking of antibodies or immunoglobulins as proteins, the class name, for example γM or γG2a (or IgM or IgG2a), is most often used. When discussing genetic (allelically-determined) structural differences between immunoglobulins or antibodies within a given class, the genetic or allotypic notation is used. For example, Ig-1 is the locus for γG2a. γG2a in the BALB/c strain is called Ig-1a and γG2a in C57BL/10 strain is called Ig-1b. Thus, although logically it would suffice to call those immunoglobulins determined at the Ig-1 locus, Ig-1 globulins, they are in any non-genetic context, and often in a genetic context as well, referred to as γG2a (or IgG2a) globulins, making it necessary for the reader to learn both class and allotypic terminology and to shift easily from one to the other if he is to make his way facilely through the mouse immunoglobulin literature*.

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

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

TABLE 13.1. Mouse immunoglobulin classes. From [5] with additions.

Class	Locus	Number of alleles known in inbred strains	Number of specificities described	Some biologic activities
γG2a (γG)*	Ig-1	8	11	Fixes complement, mediates cell lysis, fixes to tissues of other species and mediates local anaphylaxis
γA (γA)	Ig-2	5	4	Does not fix complement, secreted into milk, tears, intestinal lumen, nasal secretions
γG2b (γH)	Ig-3	6	7	Fixes complement, mediates cell lysis, passes placenta
γG1 (γF)	Ig-4	2	2	Does not fix complement, fixes to tissues of same species, mediates local anaphylaxis, passes placenta
γG3				Does not fix complement, does not fix to skin, passes placenta
γM (γm)				Fixes complement, mediates cell lysis (more efficient than γG_{2a} and γG_{2b}).

* Potter-Lieberman class notation given in parenthesis [6]

Demonstration of allelism

There are certain problems in the proof of allelism at the Ig loci which centre around the difficulty in studying a large enough number of mammalian progeny to detect crossing-over between closely-linked genetic loci. Although upwards of 2000 progeny of appropriate crosses have been examined in detail, no direct evidence of a cross-over between the loci has been found. Thus, by the original definition of a locus as a region of the chromosome defining a particular characteristic and separable by crossing-over from regions defining other characteris-

* Another notation is used by Potter, Lieberman and some other workers [6]. Although it represents a logical attempt to combine the protein and gene notation, it is not generally used because it does not accord with the World Health Organization internationally accepted nomenclature for human immunoglobulins [7].

tics, all of the Ig loci would be lumped as one and no meaningful discussion of allelism at each of the loci would be possible.

The current definition of a locus in mammalian systems, however, is not based on a demonstration of crossing-over. In analogy with the bacterial cistron, a locus is defined as a place on the chromosome at which there exists a DNA sequence (gene) coding for a polypeptide chain. Although deletions and duplications can occur, in general a haploid chromosome set in a species will carry one locus for each of the proteins produced by the species. Therefore, in a diploid organism, each parent donates one structural gene at each locus.

Variations in the DNA sequence of the structural gene, which give rise to variations in the protein end product, are called alleles. Conventionally, a locus is not *named* until at least two alleles are known for the locus, so that it can be related, by genetic testing, to other known loci*.

Four loci have been named for the four mouse immunoglobulin classes in which polymorphic variations, i.e. allotypes, have been described. For each locus, the minimal criteria for allelism were met by genetic testing as follows. Two inbred mouse strains, each with a different H-chain allotype for a given class, were crossed to obtain heterozygotes. Both allotypes were found in the heterozygotes. Heterozygotes were then crossed, either back to one of the parental strains, or to a strain carrying a third allotype, or to themselves. In all cases, the expected ratios for segregating co-dominant alleles were found, indicating that a single haploid set always carried either the allele donated by the mother of the heterozygotes or the allele donated by the father never both, never neither [5].

Demonstration of genetic linkage

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

Thus far, the testing of a large number of progeny (over 2000) has yielded no direct evidence of crossing-over between H-chain loci. Nonetheless, the existence of a number of cross-reacting allotypes at each locus are probably signs of past cross-overs. It is likely that continued progeny testing will eventually directly demonstrate crossing-over in the mouse Ig region since in humans, where the immunoglobulin genes are also closely linked, at least one family has been found where crossing-over has occurred [9].

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

Allelic exclusion

One of the fascinating peculiarities of the genetic expression of the Ig alleles is that while the heterozygote produces immunoglobulins of both allotypes, individual immunoglobulin-producing cells in the heterozygote produce either one allotype or the other. The mechanism by which one of the two immunoglobulin alleles is excluded, or, put in the positive sense, the mechanism by which only one allele is turned on in a given cell, is not understood. It is perhaps related to the fact that individual immunoglobulin-producing cells, at least when fully differentiated, appear to secrete only one species of immunoglobulin (antibody), although switching between classes may occur during differentiation to antibody production [10, 11]. No other autosomal loci are known where only one allele is expressed in a heterozygous cell.

Light chain variation

Both kappa and lambda chains are present in all classes of mouse immunoglobulins but lambda is found in only 5 per cent or less of the immunoglobulins. These chains are structurally homologous to human kappa and lambda [12]. Neither serologically detected allotypes nor amino acid compositional indicators of constant region genetic differences have been found. However, a v region genetic difference detected by peptide 'finger printing' has been found. The variant peptide is obtained in a yield of under 20 per cent, consistent with its representation on a single v region subgroup [14].

* Even more cautiously, but perhaps reasonably for immunoglobulins whose complete genetic control is to say the least still quite arcane, the reader should store the reservation that some allotype loci may be regulatory ones [8].

† Linkage to a locus for immunological responsiveness to dextran has recently been found [13].

Alleles and specificities of the Ig loci

A summary description of the six known immunoglobulin H-chain classes, including associated biological activities, is presented in Table 13.1. Both the notation we use and Potter & Lieberman's alternate notation are given. As the table shows, allelic differences are known for only four of the classes, γG2a, γA, γ2b and γG1, defining the loci Ig-1 to Ig-4 respectively. The known alleles and distribution of allotype specificities at each of the Ig loci are presented in Tables 13.2–5.

Each Ig-1 allele in Table 13.2 is assigned to a type strain whose immunoglobulins, by definition, are the standard for comparison for that allele. Since the Ig loci are closely linked genetically, and since the largest number of alleles have been described at the Ig-1 locus, the type strain assigned to each Ig-1 allele is also assigned as the type strain for the entire Ig chromosome region of which that allele is a part, and alleles at subsequently discovered loci in the Ig region are named consistently with the Ig-1 allele. For example, the BALB/c strain, which is Ig-1a, is assigned the Ig^a chromosome region, and therefore, by definition, as new Ig loci are recognized, the allele carried by BALB/c is designated by a superscript 'a', i.e., $Ig\text{-}2^a$ is the allele at the Ig-2 locus in BALB/c. It is important to note that the assignment of $Ig\text{-}1^a$ and $Ig\text{-}2^a$ to BALB/c is not in any way meant to imply that BALB/c γG2a is *structurally* more closely related to BALB/c γA than to γA molecules determined by other alleles at the Ig-2 locus.

At Ig-2 to Ig-4, although some alleles at each of the loci have been defined, allotypic differences have not, as yet, been recognized between immunoglobulins in all of the type strains. In these cases the recognized alleles are formally named as a composite of the alleles of the undifferentiated type strains, e.g., $Ig\text{-}2^{a,h}$, although in general usage $Ig\text{-}2^a$ is used to describe the allele for γA of BALB/c origin. This type of notation system was adopted, among other reasons, in the expectation that as the search for new antisera continues, some sera may be found which differentiate between the proteins produced by the different type strains; however, it is quite possible that two type strains which carry different Ig-1 alleles could have *identical* Ig-4 alleles. As yet, no example of the reverse case has been found, i.e. two strains with different alleles at Ig-2, Ig-3 or Ig-4 having the same Ig-1.

In Table 13.6, a catalogue of 70 inbred mouse strains listed according to Ig-1 allele is presented. Since, in the many cases where testing of inbred strains has been possible, the other Ig alleles have been shown to conform to the Ig-1 allotype, it is reasonable to assume that if an inbred strain is $Ig\text{-}1^a$, it is Ig^a, etc.

TABLE 13.2. The Ig-1 Locus* [5].

Type strain	Allele	Specificities										
BALB/c	$Ig\text{-}1^a$	1(G7)†	2(G8)	—	—	—	6	7	8	10(G1)	—	12(G6)
C57BL/10J	$Ig\text{-}1^b$	—	—	—	4	—	—	7	—	—	—	—
DBA/2J	$Ig\text{-}1^c$	—	2(G8)	3	—	—	—	7	—	—	—	—
AKR/J	$Ig\text{-}1^d$	1(G7)	2(G8)	—	—	5	—	7	—	—	—	12(G6)
A/J	$Ig\text{-}1^e$	1(G7)	2(G8)	—	—	5	6	7	8	—	—	12(G6)
CE/J	$Ig\text{-}1^f$	1(G7)	2(G8)	—	—	—	—	—	8	—	11	—
RIII/J	$Ig\text{-}1^g$	—	2(G8)	3	—	—	—	—	—	—	—	—
SEA/Gn	$Ig\text{-}1^h$	1(G7)	2(G8)	—	—	—	6	7	—	10(G1)	—	12(G6)

* Ig-1 determines γG2a immunoglobulin H chains.
† Potter-Lieberman determinant names given in parentheses [6].
Specificity 12 described only by Potter & Lieberman.

Allotypic specificities

Each allele at a given locus is actually defined by the unique combination of antigenic specificities found on the immunoglobulin it determines (Tables 13.2–5). These specificities represent the reactions of the immunoglobulins of a given class with a panel of anti-allotype antisera, and therefore represent structural differences between the immunoglobulin H-chains determined by the alleles at a given locus.

The specificities *for each locus* are numbered according to the order of their discovery. Therefore, specificity 3 for the Ig-1 locus has no structural relation to specificity 3 for the Ig-2 locus. When specificities at more than one locus are being dis-

cussed, the more complete designation, Ig-1·3 or Ig-3·3 should be used to prevent confusion.

Rules for defining specificities

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

sera, since this method is often more sensitive than Ouchterlony testing. Most of the other specificities have been defined by studying the nature of the inhibition of precipitation of labelled immunoglobulins when unlabelled (test) immunoglobulins, often in whole serum, from various strains are introduced into the assay. (For methods, see p. 13.8.)

In the inhibition assay, if the test (inhibitor) antigen has no specificities in common with the labelled antigen there is no inhibition of precipi-

TABLE 13.3. The Ig-2 Locus* [5].

Type strains	Alleles	Specificities			
BALB/cJ, SEA/Gn	Ig-2^{a,h}	—	2(A12)†	3(A13)	4(A14)
C57BL/10J	Ig-2^{b}	—	—	—	—
DBA/2J, RIII/J	Ig-2^{c,g}	1	—	—	—
AKR/J, A/J	Ig-2^{d,e}	—	—	3(A13)	—
CE/J	Ig-2^{f}	—	—	—	4(A14)

* Ig-2 determines γA immunoglobulin H chains.
† Potter–Lieberman determinant names given in parentheses [6].
Specificities 3 and 4 have been described only by Potter & Lieberman.

TABLE 13.4. The Ig-3 Locus* [5].

Type strains	Alleles	Specificities						
BALB/cJ, DBA/2J, SEA/Gn	Ig-3^{a,c,h}	1	2(H11)†	—	4	7	8	—
C57BL/10J	Ig-3^{b}	—	—	—	4	7	8	9(H9)
AKR/J	Ig-3^{d}	1	—	3	—	7	8	—
A/J	Ig-3^{e}	1	—	3	—	7	—	—
CE/J	Ig-3^{f}	1	2(H11)	3	4	—	—	—
RIII/J	Ig-3^{g}	1	2(H11)	—	4?	—	—	—

* Ig-3 determines γG2b immunoglobulin H chains.
† Potter–Lieberman determinant names are given in parentheses [6].
Specificity 9 has been described only by Potter & Lieberman.

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

Several of the Ig specificities have been defined by the ability of anti-allotype sera to precipitate ^{125}I-labelled immunoglobulins, either myeloma proteins or immunoglobulins isolated from normal mouse

tation. If, on the other hand, the test antigen is identical to the labelled antigen in its reactions with the antiserum (has all the specificities detected in the assay), there is complete inhibition of precipitation. In the case where the test antigen is partially identical to the labelled antigen (has some of the specificities detected in the assay, i.e. cross-reacts), there is partial (incomplete) inhibition of precipitation. When more than one specificity is detected by an antiserum, varying the combinations of labelled antigen and inhibitor antigen is often useful in reducing the number of possible specificities detected in a given assay.

Continuing with the 'rules' then: (4) the specificities detected by an antiserum in reactions with a

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

In the definition of specificities, the following notation is used [5]: An ^{125}I-labelled preparation of γ-globulin is indicated with an asterisk following the symbol of the strain from which it was prepared (for example, C3H*), while normal sera used in inhibition assays are listed by the strain symbols (e.g., C3H). The symbols C3H*-C57BL anti-C3H refer to the use of a labelled C3H γ-globulin preparation with a C57BL anti-C3H antiserum in an inhibition assay. The expression 'C3H 1' means C3H has specificity one. The expression 'C57BL-1' means C57BL does not have specificity one.

TABLE 13.5. The Ig-4 Locus*

Type	Strains	Alleles	Specificities
BALB/c AKR/J R111/J	DBA/2J A/J, CE/J SEA/Gn	Ig-4^{a,c,d,e,g,h}	1–
C57BL/10J		Ig-4^{b}	–2

* The Ig-4 locus determines γG1 immunoglobulin H chains.

Several Ig-1 specificities are here defined:

(1) C3H*-C57BL anti-C3H precipitation is not completely inhibited by DBA. C3H therefore has at least one specificity not present in DBA (C3H 1; C57BL-1; DBA-1).

(2) DBA*-C57BL anti-C3H precipitation is completely inhibited by AKR. Therefore C3H, DBA, and AKR share at least one specificity, 2 (C3H 2; C57BL-2; DBA 2; AKR 2).

(3) AKR*-C57BL anti-C3H precipitation is not completely inhibited by DBA. Therefore AKR and C3H must share at least one specificity that is not present in DBA (AKR 1).

(4) C3H*-C57BL anti-C3H precipitation is not completely inhibited by AKR. C3H therefore has at least one specificity not present in AKR (C3H 6; C57BL-6; AKR-6).

(5) Since C57BL anti-C3H recognized specificity 6, statement (2) proves that DBA does not have 6 (DBA-6).

(6) DBA*-C57BL anti-DBA precipitation is not completely inhibited by C3H or AKR. Therefore DBA has at least one specificity not present in either of these two strains (C3H-3; C57BL-3; DBA 3; AKR-3).

(7) C3H anti-C57BL precipitates C57BL*, but not C3H*, DBA*, AKR*, or A/J*. C57BL therefore has at least one specificity not present in the latter four strains (C3H-4; C57BL4; DBA-4; AKR-4; A/J-4).

(8) C3H*-C57BL anti-C3H precipitation is completely inhibited by A/J. Therefore A/J has all specificities previously assigned to C3H (A/J 1, 2 and 6).

Recognition of allotypes by electrophoretic mobility differences

As mentioned earlier, although it has been the custom with immunoglobulins to resort to serology for characterization, allotypic differences need not be recognized only by serologic methods. For a long time, no methods were found which elicited alloantisera reacting with γG1 (Ig-4) globulins. Faced with this problem, we turned to another classical criterium for demonstrating polymorphism at loci which determine protein structure electrophoretic mobility, and were able to define two alleles at the Ig-4 locus, one in BALB/c and the other in C57BL/6 [15].

For these studies rabbit antiserum for the γG1 class was raised and rendered specific by absorption with immunoglobulins of the other classes. This antiserum was used to develop immunodiffusion patterns following electrophoretic separation in agar gels of γG1 Fc fragments obtained by papain digestion.

All of the type strains and several strains from each allele group were then tested for electrophoretic mobility differences in γG1. Only two mobility types were seen. Thus, the Ig-4 locus was defined and two alleles, Ig-4^{a,c,d,e,g,h} and Ig-4^{b}, designated.

Recent innovations in techniques of immunization have now made it possible to elicit good alloantisera reacting with γG1 globulins. Nonetheless, only the two originally defined alleles at the Ig-4 locus have been recognized by these sera (see Table 13.6).

Table 13.6. Distribution of Ig-1 alleles in inbred mouse strains. From [5] with additions.

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

* Type strain for each allele is italicized.

† CWB, C3H and C3H.SW form a congenic trio C3H = $H2^k$, Ig^a; C3H.SW = $H2^b$, Ig^a; and CWB = $H2^b$, Ig^b.

‡ BAB is congenic with BALB/c: BALB/c = Ig^a; BAB = Ig^b. BAB are derived by further backcrossing from BALB/c-2, received from Dr M. Potter at the 13th backcross generation. Congenic strains are almost identical genetically except at the indicated loci.

Table 13.7a. Allotype immunization protocols

Step 1: Immunization of allotype donor				Step 2: Immunization of recipient for production of anti-allotype serum	
Antigen	Protocol No.	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 4 places s.c. Days 3 and 5: same but in 4 places s.c. Days 21 and 37: same as day 1	Pool bleed Day 25 Day 32 Day 45 Discard	Days 1, 3, 5, 22, 26, 50, 57: 10^9 bacteria + 20 μlitre 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. 10 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 class of antibody§. Pool bleed weekly	Days 1, 3, 5, 21, 37 and monthly booster. Incubate serum from Step 1 with number of bacteria just sufficient to completely absorb anti-pertussis activity, 3 hours at 37° C and overnight in cold. Wash 3X with cold saline. Resuspend in saline to 2×10^{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 μlitre antiserum from Step 1 + 20 μlitre CFA† injected i.p. and 4 places s.c. Days 21, 28 and monthly booster: 10 μlitres in 0·2 ml saline i.p.	Day 35 bleed and test. Pool bleed all positive animals weekly

* Protocol established by Dr Tohru Masuda, this laboratory.
† Complete Freund's adjuvant.
§ Incubate antiserum plus *B. pertussis*, wash. Test conjugates in radioimmune assay. Use sera whose complexes show high levels of γA and low levels of other immunoglobulins.
‖ Kindly supplied by Lederle Laboratories.

TABLE 13.7b. 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
b anti-a	(LP/J or C57BL/10)	(BALB or C3H.SW)	I	*B. pertussis*	+++†	−	+	++
b anti-a	SJL/J				+++	o.w.	+	+++
e anti-a	NZB	BALB	II	*B. pertussis*	++	++	+++	−
d anti-a	AKR				−	++	o.w.	−
b anti-a	C57BL/10				+++	++	−	++
b anti-a	C57BL/10	BALB	III	H-2	++	o.w.	o.w.	−
b anti-a	LP/J				+++	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				+++		o.w.	++
a anti-b	BALB	C57BL/10	III	H-2	+++	−	−	−
b anti-c	C57BL/10	DBA/2	III	H-2	+	++		
a anti-c	C3H				±	−		
d anti-c	AKR				±	−		
b anti-d	LP	AKR	III	H-2	++			
b anti-d	C57BL/10				++			
c anti-d	DBA/2				++			
a anti-e	BALB/c	NZB	III	H-2	++		o.w.	
b anti-e	C57BL/10	A/J			+++		o.w.	

* See Table 12.7a.

† Antibody activity was surveyed either 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.

uncommon for two different mouse strains carrying the same allotype to respond quite differently when presented with the same antigen–antibody complex in an identical protocol, suggesting that there may be genetic factors controlling the immune response to allotypic antigens [17].

From the foregoing, it is clear that the preparation of anti-allotype reagents is at present an art bordering on a science. As a guideline for the novice venturing into this field, some of the experience in our laboratory is summarized in Tables 13.7a and b. These observations are not the result of exhaustive testing and should not be considered as definitive. Minor changes in dosage or timing do not appear to be critical. A great deal of additional variable information on techniques of immunization may be found in the literature, especially in the work of Drs. Lieberman and Potter [6].

Ouchterlony analysis (double diffusion)
Miniaturized hexagonal patterns, with holes separated 5 mm from their centres are cut with a flattened 18 gauge hypodermic needle from a 6 ml bed of agar (1 per cent ionagar in ·05 M Barbital, pH 8·2) on a 2 in × 3 in microscope slide. After development of the precipitation lines, (3–6 hours at room temperature) slides are immersed briefly in water to replace the air in the wells with liquid, a piece of Whatman #1 filter paper is slid over the agar to make firm contact, and the covered slide is removed from the water, blotted and dried in a warm (not hot) airstream. When slides are dry, the paper comes off easily, leaving an agar film on the glass with the precipitate firmly imbedded in the film. Excess soluble protein is removed by soaking the slide overnight in 0·5 per cent $NaHCO_3$ and the precipitation lines permanently stained with 1 per cent Buffalo black in water–methanol-glacial acetic acid (5:5:2) (1 min in stain solution and approximately 5 min in 5 per cent acetic acid to destain).

Immunoelectrophoresis (*IEP*)
Slides for IEP are prepared as for Ouchterlony plates except that the slide is cleaned with ethanol and then dipped in 0·1 per cent agar in water and allowed to dry before the agar bed is laid onto the slide. This pretreatment prevents slippage of the agar when troughs are cut.

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

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

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

Radio immune assay for detection of antibody to immunoglobulins and for estimation of immunoglobulin levels in sera
The workhorse assay in our laboratory for the detection of anti-allotype antibody is the precipitation of isolated immunoglobulins isotopically trace labelled with ^{125}I. This same assay, with an added step in which competitive unlabelled immunoglobulins are introduced to 'inhibit' precipitation of the labelled antigen has proved quite sensitive and reliable for the quantitative estimation of immunoglobulin levels in serum or culture fluids, as well as for the recognition and characterization of allotypic determinants on various immunoglobulins.

Isolation of immunoglobulins for use as labelled antigens. Because myeloma tumours produce a large amount of a single immunoglobulin, frequently with the concurrent lowering of all other serum immunoglobulins, sera or ascites fluid from myeloma-bearing mice provide ideal starting material for the isolation of a highly homogeneous immunoglobulin with little contamination from other immunoglobulins. Therefore, whenever possible, i.e. whenever there is a passageable myeloma tumour producing a protein carrying the required antigen,

myeloma proteins are isolated for use as labelled antigens. In cases where no myeloma protein is available, a fraction of the normal serum immunoglobulins enriched for the particular class required is used.

Myeloma proteins are available for all Ig^a immunoglobulin classes but only some Ig^b and Ig^e classes [6, 18]. For Ig-1b (γG2a) we routinely use a cathodal fraction isolated from serum taken from an older SJL/J (Ig^b) mouse showing acute elevation of this class. While the elevated immunoglobulin is of restricted mobility, it does not appear to be a strictly monoclonal product and the resulting preparation is therefore more heterogeneous than isolated myeloma proteins.

Proteins are usually isolated using standard methods of column chromatography frequently with separation on DEAE-cellulose. We find it occasionally useful to separate small amounts of protein by electrophoresis in agar on a 2 in × 3 in (IEP) microscope slide. To extract the protein, the agar is cut into slices, placed in centrifuge tubes with 0·5 ml of buffer and spun at 50 000 rev/min for 30 min, which breaks the agar emulsion and frees the protein into the supernatant. This method, however, sometimes introduces contaminants which interfere somewhat with ^{125}I labelling.

Iodination with ^{125}I. Iodination of approximately 100 μg portions of γ-globulin is performed by the chloramine-T method [19] (see chapter 17) with an average labelling of less than 1 atom ^{125}I/molecule. Passage through anion exchange resin columns followed by dialysis against saline is used to remove unbound iodide. Approximately 5–30 per cent of the added iodine becomes covalently bound to the protein, and is therefore TCA precipitable. With some preparations, 10–15 per cent of the radioactivity was not easily removed by dialysis but was nevertheless not precipitable by TCA. In these cases, precipitation of the protein by 50 per cent saturated ammonium sulphate (0–10° C) was an effective means of separating the protein from the non-precipitable radioactivity.

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

For the assay, 50 μl of labelled antigen in 3 per cent BSA-tris (i.e. 0·05 M tris buffer, final pH 7·6, containing 3 per cent bovine serum albumin (BSA), diluted to contain approximately 2×10^5 cpm/50 μl, is placed in a 6 × 50 mm culture tube. To this is added, *with rapid mixing*, 50 μl of the antiserum serially diluted in S-dil, i.e. 9 parts of 3 per cent BSA plus 1 part normal rabbit serum. The reason for using a diluent containing normal rabbit serum will be discussed in the next section.

The tubes are incubated at 37° C for 3 hours, chilled to 4° C for at least 30 min, and then centrifuged in the cold at 10^4 *g* for 10 min. 50 μl of the supernatant is carefully removed and placed in a disposable counting tube containing approximately 0·5 ml of normal saline. Samples are counted in a well-type crystal, γ scintillation counter.

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

Requirement for C1q in precipitation of ^{125}I-labelled antigens. During the development of the ^{125}I-labelled antigen precipitation assay, we found that demonstration of maximal antibody titres with many antisera required the presence of a non-specific serum factor from normal rabbit serum. As a routine precaution, we therefore always included 5 per cent normal rabbit serum in the final assay mixture. Recently, consistent with findings reported by McKenzie *et al* [20], we found that the rabbit serum could be replaced by purified human C1q, a subunit of C'1, the first component of haemolytic complement [21], kindly supplied to us by Dr M. A.

Calcott in Dr Mueller-Eberhard's laboratory. This is demonstrated as follows.

Without exogenously added C1q a typical anti-allotype antiserum reaches two-thirds of maximum precipitation at 0·3 μl antiserum/assay tube. With 5 per cent normal rabbit serum in the incubation mixture, the same endpoint is reached at ·05 μl/tube. Depletion of C1q from the antiserum by inactivation at 56° C for 30 min reduces the titre in the absence of exogenously added C1q such that two-thirds maximum is not even approached at 5 μl/tube, but the titre is restored to maximum again by the presence of 5 per cent normal rabbit serum. Complement inactivated (56° C for 30 min) rabbit serum will not restore the titre. Finally, 0·3 μg isolated human C1q restores the titre as well as the normal rabbit serum.

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

The dependence on C1q for precipitation may mean that antibody which does not bind complement will not be detected in this kind of assay. Some circumstantial evidence points to the existence of such antibody in some sera [22], but we have not explored this question at any length.

The C1q in mouse, human, rabbit, goat and guinea-pig sera seem about equally effective in precipitating labelled complexes, however, without a measure of C1q levels in these sera, no definite statement can be made. Isolated human C'1, kindly supplied by Dr Dean Linscott, was only marginally active until treated with EDTA to separate the C1q from the other C'1 subunits. The whole serum samples also increased in activity after EDTA treatment suggesting that only C1q works to facilitate precipitation.

The facilitation also demonstrates a salt concentration sensitivity. At a NaCl concentration equivalent to that in normal serum (0·15 M) no precipitation occurs at all. Somewhere below 0·1 M, precipitation begins to occur and is maximal at about 0·07 M. Assays are routinely run therefore at 0·05 M Tris with no added sodium chloride.

We have bound partially purified C1q to Sepharose using the Cyanogen–Bromide method [23] to make a C1q-Sepharose column (see insoluble immunoabsorbants, p. 13.16). The bound material retains preformed antigen–antibody complexes when these are passed through the column at low salt concentration and releases the complex still intact at 0·15 M NaCl. Elution of the complexes from the column regenerates the column, and it once again binds complexes when the salt concentration is reduced.

Quantitation of immunoglobulins by radioimmune assay

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

The inhibition assay is performed as follows (slightly modified from [24]):

As in direct precipitation, 50 μl of labelled antigen diluted in 3 per cent BSA-tris such that 50 μl contain approximately 2×10^5 cpm, is added first. Next 5 μl of unlabelled test or standard antigen at varying dilutions in 3 per cent BSA-tris is added and the tubes mixed. Last, 50 μl of antiserum at a dilution (in S-dil) chosen such that the amount added is sufficient to precipitate about two-thirds of the maximum number of counts precipitable by that antiserum is added (Fig. 13.1). Since rapid mixing is essential, antiserum is generally added to two tubes at a time and those are then immediately mixed with a Vortex Junior mixer (Scientific Industries, Springfield, Mass.). Incubation and sampling is as described above in the section on precipitation of labelled antigen. All tests are done in duplicate.

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

Specialized laboratory methods

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

Production of antisera

Rabbit antisera

Rabbit antisera are made against mouse whole sera, partially purified normal immunoglobulins, and purified myeloma proteins and fragments (papain digestion products, i.e. Fc and Fab pieces) of immunoglobulins. Two injections, each of approximately 100 μg of purified protein (50 μg in the case of the papain fragments) or 100 μl of whole serum, evoke antisera which give strong precipitin arcs in immunoelectrophoresis.

The first injection of the antigen emulsified in complete Freund's adjuvant made with oil to water, 3:1 is given in the foot pads and in several subcutaneous sites using about 0·1 ml/site. After approximately 4 weeks, a second injection of the antigen, without adjuvants, is given intramuscularly. Starting at 1 week after the second injection and continuing weekly thereafter for several weeks, 30–60 ml of blood are drawn from the heart.

Preparation of antisera specific for a given immunoglobulin class often requires absorption of the antisera as well as careful isolation of the immunizing protein or fragment. Methods for absorption are presented in a later section.

Mouse anti-allotype sera

Although normal serum or normal γ-globulin can be used as antigen to stimulate anti-allotype antibody, far better results have been obtained by using, as the antigen, sera or immunoglobulins from animals which contain antibodies directed to some tissue or protein component in the recipient strain. For example, whereas BALB/c mice immunized with normal (C57BL/6) serum failed to produce any detectable anti Ig-1b, immunization of the same strain with C57BL/6 anti DBA/2-spleen-antiserum (which has a high anti H-2d titre) was quite successful (BALB/c and DBA/2 are both H-2d). Similarly, an antiserum to a complement-component antigen, Hc^1 [16], was also effective in stimulating anti-allotype serum. In many cases the first antigen injection was in complete Freund's adjuvant but boosts were aqueous.

In our hands this method of immunization is particularly effective for raising antibody reactive with Ig-1 (γG2a) allotypes, but only marginally useful for raising antibody to allotypes on other classes. With the exception of the immunization of C57BL/6 with DBA/2, where antisera often react well with Ig-2 (γA) as well as Ig-1, most immunizations by this method result in low titres in occasional animals for all antigens other than the Ig-1 (γG2a) allotypes.

More effective immunization for other classes has been obtained by challenging the animal with immunoglobulins in the form of an externally created antigen–antibody complex. Recently, we have used a method established by Dresser, Taylor & Wortis (personal communication) in which *Bordetella pertussis* vaccine is used to immunize mice from one strain, these mice are bled, and their sera incubated *in vitro* with *B. pertussis* to allow complex formation. The whole mixture is then injected into a second mouse strain of a different allotype to generate an anti-allotype response. This method has proved excellent for obtaining antibody reactive with Ig-4 (γG1) allotypes as well as antibodies reactive with Ig-1 (γG2a) allotypes. To a lesser extent, it has been useful for producing antibody to Ig-3 (γG2b) allotypes. Strangely, we found no antibody produced reactive with Ig-2 (γA) allotypes.

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

Not only, however, is it important to choose an appropriate protocol for preparing the immunogen to elicit antibody to a particular anti-allotype, but the choice of recipient mouse strain can considerably influence the success of the anti-allotype immunization. As many investigators involved in preparation of anti-allotype sera, notably Dr Rose Lieberman and Dr Michael Potter, have pointed out, it is not

fore the amount of labelled antigen precipitated by the antiserum in the absence of inhibitor (maximum precipitation control).

A series of dilutions of a known inhibitor protein or normal serum pool carrying the allotype under test is always included in each assay, to provide data for a standard curve by comparison with which the allotype levels in the unknowns are determined. For convenience in interpolating values when reading

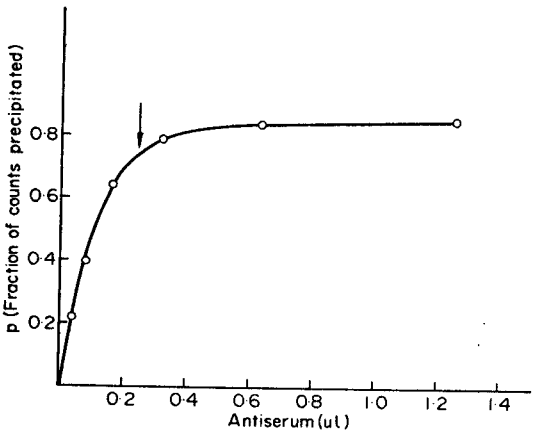

FIG. 13.1. Precipitation of ^{125}I-labelled antigen by anti-allotype serum. *P*: fraction of counts precipitated. Each tube contains ~0·013 μg of ^{125}I-labelled γb2a myeloma protein, GPC-8, in 50 μl 3 per cent BSA-tris plus Igb anti Iga (LP anti BALB/c-pertussis) diluted in 50 μl S-dil. Arrow indicates amount of antiserum (0·25 μl) chosen for use in quantitative inhibition of precipitation assay presented in Fig. 12.2. Better linearity in the inhibition assay would be obtained choosing ~0·18 μl antiserum.

from this curve, the *reciprocal* of the percentage of labelled antigen precipitated is plotted as a function of inhibitor concentration. This simple manoeuvre yields a standard curve which is linear over a wide range of concentrations.

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

Put into mathematical terms, if for each assay tube:

P: fraction of radioactivity precipitated,
Ab: μg of antigen precipitated (bound) by the amount of antibody used,
Ag: μg of labelled antigen used,
*Ag**: number of counts/min in Ag, and
u: varying amount (in μg) of unlabelled (inhibitor) antigen added

then

$\frac{Ag^*}{Ag}$: specific radioactivity of the labelled antigen preparation and

$\frac{Ag^*}{u+Ag}$: specific radioactivity of total antigen in tube when varying amounts of unlabelled antigen are added.

When no inhibitor is present,

$$P = \frac{Ab.\frac{Ag^*}{Ag}}{Ag^*} = \frac{Ab}{Ag} = \text{maximum fraction of counts}$$

precipitated in the assay.

When varying amounts of inhibitor (*u*) are present,

$$P = \frac{Ab.\frac{Ag^*}{u+Ag}}{Ag^*} = \frac{Ab}{u+Ag}$$

and, taking the reciprocal

$$\frac{1}{P} = \left(\frac{1}{Ab}.u\right) + \frac{Ag}{Ab}$$

This is in the slope intercept form of the equation for a straight line when $1/P$ is plotted against *u*. The inverse of the ordinate intercept of this line is equal to Ab/Ag, the maximum fraction of labelled antigen which is precipitable by the amount of antibody used. The slope, $1/Ab$, decreases as the absolute amount of antigen precipitated increases, hence as the amount of antibody used increases.

Data showing a typical reciprocal plot (linear) standard curve for an inhibition assay are presented in Fig. 13.2a. For comparison, the same data with percentage of labelled antigen plotted directly as a function of amount of inhibitor are presented in Fig. 13.2b. Although the data in both figures are

presented in terms of μg of inhibitor added, it is, of course, equally possible to use a standard of unknown inhibitor concentration, e.g. normal serum, and express values for unknowns as μl of standard/μl unknown.

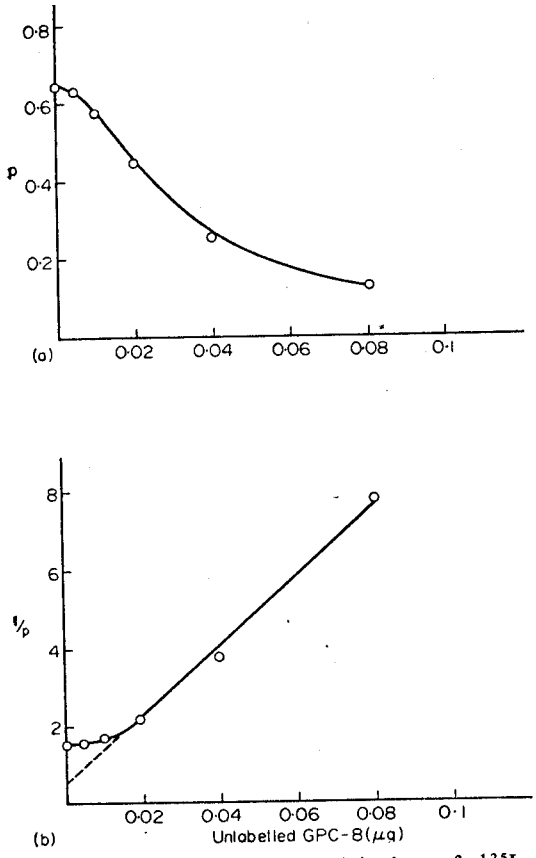

FIG. 13.2. Inhibition of precipitation of ^{125}I-labelled antigen by homologous unlabelled antigen. *P*: fraction of counts precipitated. Each tube contains ~0·013 μg of labelled γG2a myeloma protein, GPC-8, in 50 μl 3 per cent BSA-tris, 0·25 μl Igb anti Iga (LP anti BALB/c-pertussis) in 50 μl S-dil and 5 μl unlabelled GPC-8 diluted in 3 per cent BSA-tris.

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

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

Sensitivity of the assay varies according to the concentration of antibody and labelled antigen. Generally, the minimum amount of immunoglobulin carrying a particular allotypic antigen detectable is in the region of 0·02 mg/ml serum, although if need be the lower limit can be decreased by as much as an order of magnitude. Determinations may be carried out without interference even in the presence of a 20 000-fold excess of a non-cross-reacting immunoglobulin. Thus, as little as 10 μg of allotype-carrying immunoglobulin may be detected in a mouse with roughly 30 mg of total immunoglobulin.

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

(1) avoid the introduction of a large amount of extraneous antigen–antibody complex, which will non-specifically consume C1q and therefore prevent complete precipitation of the labelled complex (see C1q p. 13.12), and

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

Linearity for a cross-reacting standard may be obtained by subtracting the fraction of radioactivity

precipitation not inhibitable from the total fraction precipitated before converting to the reciprocal. It is, however, more satisfactory to choose appropriate combinations of antiserum and labelled antigen or to absorb the antiserum first, so that such manipulations are unnecessary.

Absorption of antisera: isolation of specific antibody from multi specific antisera using Sepharose conjugated immunoabsorbents

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

The data in Table 13.8 illustrate the isolation of two anti-allotype antibodies using antigen-Sepharose column adsorptions and elutions. In the first step, passage through a Sepharose Ig-1a column removed virtually all of the anti Ig-1a antibody from a complex antiserum containing anti Ig-1a and anti Ig-4a while nearly all of the anti Ig-4a activity was recovered at the column front. The bound anti Ig-1a activity was then eluted with 0·1 M acetic acid. Finally a small amount of contaminating (probably non-specifically trapped) anti Ig-4a activity found in the eluate was removed by passing the eluate through a Sepharose Ig-4a column. Recoveries, calculated in antigen binding units, were excellent, running from at least 50 per cent to as good as 90 per cent. The resolved components were each specific for the appropriate antigen within the limits of the assay.

For the separation described above, the antiserum used was an Igb anti Iga, prepared by immunizing LP mice with BALB/c antipertussis conjugated to pertussis organisms p. 13.8. The serum was loaded onto a Sepharose-Ig-1a column* PBS (0·01 M phosphate, pH 7·1, in 0·15 M NaCl) was then passed through the column and fractions collected until no more 280 nm absorbing material came off.† The initial fractions collected accounted for 90 per cent of the anti Ig-4a activity in the original serum, while the anti Ig-1a activity recovered (0·2 per cent of original anti Ig-1a) bordered on the limits of detectability. Thus, in one pass the column specifically removed (adsorbed) essentially all the anti Ig-1a activity from the serum.

To recover the bound anti Ig-1a, 0·1 M acetic acid was passed through the column. A protein peak moved at the acid front. 2·5 ml fractions were collected into tubes containing 2·5 ml 2X PBS in 3 per cent BSA which immediately brought the pH of the eluted material near neutrality. The pooled material collected under the protein peak accounted for 51 per cent of the original anti Ig-1a activity. In addition, a small amount (5 per cent of original) of anti Ig-4a activity appeared which was either trapped non-specifically or able to react with Ig-1a as well.

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

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

Acknowledgments

The studies described in the sections on the requirement for C1q in precipitation of ^{125}I-labelled anti-

* Before use, the column was washed with 0·1 M acetic acid to remove any extraneous protein, then PBS to return to neutrality. Then, to block non-specific absorption, 2 ml of LP normal serum was run through, followed by PBS washing until no more 280 nm absorbing material appeared in the effluent.

† Recent experience indicates that C1q helps to stabilize the antigen–antibody complex on the column. Therefore, 0·05 M Tris is probably a better buffer for washing (p. 13.12).

TABLE 13.8. Recoveries of anti-Ig-1a and anti-Ig-4a activity.

Fraction		Anti-Ig-1a			Anti-Ig-4a		
Step	Description	Units/ml*	Total units	% of original	Units/ml	Total units	% of original
	Original serum Ig^b anti Ig^a . . . ('A')	12·5	130	100	4	42	100
Passed 'A' through Ig-1a Sepharose†	Specific anti Ig-4a	0·3	0·2	0·2	5·8	38	90
Column eluted with 0·1 M acetic acid	Activity bound to Ig-1a Sepharose . . . . . ('B')	6·3	66	51	25	2·1	5
Passed 'B' through Ig-4a Sepharose‡	Specific anti Ig-1a	3·2	67	51	0·02	0·4	0·1
Column eluted with 0·1 M acetic acid	Activity bound to Ig-4a Sepharose	0·7	1·1	0·8	0·5	0·8	2

* 1 unit of antibody = that volume able to precipitate two-thirds of maximum precipitable ^{125}I antigen precipitated by the original serum $\times 10^{-3}$.

† 5 ml column bed, with total of 10 mg bound RPC-5 (Ig-1a myeloma protein).

‡ 0·5 ml column bed, with total of 1 mg bound S-8 (Ig-4a myeloma protein).

gens and the absorption of antisera with Sepharose-bound (insoluble) immunoadsorbants, published for the first time here, were undertaken in our laboratory in collaboration with Mr Derek Hewgill. In addition, the authors wish to thank the entire staff of our laboratory, and in particular, Mrs Myrnice Ravitch and Mrs Phillipa Meyering, for their patient help in the preparation of this manuscript.

References

[1] EDELMAN G.M. & GALL W.E. (1969) The antibody problem. *Ann. Rev. Biochem.* **38,** 415

[2] FAHEY J.L. (1962) Heterogeneity of γ-globulins. *Advanc. Immunol.* **2,** 41

[3] FAHEY J.L., WUNDERLICH J. & MISHELL R. (1964) The immunoglobulins of mice. II. Two subclasses of mouse 7S γ_2 globulins: γ_{2a} and γ_{2b} globulins. *J. exp. Med.* **120,** 243

[4] GREY H.G., HIRST J.W. & COHN M. (1970) A new mouse immunoglobulin: IgG3. *J. exp. Med.* **133,** 289

[5] HERZENBERG L.A., MCDEVITT H.O. & HERZENBERG L.A. (1968) Genetics of antibodies. *Ann. Rev. Genet.* **2,** 209

[6] POTTER M. & LIEBERMAN R. (1967) Genetics of immunoglobulin in the mouse. *Advanc. Immunol.* **7,** 92

[7] Nomenclature for immunoglobulins (1964) *Bull. Wld. Hlth Org.* **30,** 447. Extensions are published about yearly in *Bull. Wld Hlth Org.*

[8] TODD C.W. (1972) Genetic control of H chain, biosynthesis in the rabbit. *Fed. Proc.* **31,** 188

[9] KUNKEL H.G., JOSLIN F.G., PENN G.M. & NATVIG J.B. (1970) Genetic variants of γG_4 globulin. A unique relationship to other classes of γG globulins. *J. exp. Med.* **132,** 508

[10] PERNIS B., FORNI L. & AMANTE L. (1972) Immunoglobulins as cell receptors. *Ann. N.Y. Acad. Sci.* **190:** 420–431

[11] WANG A.C., WILSON S.K., HOPPER J.E., FUDENBERG H.H. & NISONOFF A. (1970) Evidence for control of synthesis of the variable regions of the heavy chains of immunoglobulins G and M by the same gene. *Proc. nat. Acad. Sci.* (*Wash.*) **66,** 337

[12] APPELLA E. & PERHAM R.N. (1967) The structure of immunoglobulin light chains. *Cold Spr. Harb. Symp. quant. Biol.* **32,** 37

[13] WEIGERT M. (personal communication) (1972)

[14] EDELMAN G.M. & GOTTLIEB P.D. (1970) A genetic marker in the variable region of light chains of mouse immunoglobulins.

[15] HERZENBERG L.A., MINNA J.D. & HERZENBERG L.A. (1967) The chromosome region for immunoglobulin heavy chains in the mouse: allelic electrophoretic mobility differences and allotype suppression. *Cold Spr. Harb. Symp. quant. Biol.* **32,** 181

[16] ERICKSON R.P., TACHIBANA D.K., HERZENBERG L.A. & ROSENBERG L.T. (1964) A single gene controlling hemolytic complement and a serum antigen in the mouse *J. Immunol.* **92,** 611

[17] LIEBERMAN R. & HUMPHREY W., Jr. (1971) Association of H-2 types with genetic control of immune responsiveness to IgA allotypes in the mouse. *Proc. nat. Acad. Sci.* (*Wash.*) **68,** 2510

[18] WARNER N.L. (personal communication) (1971)

[19] GREENWOOD F.C., HUNTER W.M. & GLOVER J.S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89,** 114

[20] MACKENZIE M.R., WARNER N.L., LINSCOTT W.D. & FUDENBERG H. (1969) Differentiation of human IgM subclasses by the ability to interact with a factor resembling the first component of complement. *J. Immunol.* **103,** 607

[21] MULLER-EBERHARD H.J. (1968) Chemistry and reaction mechanisms of complement. *Advanc. Immunol.* **8,** 30

[22] BAIRD S., SANTA J. & WEISSMAN I. (1971) Anti-Theta antisera may contain antiallotype contamination. *Nature–New Biology* **232** 56

[23] CUATRECASAS, F., WILCHEK M. & ANFINSEN C.B. (1968) Selective enzyme purification by affinity chromatography. *Proc. nat. Acad. Sci.* (*Wash.*) **61,** 636

[24] HERZENBERG L.A. & WARNER N.L. (1968) *Genetic control of mouse immunoglobulins in regulation of the antibody response*, ed. CINADER B., p. 322. Springfield: C.C. Thomas