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IMMUNOGLOBULIN ISOANTIGENS (ALLOTYPES) IN THE MOUSE

III. Detection of Allotypic Antigens with Heterologous Antisera[1]

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The immunoglobulins of many species have been subdivided into separate classes on the basis of differing functional, chemical and antigenic properties (1, 2). The antigens which are associated with only one class of immunoglobulin are located on the H-chain, and usually on the Fc fragment of that chain. Detection of these antigens is made with antisera elicited in animals of a different species (heteroantisera). Superimposed upon this variation from class to class within an individual is the antigenic variation of a particular immunoglobulin class between individuals. This latter type of variation (allotype) (3, 4) is usually determined with antisera elicited in animals of the same species (isoantisera).

Recently it has been shown that some heteroantisera contain antibodies which recognize allotypic antigens. This has been demonstrated for rabbit γ-globulin (5), human γ-globulin (6–8), human β-lipoprotein (9) and human α2-macroglobulin (10).

Genetically controlled allotypes of three (11–19) of the five mouse immunoglobulin classes (20, 21) have been demonstrated using isoimmune sera elicited in various inbred mouse strains. The present article reports the detection in rabbit antimouse immunoglobulin sera of antibodies detecting several different allotypic antigens.

MATERIALS AND METHODS

Antigens for immunization. Rabbit antisera were prepared against normal mouse immunoglobulin fractions and against microsomes obtained from transplantable plasma cell tumors arising and maintained in hybrid mice. The rabbit antisera and the antigens used for immunization are as follows: *1)* Rabbit antimouse γ-globulin (RaMγ); a sample of pooled C3H.SW and C57BL/10 normal mouse serum was separated by electrophoresis on starch-pevikon block (pH 8.6, 0.05 M barbital), and the broad slow migrating cathodal fraction was used. *2)* Rabbit antimouse γ-globulin Fc fragment (RaFc); normal C57BL/10 mouse serum was separated by electrophoresis on starch-Pevikon block and the broad cathodal fraction obtained by elution was digested with papain for 16 hr by the method of Porter (22). The digest was fractionated by column chromatography on carboxymethyl cellulose. The first peak from this column was further resolved by a diethylaminoethyl (DEAE) cellulose column into two fractions, which on testing with specific antisera were shown to be the Fab and Fc fragments of mouse γ-globulin. *(3, 4)* Rabbit antimouse myeloma protein; microsome preparations were made from two plasma cell tumors arising in (NZB $\times$ BALB/c)F_1 hybrid mice, namely GPC-7 and 5. The method of isolation of microsomes has previously been described (19).

Immunization schedule. Each rabbit received multiple site primary subcutaneous injections of either immunoglobulin protein (100 μg) or microsomes (100 μg), combined with complete Freund's adjuvant. The rabbits were boosted with antigen 4 weeks later and bled the following week.

Precipitation technique. The basic method used for detecting antigen antibody reactions involves labeling of a purified immunoglobulin with Iodine^{125}, incubation with antiserum in the presence or absence of inhibitor proteins or serum, centrifugation and counting of supernatant samples to determine percent of label (antigen) precipitated. The techniques for purifying the antigens used in these *in vitro* tests and the many details of the method discussed above have all been previously described (16, 18).

Normal mouse sera and myeloma proteins. Nor-

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mal sera from inbred strains were obtained by tail bleeding and were stored at −20°C until used. The myeloma proteins used in this study, with the immunoglobulin class and strain of origin of each, are as follows: γG_{2a}-immunoglobulins: 5563 (C3H), RPC-5 (BALB/c), GPC-7 (NZB type from (BALB/c × NZB)F_1); γG_{2b}-immunoglobulins: MPC-11 and MPC-31 (BALB/c); γG1: MPC-25 (BALB/c); γA: MPC-1 (BALB/c); γM: MOPC-104 (BALB/c), and GPC-5, a presumed recombinant type between NZB types γG_{2a} and γG_{2b} ((BALB/c × NZB) F_1) (18).

Absorption of sera with myeloma proteins. Rabbit antisera were absorbed with various immunoglobulins conjugated to polyaminopolystyrene (PAPS), by a method also previously described (19).

Expression and interpretation of results. The results obtained from each inhibition assay are presented either graphically or in tabular form, and give the percent of inhibition of the particu-

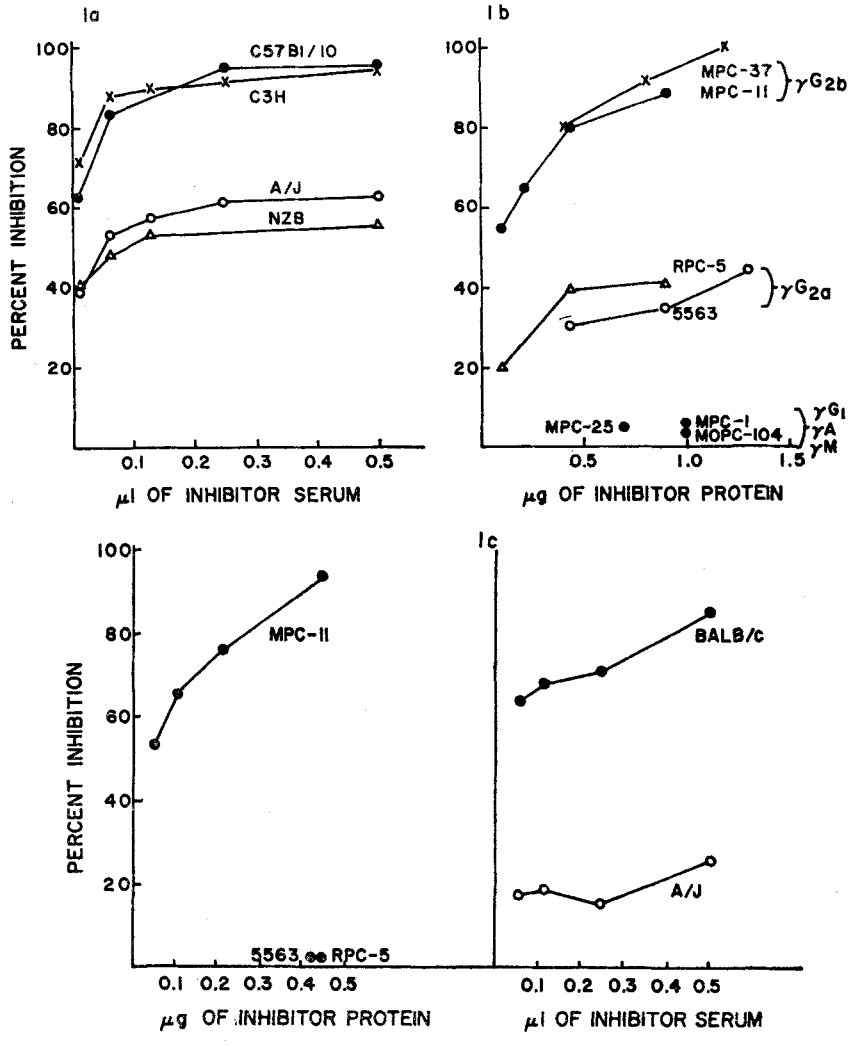

Figure 1. Inhibition of precipitation of I^{125} labeled MPC-31 myeloma protein by whole mouse sera (1*a*) and purified myeloma proteins (1*b*). Antiserum used was rabbit antimouse immunoglobulin Fc fragment. In Figure 1*c*, this antiserum had first been absorbed consecutively with two Ig-1^a allotype, γG_{2a} myeloma proteins (5563, and RPC-5) conjugated to PAPS. C3H and BALB/c have been used interchangeably as they have the same Ig-1 and Ig-3 allotypes.

lar reaction with each concentration of each inhibitor used. With an I^{125} labeled antigen, A, and a rabbit antiserum which precipitates A, an excess of unlabeled A will give 100% inhibition of precipitation of I^{125}. An immunoglobulin preparation from another strain, B, which lacks an allotypic specificity present on A and detected by anti A, will even in large excess usually give only a partial inhibition. The uninhibitable precipitation is due to (an) antigenic determinant(s) present in A and absent in B. (A complete discussion of the premises used in this interpretation has been offered (16, 19).)

RESULTS

Detection of allotypic antigens on γG_{2b}-immunoglobulins. Precipitating assays were set up with several rabbit antisera and labeled mouse myeloma protein MPC-31 (BALB/c type γG_{2b}). Normal sera from various mouse strains were tested for their ability to inhibit these precipitations. A typical experiment is shown in Figure 1. In this, and all following figures, percent of inhibition of precipitation of the particular antigen antibody reaction is plotted against the concentration of inhibitor protein or serum. The results in Figure 1*a* show that at least two antigenic determinants are being detected in this assay (see interpretation of results in Method section). One is absent from A/J and NZB and the other is present on all four sera tested. Figure 1*b* shows that two γG_{2a} myeloma proteins inhibit and therefore cross-react in this system but that γM, γA and γG_1 proteins do not carry the H-chain specificities thus detected. After absorption of the antiserum by these two γG_{2a} proteins (completeness of absorption shown in Figure 1*c* by the lack of inhibition by proteins used for absorption) the antiserum still reacts with γG_{2b} proteins. That the absorbed antiserum still contains the antiallotype antibodies is shown by the continued inability of A/J serum to inhibit completely while BALB/c does completely inhibit precipitation of the γG_{2b} labeled antigen.

The absence of the allotypic antigen from the

TABLE I

Effect of absorption of rabbit antimouse γ-globulin with A/J whole serum

Inhibitor Serum	Volume Added	Inhibition	
		Antiserum unabsorbed	Antiserum absorbed with A/J whole serum[a]
	μl	%	%
C3H[b]	1.00	100	100
	0.06	48	100
C57BL/10	1.00	92	100
	0.06	45	100
A/J	1.00	28	0
	0.06	21	0
NZB	1.00	30	0
	0.06	21	0

Precipitating assay: MPC-31 (I^{125} labeled)-rabbit antimouse γ-globulin.

[a] A lower percentage of precipitation of the labeled antigen was used in this assay, and hence an increased sensitivity was observed, i.e., for C3H and C57BL/10, more inhibition was obtained with 0.06 μl than in the assay with unabsorbed serum.

[b] C3H has the BALB/c allotype.

TABLE II

Rabbit antimouse immunoglobulin sera tested for antibody to γG_{2b} allotypic specificity

Sera Containing Antiallotype Antibody	Sera Not Containing Antiallotype Antibody
Rabbit anti-normal mouse γG2 (RaMγ pool A)	Rabbit anti-normal mouse γG2 (pools B and C)
Rabbit antimouse Fc fragment (RAFc-1, RAFc-2)	Rabbit antimouse Fab fragment (RASP-1).
Rabbit anti MPC-11 myeloma	
Rabbit anti MPC-37 myeloma	
Total: 5 sera	Total: 3 sera

TABLE III

Inhibition of precipitation of labeled MPC-31-RaFc by various mouse sera

Complete Inhibition		Partial Inhibition	
Strain	Ig-1 Allele	Strain	Ig-1 Allele[c]
BALB/C, C3H, NZC	a	AKR, AL/N	d
C57BL/10	b	A, NZB, NZO, NZW	e
DBA/2	c		
CE	f		
RIII	g		
SEA	h		

whole serum of A/J mice was also shown by absorption of another rabbit antiserum to mouse γ-globulins with whole A/J serum conjugated to PAPS. After absorption, this antiserum still precipitates labeled MPC-31. This precipitation is not inhibited by A/J or NZB serum, but is inhibited by other mouse sera (Table I).

Six other such sera, a total of eight rabbit antisera made to different preparations of mouse γG_2 immunoglobulins of BALB/c or C57BL/10 type, have been tested for the presence of antibody to this allotypic antigen. Five of the eight were found to contain this antibody (Table II).

Other inbred mouse sera were tested for their ability to inhibit the reaction shown in Figure 1a. Included were sera of the eight type-strain alleles for the Ig-1 locus (16). Six mouse sera were found only partially to inhibit and therefore lack the antigen present in BALB/c and C3H and absent in A/J and NZB (Table III). All of these are of (Ig-1) allelic types d or e and in fact are the only six mouse strains known at present to carry these d and e alleles.

The current list of antigenic specificities for the Ig-1 and Ig-3 loci (as defined by mouse isoantisera) is given in Table IV. One of these specificities, Ig-3.4, shows the same strain distribution as that recognized by the rabbit antisera described in this section.

Detection of allotypic antigens on γG_{2a}-immunoglobulins. The precipitation of labeled RPC-5 (γG_{2a}) myeloma protein by RaMγ was completely inhibited by mouse sera from all type strains (listed in Table IV), except C57BL/10 (Fig. 2).

TABLE IV

Allotype specificities of mouse γG_{2a} and γG_{2b} immunoglobulins as defined by mouse isoantisera

Type Strain	Allele	Ig-1 Locus (γG_{2a})	Ig-3 Locus (γG_{2b})
BALB/c[a]	a	1 2 – – – 6 7 8 9 10 –	1 2 – 4 5 6 7 8
C57BL/10	b	– – – 4 – – 7 – 9 – –	– – – 4 – – 7 8
DBA/2	c	– 2 3 – – – 7 – 9 – –	1 2 – 4 5 – 7 8
AKR	d	1 2 – – 5 – 7 – – – –	1 – 3 – 5 6 7 or 8
A/J	e	1 2 – – 5 6 7 8 – – –	1 – 3 – 5 6 7 –
CE	f	1 2 – – – – – 8 9 – 11	1 2 – 4 5 6 – –
RIII	g	– 2 3 – – – – – 9 – –	1 2 – 4 5 6 ? –
SEA	h	1 2 – – – 6 7 – 9 10 –	1 2 – 4 5 6 7 8

[a] Indistinguishable allotypically from C3H.

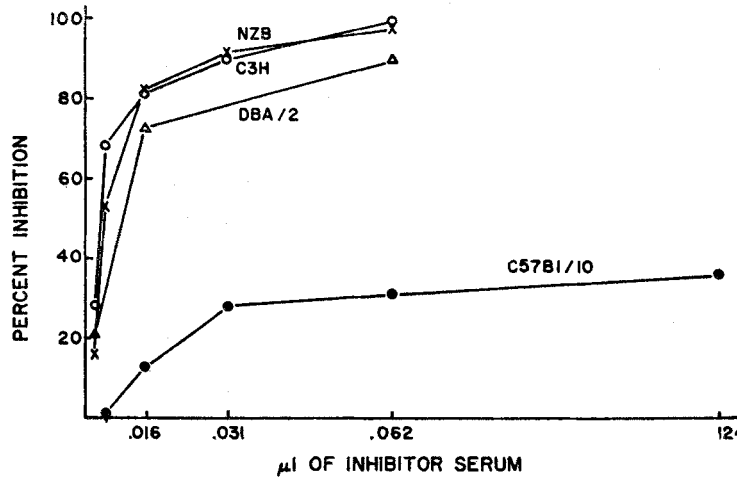

Figure 2. Inhibition of precipitation of I^{125} labeled RPC-5 myeloma protein by whole mouse serum. Antiserum used is rabbit antimouse γ-globulin no. 1.

This allotypic specificity was still detected after absorption of the antiserum with MPC-25 myeloma (γG_1). Since this particular allotypic specificity was not detected when this serum was used with a labeled γG_{2b} myeloma (see previous section), it can be concluded that this antiserum is detecting a γG_{2a} allotypic specificity present in all type strains except C57BL/10. Allotypic specificity Ig-1.2 corresponds to this strain distribution.

Another antimyeloma antiserum, rabbit anti-GPC-7 (this tumor is γG_{2a} of Ig-1^e allotype), was also found to detect this allotypic specificity after absorption of the antiserum with a γG_{2b} myeloma (MPC-11). Additional allotypic specificities were also detected with this antiserum, as type strains other than C57BL/10 also failed to inhibit completely. To determine the distribution of these specificities, this antiserum (RaGPC7) was tested with a γG2 preparation of four of the type strains and the inhibition patterns with all eight type-strain sera were determined. The results, presented in Table V, show a similar pattern of inhibition with labeled C3H and SEA and for labeled DBA/2 and RIII. When these latter two are used as labeled antigen, C57BL/10 is the only type-strain serum which fails to inhibit completely, and this would correspond to Ig-1.2. However, when C3H or SEA are used as labeled antigens, DBA/2 and RIII also fail to inhibit completely. This indicates another specificity with the strain distribution of Ig-1.1 detected by this antiserum. Since the antiserum used in these assays was one absorbed with a γG_{2b} myeloma, these are γG_{2a} specificities.

TABLE V

Inhibition of precipitation of labeled γG2 globulin from various mouse strains with sera from the eight type strains of the Ig-1 locus

Inhibitor Serum	Allelic Type	Inhibition with Various Labeled Antigens			
		C3H[a]	DBA/2	RIII	SEA
		%	%	%	%
BALB/c	a	100	100	100	100
C57BL/10	b	50	39	40	27
DBA/2	c	81	100	100	81
AKR	d	100	100	NT[b]	100
A/J	e	100	100	NT	100
CE	f	100	100	NT	100
RII	g	79	100	100	86
SEA	h	100	100	100	100

Antiserum used was rabbit anti-GPC-7 absorbed with a γG_{2b} myeloma protein (MPC-11).

[a] C3H is of BALB/c allelic type.

[b] Not tested.

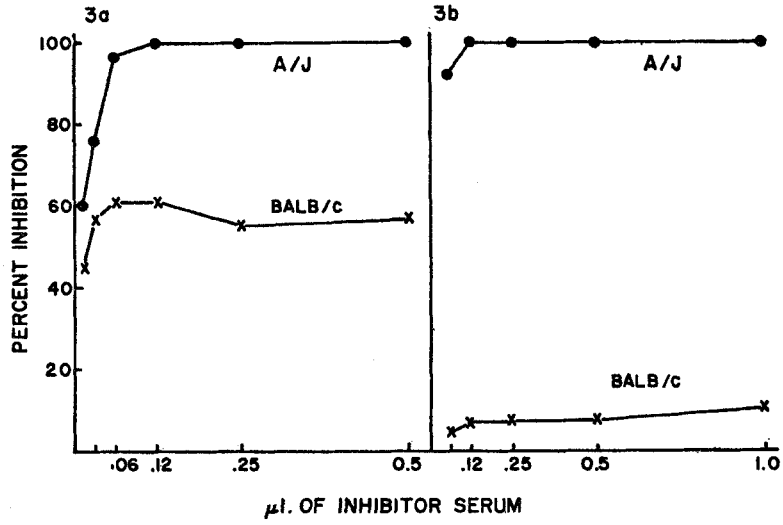

Figure 3. Inhibition of precipitation of labeled myeloma proteins by BALB/c and A/J normal serum. 3*a*: GPC-5-rabbit anti-GPC-7; 3*b*: GPC-7-rabbit anti-GPC-5. Each antiserum was previously absorbed with a γ G_{2b} myeloma (MPC-11) conjugated to PAPS. The greater degree of inhibition with BALB/c serum obtained in 3*a* as compared to 3*b* indicates a relatively greater amount of the anti-allotype antibody in the antiserum used in 3*b* than that used in 3*a*.

One more specificity was detected both by RaGPC7 tested with labeled GPC-5 myeloma protein, and by a rabbit anti GPC-5 serum used with labeled GPC-7 myeloma protein (both antisera first absorbed with a BALB/c γG_{2b} myeloma). The testing of these antisera with their nonhomologous antigens was to avoid the detection of "myeloma specific" antigens which are readily detected by these antisera. The results in Figure 3 show that both these assays detect a γG_{2a} allotypic specificity present in A/J and absent from BALB/c. Of the known γG_{2a} allotypic specificities, only Ig-1.5 has this distribution.

It might be noted that one antiserum, RaGPC-7, has been shown to contain at least three populations of antibodies directed to allotypic antigens. These three populations of antibodies form only a minor proportion of the total antibody population in the unabsorbed serum. Higher titers of antibodies were found directed to *1*) "myeloma specific" antigens, *2*) class specific antigens and *3*) antigens common to several H-chain classes. In fact, when the unabsorbed antiserum was used with a labeled γG_{2a} myeloma, the allotypic antigens could not be detected owing to the very minor involvement of the antiallotypic antibodies relative to the above-mentioned antibodies in this precipitation.

DISCUSSION

The main finding presented in this article is that various rabbit antimouse γ-globulin sera do contain antibodies directed to allotypic antigens as well as those antibodies which define the H-chain classes. This observation, although not previously recorded for mouse allotypic antigens, has been made in both rabbit (5) and human (6-8) γ-globulin allotypic systems. The results of the present study indicate the relative difficulty of detecting these antibodies in the presence of the much greater amounts of antibodies directed to class specific antigens.

Since each assay used was carefully tested to ensure that only one class of immunoglobulin was involved, a comparison with the current list of mouse allotypic specificities (as defined with isoantisera) is valid. It is probably more than coincidental that a corresponding strain distribution could be found for each rabbit-defined allotypic antigenic specificity. No rabbit-defined allotypes have so far been found for γM, γA or γG_1. This is in line with the results obtained with mouse isoantisera, of which only a very few have been found to detect γA allotypes and none for γM or γG_1. This correspondence between the rabbit and the mouse antisera, particularly in the similar strain distribution of the allotypic antigenic specificities, probably means that the same antigenic determinant is responsible for the elicitation of the antibodies in both iso- and heteroimmunizations. However, this cannot be definitely stated, since two different determinants which happen to have identical strain distributions may be involved.

Although careful quantitation of the amounts of different antibodies in the rabbit antisera were not made, it appeared from the titration of these sera before and after various absorptions that a much greater amount of antibody was directed to the class specific antigens (isotypic antigens, in the terminology of Oudin) (5) than to the allotypic antigens. We might speculate that this difference is due to either *1*) a greater number of actual antigenic determinants involved in the class specific antigen, each of which gives rise to a separate antibody population, the sum of which is detected as "class defining antibodies"; or *2*) a fundamental difference in the nature of the chemical site of these two types of antigens, which greatly affects the immunogenicity of the two types of antigens.

It appears that the allotypic antigenic determinant may be associated with only very minor differences, as recent evidence has been obtained for the human Inv determinant indicating a single amino acid substitution (23, 24). Peptide mapping of the tryptic digests of mouse immunoglobulin Fc fragments has, however, indicated many differences between different classes of immunoglobulins (25). This might be construed to favor the first explanation, but clearly we must await a further chemical description of the antigenic sites in order to decide between these and perhaps among other explanations.

This study indicates that with appropriate methods antiallotypic antibodies can be detected in heterologous antisera. This type of antiserum clearly merits further study, since it has several advantages over the isoimmunization system, particularly with mice, in that *1*) larger quantities of antisera might be made available, and *2*) since all antisera are made in a different species, mixing of the antisera can be done without the problem of interactions between the antisera. Finally,

it may be possible to produce antibodies to determinants previously unrecognized, as has in fact been shown recently with rabbit antihuman immunoglobulins (26).

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SUMMARY

Rabbits were immunized with either normal mouse immunoglobulins or isolated mouse myeloma proteins from BALB/c or (BALB/c × NZB)F_1 plasma cell tumors. The antisera were tested for antibodies directed to allotypic antigenic specificities by the method of inhibition of precipitation of I^{125} labeled antigens.

Three γG_{2a} and one γG_{2b} allotypic specificities were detected with one or another of these rabbit antisera. These specificities each corresponded in mouse strain distribution with one of the allotypic specificities previously defined through the use of mouse isoantisera.

The possible nature of the allotypic sites versus the class specific antigen sites was briefly discussed and the potential usefulness of these heterologous antisera in allotypic systems noted.

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