Detection of Rabbit Allotypes by Hemagglutination* (34115)

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Both the heavy (H) and light (L) chains of rabbit immunoglobulins[3] bear genetic markers which Oudin has named allotypes (1). The Fd portions of the H chains of the IgG, IgM (2–4), and IgA (5–9) classes share the group a (10) specificities, a1, a2, and a3 (11). The group b specificities, b4, b5, b6, and b9, are present on the κ L chains, and the specificity c7 is present on the λ L chains (12). Each of these specificities is detectable by precipitation techniques. Specificities A8 and A10 are on the Fc region of some H chains bearing the a1 specificity (13). They are coprecipitable with specificity a1, but by themselves inhibit precipitation. Recently a new specificity, A11, detectable by hemagglutination, but not by precipitation, has been described (14, 15). This specificity appears to be present in the hinge region of the IgG H chains (16).

The present paper describes the detection of the groups a and b allotypic specificities by hemagglutination. This method permits the detection of these specificities at dilutions much greater than is possible by precipitation techniques. It has proved of particular value in characterizing antibody directed against allotypic specificites (18). The method employs rabbit type F erythrocytes (19) coated with IgG using chromic chloride as a coupling agent (20, 21) and agglutinated by antisera to the allotypic specificities of the coat IgG.

Materials and Methods. The anti-allotype sera were prepared by injection of rabbits with rabbit antiovalbumin specific precipitate following the method of Oudin (22). Rabbit IgG was isolated by precipitation with 1.75 *M* ammonium sulfate followed by chromatography over diethylaminoethyl cellulose (DEAE) in 0.0175 *M*, pH 6.5, phosphate buffer. Rabbit $(Fab')_2$ was obtained by pepsin hydrolysis of IgG (23). The corresponding Fab′ fragment was obtained by reduction with mercaptoethanol and subsequent reaction with iodoacetamide (24).

Rabbit type F erythrocytes (19) were collected in heparin, washed in saline, and coated with rabbit IgG by the chromic chloride method of Gold and Fudenberg (20, 21). Optimum conditions for coating were determined using solutions of chromic chloride ranging from 0.06 to 2.0 mg/ml and solutions of IgG ranging from 0.04 to 5.0 mg/ml in saline. To 0.5 ml of a chromic chloride solution was added 1.0 ml of an IgG solution followed by prompt mixing and addition of 0.25 ml of 50% suspension of thrice-washed erythrocytes in saline. The mixture was gently agitated for 15 min. The cells were washed three times in saline and resuspended in saline to give a 2% suspension. The cells were checked for coating by gentle agitation of equal volumes of coated erythrocytes and goat anti-rabbit IgG serum (absorbed with rabbit F erythrocytes) or rabbit anti-allotype serum on a hemagglutination test plate. The plates were examined for agglutination microscopically at low magnification. In this way titers for the various antisera to the coat

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[3] The system of nomenclature recommended by WHO will be used for the immunoglobulin classes and fragments (17). For those specificities which have been assigned to a definite locus the initial A has been omitted; *i.e.*, a1 in lieu of Aa1.

TABLE I. Agglutination of Coated Cells by Anti-Allotype Sera.[a]

Allotype of coat	Specificity of antisera (titer[b] × 10^{-2})						
	a1	a2	a3	b4	b5	b6	b9
a1, b4	163	0	0	1310	0.08	0.16	0
a1, b4	40	0	2.6[b]	40	0	0	0
a2, b5	0	10	0	0	40	0	0
a3, b6	0	0	10	0	0.08	10	0
a3, b4			20	40		0	
a2, a3, b9	0	20	20	0	0	0	40
a1, a3, b4, b5	20	0	10	20	40	0.04	0

[a] The titer is the reciprocal of the highest dilution causing agglutination.

[b] This anti-a3 serum was shown to have agglutinating activity for A11, which was present on the IgG used as a coat.

IgG allotypes were determined and expressed as the reciprocal of the highest dilution agglutinating coated cells.

Dilutions of antiserum still giving a strongly positive agglutination, usually two doubling dilutions less than the end point of titration, were used for inhibition assays. Sera of allotypy determined by interfacial precipitin tests, IgG, and the $(Fab')_2$ and Fab′ fragments derived from it were tested for ability to inhibit agglutination of the coated cells by homologous anti-allotype sera as follows. Equal volumes of diluted inhibitor and anti-allotype serum were incubated on a test plate in a moist atmosphere at 37° for 1 hr. Coated cells were then added. The mixture was shaken for 15 min at room temperature and read for agglutination. Results are expressed as the reciprocal of the highest dilution of inhibitor inhibiting agglutination.

Results. Optimum concentrations of $CrCl_3 \cdot 6H_2O$ and IgG for coating were found to be 1.0 mg/ml and 2.5 mg/ml respectively.

Table I lists the titers obtained for agglutination of coated cells by homologous antisera. When the antiserum and coat are

TABLE II. Inhibition of Agglutination by IgG.

Allotype of coat IgG	Antiserum	Dilution[a]	Allotypes of inhibitor IgG (titer[b] × 10^{-2})				
			a1, b5	a1, b4	a2, b5	a3, b4	a2, a3, b9
a1, b4	a1	2000	41			0	
	b4	8200	0			21	
a2, b5	a2	250	0			0	1.3
	b5	1000	10		10	0	
a3, b6	a3	250				2.6	
	b6	250				0	
a2, a3, b9	a2	250					0.6
	a3	500				5	
	b9	1000					1.3
a1, a3, b4, b5	a1	500	2.6	2.6		0	
	a3	250				1.3	
	b4	500		5		0.6	
	b5	2000	10		5	0	
a3, b4	a3	500	0			2.6	

[a] This figure is the reciprocal of the dilution used for agglutination.

[b] The titer is the reciprocal of the highest dilution of a solution of IgG containing 5 mg/ml inhibiting agglutination.

TABLE III. Inhibition of Agglutination by Serum.

Allotype of coat IgG	Antiserum	Dilution[a]	Allotypes of inhibitor serum (titer[b] × 10^{-2})				
			a1, b5	a2, b6	a3, b4	a2, b9	a2, a3, b9
a1, b4	a1	2000	327	0	0		0
	b4	8200	0	0	10		0
a2, b5	a2	250	0	2.6	0	5	10
	b5	1000	163	0	0	0	0
a3, b6	a3	250	0	0	5		
	b6	250	0	2.0	0		
a2, a3, b9	a2	250	0	2.6	0	10	
	a3	500	0		10	0	1.3
	b9	1000	0	0		5	
a1, a3, b4, b9	a1	500	10		0		
	a3	250	0		2.6	0	
	b4	500	0		5		
	b5	2000	20		0		
a3, b4	a3	500	0		10		

[a] This figure is the reciprocal of the dilution used for agglutination.

[b] The titer is the reciprocal of the highest dilution of normal serum inhibiting agglutination.

properly matched, titers of several thousand are usually obtained. Of course, antisera differ in strength and in some experiments titers in excess of 10^6 have been obtained with anti-a1 and anti-b4. The cross reactivity occasionally noted at low dilutions will be discussed below. At titers in excess of 10^3, the method has been specific with IgG coats obtained from rabbits either homozygous or heterozygous with respect to allotypic specificity.

Allotypic specificities of sera, IgG, or fragments of IgG can be determined by their ability to inhibit agglutination exactly as is done for the Gm system in man (25). Table II presents results obtained by inhibition with IgG. The allotypic specificities of groups a and b present on IgG can be detected in solutions containing as little as 1–50 γ/ml. Similarly, as seen in Table III, the allotypic specificities of sera can be detected even though the sera are diluted from 130- to over 30,000-fold. As would be expected from the presence of the specificities of both groups a and b on the Fab′ fragment (26, 27), this system can also be used to detect the allotypy of these fragments, as exemplified in Table IV.

Discussion. The purpose of this report is threefold: to describe a method used by us to

TABLE IV. Inhibition of Agglutination by IgG Fragments.

Allotype of coat IgG	Antiserum	Dilution[a]	a3, b4 Inhibitor (titer[b] × 10^{-2})			
			Serum	IgG	$(Fab')_2$	Fab′
a3, b4	a3	500	10	2.6	10	10
	b4	500			20	2.6
a1, b4	a1	1000	0	0	0	0
	b4	2000	10	5	5	1.3

[a] This figure is the reciprocal of the dilution used for agglutination.

[b] Titer is the reciprocal of the highest dilution of normal serum or of a solution containing 5 mg/ml of IgG, $(Fab')_2$, or Fab′ which inhibits agglutination.

follow the concentration of anti-allotype antibody (18), to give details on the sensitivity with which this method can be used to detect immunoglobulin-bearing groups a and b allotypic specificities, and to point out possible sources of error which may complicate the interpretation of results obtained using the method.

Several agents have been used to attain passive sensitization of erythrocytes for general use in hemagglutination. Chou *et al.* (28) have used IgG coupled to red blood cells with bis-diazotized benzidine, and Kelus and Gell (29) have mentioned the use of sheep erythrocytes sensitized with rabbit antisera to detect allotypic specificities, but few details have been supplied. We have found the use of chromic chloride to couple IgG to the red blood cells as described by Gold and Fudenberg (20) convenient, reliable, and rapid. Such coated cells in conjunction with appropriate anti-allotype sera detect allotypic specificities with high sensitivity.

In applying this method it is important to recognize some sources of error. A minor problem is the low degree of cross reactivity of the group b specificities as exemplified in Table I. This probably reflects the cross reactivity observed in precipitation reactions. We have observed that hyperimmune antisera directed to any of the group b specificities will often react weakly with other group b specificities not present in the immunized rabbit, the precipitating cross reactivity between b5 and b6 being particularly strong. As seen here, this cross reaction disappears even at low dilutions of the anti-allotype sera.

A second source of error is exemplified in Table I; cells coated with a1, b4 IgG were agglutinated by anti-a3 even though the coat did not possess the group a allotypic specificity against which the antiserum was directed. This effect has been traced to the presence of A11 (14) specificity on the coat IgG and the presence of A11 agglutinator in the anti-a3 serum. The A11 agglutinator in turn resulted from the presence of the A11 specificity in the ovalbumin antiserum used to invoke the anti-a3 in a rabbit which lacked this specificity. These titers are usually lower than those resulting from reaction of group a or b specificities with homologous antiserum. The effect has been encountered less often than one might expect on a statistical basis. This low frequency is attributed to the observation that rabbits producing antisera to A11 usually cease production of agglutinator serum after several weeks, whereas rabbits will continue to make antiserum directed against groups a or b specificities for many months.

As an extension of this effect a serum possessing the specificity a3 was found unable to inhibit the agglutination of cells coated with a3 IgG by anti-a3 at agglutinator dilutions of less than 1/32. This effect resulted from the absence of A11 in the inhibitor serum, its presence on the coat IgG, and homologous antibodies in the anti-a3.

Summary. Genetic typing of rabbit sera and IgG has been successfully achieved by inhibition of passive hemagglutination using rabbit type F cells coated with IgG of known allotype by chromic chloride. The method is particularly sensitive and is superior to the usual interfacial precipitation or gel diffusion tests for detection of allotypes on small quantities or high dilutions of material.

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