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Haemagglutination Inhibition Assay of the Common Determinants and Subspecificities of Australia Antigen

M. IMAI, Y. YAMASHITA, Y. MIYAKAWA AND M. MAYUMI

Immunology Division, Jichi Medical School, Tochigi-Ken, and Third Department of Internal Medicine, Faculty of Medicine University of Tokyo, Japan

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Summary. Antigenic determinants of Australia antigen (AuAg) may be determined by haemagglutination inhibition with ease and high sensitivity. The reaction depends on the inhibitory effect of the test antigen-positive sample on the agglutination by specific antibody of glutaraldehyde-fixed sheep erythrocytes which have been tanned and coated with AuAg of different specificities. The standard monospecific antibody reactants are directed against one or other of the common antigens, *a* and *Re*, or against the subtypic determinants, *d*, *y*, *w* and *r*. Because it is more sensitive than conventional immunodiffusion or electrosyneresis, and more convenient than radioimmunoassay, haemagglutination inhibition is most suited to large-scale determinations of AuAg subtypes in samples of relatively low antigenic activity.

INTRODUCTION

Using immunodiffusion, Le Bouvier (1971) found two different, mutually exclusive antigenic determinants, *d* and *y*, on the surface of Australia antigen (AuAg) particles, in addition to the commonly shared determinant detected and named *a* by Levene and Blumberg (1969).

Bancroft, Mundon and Russell (1972) recently identified, also by immunodiffusion, the *w* and *r* determinants, one or other of which is similarly carried by individual AuAg, in addition to either *d* or *y*. Thus, at the present time, at least four subtypes of AuAg are recognized, that is, *adw*, *adr*, *ayw* and *ayr*; and these antigenic subtypes appear to be specified by four distinct genotypes of hepatitis-B virus. The significance of determining AuAg subtypes is increasingly evident, because of their differing distribution in epidemiological studies (Mazzur, Falker and Blumberg, 1973) and their usefulness in tracing routes of virus transmission (Mayumi and Nakajima, 1973), and in view of their possible relationship to particular manifestations of liver diseases (Nielsen, Le Bouvier and the Copenhagen Hepatitis Acute Program, 1972). However, because of the limited sensitivity of immunodiffusion for detecting either antigen or antibody, it has not always been possible to characterize the subtype specificities in known antigen-positive materials.

The present report describes a rapid, simple and sensitive method for detecting the individual determinants of AuAg by means of haemagglutination inhibition. It depends

The letters denoting antigenic determinants, such as *a*, *Re*, *d*, *y*, *w* and *r*, are italicized for the sake of visual clarity. In the present context, however, they refer to the AuAg phenotype, and not to the postulated HBV genotype.

Correspondence: Dr M. Mayumi, Immunology Division, Jichi Medical School, Tochigi-Ken, Japan 329-04.

on the absorption by the antigen sample of a known amount of antibody directed against one or other subspecific antigenic configuration. The absorption is reflected in the failure of the antibody to agglutinate glutaraldehyde-fixed sheep erythrocytes coated with AuAg bearing the homologous determinant. Besides these subspecificities, the common determinant *a* is also measured, as well as another common reactivity, *Re*, recently described by us (Imai, Gotoh, Nishioka, Kurashina, Miyakawa and Mayumi, 1974), which is resistant to reduction and alkylation. Thus, a total of six antigenic determinants of AuAg have been individually detected and assayed by the method of haemagglutination inhibition.

MATERIALS AND METHODS

Preparation of AuAg

AuAg particles were separated from the plasma of healthy carriers by ultracentrifugation or by affinity column chromatography. Plasma was ultracentrifuged in concentration gradients of CsCl and sucrose after the method of Gerin, Holland and Purcell (1971). For affinity chromatography, horse anti-Au antibodies were bound to Sepharose by cyanogen bromide and packed in a column of 2·6 × 40 cm (bed volume 200 ml). After applying 2 l of antigen-positive plasma, antigens were eluted with 5 M $MgCl_2$ solution and concentrated. Antigens were separated from contaminating proteins by ultracentrifugation. By either of these methods, the purified AuAg was free of any serum component as tested by immunodiffusion using goat antiserum to whole human serum. The affinity chromatography method had the advantage over zonal ultracentrifugation in greater ease of operation and less cost, and the same column could be reused almost indefinitely.

Reduction and alkylation of AuAg

Details have been described elsewhere (Imai *et al.*, 1974). Briefly, purified AuAg particles were suspended in 0·55 M Tris–HCl buffer (pH 8·2) at a concentration of 1 mg/ml (estimating $E^{1\,cm}_{280\,nm}$ 0·1 per cent value at 3·72 (Vyas, Williams, Kraus and Bond, 1972)) and reduced in the presence of 0·1 M dithiothreitol at 25° for 1 hour. The antigen was then alkylated with an excess amount of iodoacetamide and dialysed against physiological saline.

Buffers

Phosphate-buffered saline (PBS) contained 4·5 g of NaCl, 3·0 g of KH_2PO_4 and 7·5 g of Na_2HPO_4 in 1000 ml of distilled deionized water (pH 7·2 at 24°). 'PHA buffer' was prepared by adding 10 g of sucrose and 10 ml of normal rabbit serum to PBS to make the final volume of 1000 ml. A concentration of 0·2 per cent of NaN_3 was maintained to prevent bacterial growth.

Preparation of antigen-coated sheep erythrocytes

Sheep erythrocytes (SRBC) were fixed and coated with AuAg by the following method (Table 1). Blood was collected and stored in sterile Alsever's solution at 4° for a week. SRBC were washed four times with 20 volumes of PBS and finally suspended in the same buffer at a concentration of 5 per cent (v/v). Washed SRBC were resuspended at 5 per cent in PBS, mixed with glutaraldehyde (final concentration 0·4 per cent (w/v))and held at 24° for 1 hour. The fixed SRBC were washed four times and a 5 per cent suspension was

made in PBS. An equal volume of tannic acid solution (5 mg per cent in PBS) was added to the SRBC suspension and the mixture was incubated at 37° for 10 minutes with constant agitation. The tanned SRBC were washed again four times in PBS and resuspended at 5 per cent. Equal volumes of tanned SRBC and purified AuAg particle suspension (at a concentration of 10–100 μg/ml in PBS) were then mixed and incubated at 24° for 1 hour with shaking. AuAg-coated SRBC were again washed four times in PBS and suspended in PHA buffer at 10 per cent as a stock solution. The stock solution of AuAg-coated SRBC can be stored at 4° for more than 6 months without any loss of reactivity; it is diluted to 1 per cent by adding 9 volumes of PHA buffer before use.

TABLE 1

PREPARATION OF AuAg-COATED SHEEP ERYTHROCYTES

Sheep erythrocytes (SRBC)
↓ wash four times with PBS
Washed SRBC (5 per cent suspension), 1 volume +glutaraldehyde (2·5 per cent), 1/5 volume
↓ 24° for 1 hour ↓ wash four times with PBS
Fixed SRBC (5 per cent suspension), 1 volume +tannic acid (5 mg/100 ml), 1 volume
↓ 37° for 10 minutes ↓ wash four times with PBS
Tanned SRBC (5 per cent suspension), 1 volume +AuAg (10–100 μg/ml), 1 volume
↓ 24° for 1 hour ↓ wash four times with PBS
AuAg-coated SRBC

Preparation of monospecific antisera to subtype-specific determinants of AuAg

Antisera to AuAg were obtained from antibody-positive blood donors or by immunizing animals. Rabbits and guinea-pigs were immunized in the foot pads and intracutaneously. Each received 0·5–1·0 mg of purified AuAg emulsified in Freund's complete adjuvant four times at monthly intervals. Antisera were collected 2 weeks after the last inoculation These antisera usually contained antibodies of different specificities in varying proportions. To obtain monospecific antibody to a particular determinant, either of the following two methods was used. Antisera were diluted so as to dilute out the minor antibody components, while preserving only the major component directed against a single specificity. Alternatively, multispecific antisera could be absorbed by adding an excess amount of the AuAg preparation, carrying all but one of the determinants corresponding to the specificities of the antiserum. Antibodies were titrated in microtitre plates by the passive haemagglutination method using AuAg-coated SRBC. One haemagglutinating unit was defined

as the least amount of antibody required to agglutinate 25 μl of AuAg-coated SRBC suspended at 1 per cent in PHA buffer.

Monospecific anti-*a*, anti-*Re*, anti-*w* and anti-*r* antisera were prepared from human sera of blood donors with high anti-Au antibody titre. Anti-*d* and anti-*y* were obtained from immunized rabbit or guinea-pig sera.

Standard antigen panel

From the blood donors of the Japanese Red Cross Association eleven healthy antigen carriers were selected and their sera were used as the standard Jichi antigen panel. It consisted of AuAg of different subtypes (*adw*, 3; *adr*, 3; *ayw*, 2; and *ayr*, 3). Standard antigen panels were also obtained from Dr P. V. Holland, Blood Bank Department, Clinical Center, NIH (*adw*, 2; *adr*, 2; *ayw*, 2; and *ayr*, 1) and Dr G. Irwin, Department of Virus Diseases, Walter Reed Army Institute of Research (*adw*, 1; *adr*, 1; and *ayw*, 1).

RESULTS

ASSAY OF AuAg ANTIGENIC DETERMINANTS BY HAEMAGGLUTINATION INHIBITION (HI)

The presence and activity of each AuAg antigenic specificity in the test sample were determined by the following method (Table 2). In a polystyrene V-bottom microtitre

TABLE 2

PROCEDURE OF HAEMAGGLUTINATION INHIBITION*

Serum, 25 μl, two-fold serial dilutions†
+Ab, 25 μl (monospecific, two haemagglutination units)
↓ mix
↓ 37° for 2 hours
+AuAg-coated SRBC, 25 μl, 1 per cent suspension
↓ mix
↓ 24° for 1 hour
Observation

* Reactions were carried out in PHA buffer (see text).
† Another series of serum dilutions is set up and AuAg-coated SRBC are directly added to it for detection of anti-Au antibodies.

plate (Cooke Engineering Company, Alexandria, Virginia), the test sample (serum) was serially diluted in PHA buffer. To each well was added 25 μl of PHA buffer containing two haemagglutination units of monospecific antiserum directed against the antigenic determinant to be tested. The plate was agitated for 30 seconds with Micro-mixer (Kowa Kizai Company, Tokyo) and incubated at 37° for 2 hours. Then 25 μl of 1 per cent target cell suspension was added to each well and incubated at 24° for 1 hour after mixing. Target cells were SRBC coated with AuAg bearing the corresponding antigenic determinant. The plates were observed, without centrifugation, on the observation apparatus (opalescent

glass placed over fluorescent lamps) and the inhibition of haemagglutination was determined: haemagglutination indicated the absence of the antigenic determinant in the test sample, whereas no haemagglutination indicated its presence (Fig. 1). For an antigen-positive sample, the highest dilution giving complete inhibition of haemagglutination was determined and the antigenic titre of the sample was expressed as the reciprocal of this dilution.

Usually, an additional series of dilutions of the test sample was set up in parallel, to which target cells were added directly for the detection of anti-AuAg antibody.

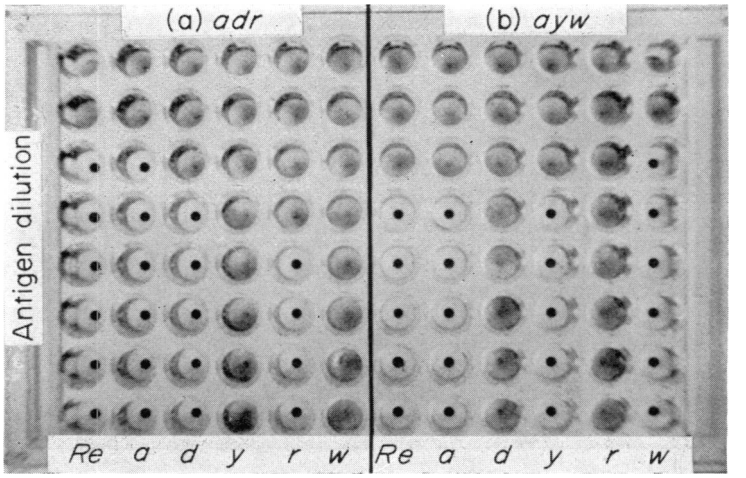

FIG. 1. Haemagglutination inhibition assay of Australia antigen in sera bearing subspecificities of (a) *adr* and (b) *ayw*. Central dark spots indicate the inhibition of haemagglutination.

It was found that the antibody reactant need not necessarily be monospecific, provided any 'contaminating' antibodies were not directed against the other antigenic determinants of the particular AuAg used to coat the target SRBC. Thus the antibody reactant containing anti-*d*+anti-*r* could be used to measure the *d* determinant, when the target cells were coated with AuAg of *adw* subtype. Only that antigenic determinant was detected by HI, whose specificity was possessed both by the antibody used and by the AuAg-coated cells. These situations allowed the use of some multispecific antisera to determine only one antigenic determinant by the HI method.

COMPARISON OF THE RESULTS OF SUBTYPING BY HAEMAGGLUTINATION INHIBITION AND IMMUNODIFFUSION

The results of subtyping and subdeterminant titrations by the HI method, as well as the assays of the common antigenic reactivities, *a* and *Re*, are given in Table 3. The same antigen panel prepared in our laboratory and subtyped by HI was distributed to Dr G. L. Le Bouvier of Yale University, to Dr P. V. Holland of National Institute of Health, Bethesda, and to Dr G. Irwin of the Walter Reed Army Institute of Research, for subtyping by their own techniques of immunodiffusion. Their results and ours were completely in accord.

TABLE 3

DETERMINATION OF ANTIGENIC ACTIVITIES OF JICHI ANTIGEN PANEL BY HAEMAGGLUTINATION INHIBITION

Serum sample	Subtype of antigen†	Titre* of haemagglutination inhibition					
		Common specificity		Subtypic specificity			
		a	*Re*	*d*	*y*	*w*	*r*
1. H-34	*adw*	1024	1024	1024	—‡	2048	—
2. H-45	*adw*	1024	2048	1024	—	2048	—
3. H-72	*adw*	1024	1024	512	—	2048	—
4. H-41	*adr*	2048	2048	1024	—	—	256
5. H-49	*adr*	2048	2048	2048	—	—	256
6. H-70	*adr*	2048	1024	2048	—	—	128
7. J-1	*ayw*	64	128	—	32	64	—
8. J-48	*ayw*	128	128	—	64	128	—
9. J-161	*ayr*	1024	512	—	512	—	128
10. J-165	*ayr*	2048	2048	—	1024	—	256
11. J-185	*ayr*	2048	2048	—	1024	—	64

* Reciprocal of the highest dilution of the sample that completely inhibited haemagglutination by two haemagglutinating units of monospecific antibody.

† Determined unanimously by Dr Le Bouvier of Yale University, Dr Holland of NIH, and Dr Irwin of Walter Reed Army Institute of Research.

‡ No inhibition was observed at 1:2 dilution.

TABLE 4

DETERMINATION OF ANTIGENIC ACTIVITIES OF NIH AND WALTER REED ANTIGEN PANELS BY HAEMAGGLUTINATION INHIBITION

Serum sample	Subtype of antigen	Titre* of haemagglutination inhibition					
		Common activity		Subtypic activity			
		a	*Re*	*d*	*y*	*w*	*r*
NIH Panel							
1. J.M.	*adw*	5120	2560	2560	—†	2048	—
2. X50445	*adw*	640	1280	320	—	1280	—
3. BE	*adr*	1280	1280	1280	—	—	160
4. KI	*adr*	640	640	640	—	—	80
5. LE	*ayw*	2560	1280	—	2560	10240	—
6. GR	*ayw*	320	640	—	160	640	—
7. CH	*ayr*	80	40	—	20	—	20
WRAIR Panel							
1. EC 318	*adw*	640	640	1280	—	2560	—
2. EH 17	*adr*	2560	2560	1280	—	—	160
3. EA 47	*ayw*	640	160	—	160	2560	—

* Reciprocal of the highest dilution of the sample that completely inhibited haemagglutination by two haemagglutinating units of monospecific antibody.

† No inhibition was observed at 1:2 dilution.

As a further comparative test, selected antigen reactants of known subtype received from Dr Holland (NIH panel) and from Dr Irwin (WRAIR panel) were also analysed for their subspecific reactivities by the HI method. The subtyping results are shown in Table 4, along with the antigenic titres of the various determinants. As before, HI was shown to be able to detect every antigenic determinant that had been ascertained by immunodiffusion.

DISCUSSION

We have described a method of haemagglutination inhibition for detecting six antigenic determinants of AuAg, both common—*a*, *Re*—and subtypic—*d*, *y*, *r* and *w*. This procedure is rapid, sensitive, reproducible, and easy to perform, and large numbers of samples can be handled in a limited time. The specificity of the method was confirmed by testing the same panels of antigen reactants in different laboratories. The HI method adapted to detect the AuAg antigenic determinants has a number of advantages over other techniques using immunodiffusion and radioimmunoassay. HI is generally accepted as being at least 100-fold more sensitive for AuAg detection than standard immunodiffusion, 10-fold more sensitive than electrosyneresis or rheophoresis, and nearly as sensitive as radioimmunoassay. Because of its high sensitivity, the amount of antibody used for a test can be cut down to a minimum and standard reagents can be spared. The minimal expenditure of standard antibody, coupled with the stability of target cells (more than 6 months at 4° without loss of activity) would permit the use of the HI method as a means of standardizing the assay of the individual AuAg determinants in many different laboratories. Such standardization becomes increasingly necessary, as the world-wide prevalence of asymptomatic and clinically apparent HBV infections causes more and more laboratories to enter this field of investigation. With reference centres supplying the standard antibody and antigen-coated cells wherever required, HI is likely to become a standard procedure for the determination of AuAg subtypes. Its versatility makes subtyping almost universally practicable, and gives it an advantage over radioimmunoassay, with its requirement for specialized facilities.

Vyas and Shulman (1970) introduced the HI method to detect AuAg, and Prince, Szmuness, Brotman and Ikram (1973), successfully applied the technique to the identification of the *d* and *y* determinants, stressing the usefulness of subtyping by HI as a sensitive identity test for AuAg. The present method differs from theirs in that sheep erythrocytes, fixed with glutaraldehyde, are used as the target cells, and tannic acid is employed in coating them with AuAg. In our experience in haemagglutination inhibition assay of AuAg subtypes, the sedimentation pattern of sheep erythrocytes is clearer and easier to read than that of human erythrocytes. Furthermore, after the cells have been fixed with glutaraldehyde, natural antibodies to sheep erythrocytes in human sera do not disturb the result.

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