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Specificity control in solid-phase enzyme immunoassay for HB_sAg by one-step *in situ* blocking with human anti-HB_s

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The solid-phase enzyme immunoassay (EIA) for HB_sAg², described by Wolters *et al.* (1976), has a similar sensitivity as a commercially available sandwich-type solid-phase radioimmunoassay (RIA). In the same paper, a method for the confirmation of presumptive positives was described. As this method is laborious, we have attempted to develop a simpler one, which is based on the *in situ* blocking of the antigen bound to the solid-phase antibody by human antibodies against HB_sAg (anti-HB_s). Essentially the same principle has also been reported for the specificity control of presumptive positives in RIA (Ling *et al.*, 1975).

Human anti-HB_s is often preferred as neutralising antibody because the risk of neutralising non-specifically reacting factors is small. However, one must be sure that HB_sAg of one subtype, for example, *ay*, will be neutralised by antibodies against the other subspecificity (*ad*) or by antibodies directed only against the main antigenic determinant *a*. We describe the results obtained with two different human anti-HB_s sera comparing the two different specificity control methods mentioned above.

Material and methods

Two human anti-HB_s sera were used in this study. One had the subspecificity of anti-*ay* as demonstrated by immunodiffusion. The subspecificity of the other anti-HB_s serum could not be determined by this technique. Both antisera were further characterised by determining the highest dilution that still reduced the reaction of HB_sAg of either subtype in EIA. It was found, for the anti-*ay* serum, that the ratio

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anti-*ad* titre: anti-*ay* titre was 1:2.5. For the other anti-HB_s serum this ratio was 2.1:1. We consider this indicates that the latter antiserum contains antibodies to the *d*-determinant. For the sake of clarity, we shall designate this antiserum as 'anti-*ad*' in the following text. Both antisera were compared at the same strength of anti-HB_s, that is, diluted on the basis of the 'average' titre:

$$\sqrt{(\text{anti-}ad \text{ titre}) \times (\text{anti-}ay \text{ titre})}.$$

The first EIA confirmation method (hereafter referred to as 'dilution method') was performed as described by Wolters *et al.* (1976) with some minor changes: A presumptive positive sample was serially diluted and incubated for 16 hours at room temperature in glass tubes with human anti-HB_s of either subspecificity (Table 1, anti-HB_s columns I (*ay* and *ad*)).

A sham neutralisation was performed with normal human serum (NHS) 1:10 (free from HB_sAg and anti-HB_s) on an identical dilution series of the same sample (Table 1, columns II). After the neutralisation procedure, each dilution was tested according to the screening procedure in the anti-HB_s-coated microtitre wells (Wolters *et al.*, 1976).

Untreated (undiluted) samples were retested simultaneously in order to check the results of the screening (Table 1, columns III). If read with the naked eye, a titre reduction of at least two dilution steps after anti-HB_s treatment, as compared with the control treatment, was considered specifically positive. Objective reading was done by measuring extinction at 492 nm (E₄₉₂). Solutions with E₄₉₂ ≥ 2 were diluted 10 × in 1.33 N sulphuric acid and measured again. The percentage of neutralisation by anti-HB_s was calculated as follows:

$$\% \text{ neutralisation} = \frac{(E_{492} \text{ II}) - (E_{492} \text{ I})}{(E_{492} \text{ II}) - (E_{492} \text{ neg. control})} \times 100\%,$$

where

E₄₉₂ I = extinction after treating the sample with anti-HB_s

E₄₉₂ II = extinction after treating the sample with NHS

E₄₉₂ neg. control = average extinction of at least five negative control sera tested according to the normal screening procedure.

The performance of the *in situ* blocking with human anti-HB_s (hereafter referred to as 'blocking

Table 1 Comparison of 'dilution' and 'blocking' methods for specificity control in dilution series of sera containing HB_sAg of the ay and ad subtypes

HB _s Ag (μ g/ml)	'Dilution' method						'Blocking' method					
	% Neutralisation, colorimetric reading		Eye reading				% Neutralisation, colorimetric reading		Eye reading			
	Anti-HB _s		Anti-HB _s				Anti-HB _s		Anti-HB _s			
	ad	ay	I ad	I ay	II	III	ad	ay	I ad	I ay	II	III
ay subtype												
99.4 u	0	0					87	91				
49.7 .	0	2					70	87				
24.8 .	0	0					71	89				
12.4 .	0	0					73	90				
6.2 .	0	0					70	87				
3.1 *	0	0					66	86				
1.5 *	0	4					69	86				
0.8 *	6	2					70	86				
0.4 *	22	30					74	87				
0.2 *	21	80					86	96				
0.09 *	87	98					95	99				
ad subtype												
13.7 u	0	0					93	89				
6.8 .	0	0					96	93				
3.4 .	14	0					98	94				
1.7 .	0	4					99	96				
0.9 *	5	9					98	96				
0.4 *	20	19					98	97				
0.2 *	54	68					99	97				
0.1 *	94	92					100	98				
0.05 *	101	100					100	98				
0.03 *	104	103					100	97				
0.01 *	99	113					103	92				

u = undiluted serum

. = serum diluted in saline

* = serum diluted in saline containing 0.1% BSA

= dark yellow-orange
 = light yellow
 = colourless

method") was as follows (see Figure): 100 μ l of test sample was added to four different wells of an antibody-coated microtitre plate (I *ad*, I *ay*, II, and III). NHS was included as a negative control and treated further in the same manner as the sample in well III. The plates were then covered with parafilm and incubated for 16-24 hours at room temperature. Wells I and II were emptied by aspiration (well III and the control wells were left untouched) and washed three times. 150 μ l (or four drops) of human anti-HB_s of either subspecificity were dispensed into corresponding wells labelled I *ay* and I *ad*; 150 μ l (or four drops) of NHS 1:10 were dispensed into well II. The plate was then covered and incubated for two hours at 37°C, after which all wells were aspirated and washed three times. Thereupon the procedure for screening was resumed, as described previously (Wolters *et al.*, 1976). When read by eye, the interpretation of the results was as follows:

if the colour developed in well I was weaker than in wells II and III, the sample was regarded as specific positive;

if the colour developed in all four wells was equally intensive (and weak), the sample was regarded as false positive;

if no colour developed in any of the four wells the sample was also regarded as false positive ('repeat negative'). All other combinations of colours rendered an inconclusive result. When read colorimetrically, the percentage of neutralisation in well I was calculated by the same formula as described above.

The total amount of antibody used per test sample was about the same for both methods.

Both methods were studied with two sera containing HB_sAg, one of the *ay* subtype and the other of the *ad* subtype. Serial dilutions were made in 0.9% NaCl (or 0.9% NaCl containing 0.1% bovine serum albumin). The concentration of HB_sAg was determined using the German Reference Panel for HB_sAg as a standard (Gerlich *et al.*, 1976) and was 99.4 μ g/ml for *ay* and 13.7 μ g/ml for *ad*. Each of these serum dilutions was neutralised with both human anti-*ay* and anti-*ad*.

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Table 2 Comparison of the 'blocking' method for specificity control of EIA for HB_sAg with Ausria II screening and confirmation tests

Sample No.	EIA		Confirmation	Ausria II	
	Screening			Screening (ratio)	Confirmation
	Eye reading	E ₄₉₂ (ratio)			
1	+	3.76	S	4.93	S
2	+	2.22	?	3.15	S
3	+	2.15	S	5.50	S
4	+	3.29	NS	2.33	S
5	+	2.89	S	1.56	—
6	+	3.21	S	5.17	S
7	+	2.86	S	11.92	S
8	+	2.87	S	11.14	S
9	+	2.67	S	5.15	S
10	+	2.28	S	7.26	S
11	+	3.29	S	7.30	S
12	+	3.96	NS	1.93	—
13	+	2.81	?	2.93	S
14	+	3.37	S	5.42	S
15	+	2.09	RN	1.02	Not done
16	+	6.77	NS	1.18	” ”
17	+	4.95	NS	0.92	” ”
18	+	2.66	NS	1.05	” ”
19	+	5.61	NS	1.15	” ”
20	+	5.20	NS	0.75	” ”
21	+	4.72	NS	1.04	” ”
22	+	3.20	RN	0.82	” ”
23	+	4.84	NS	1.51	” ”
24	+	2.33	NS	0.87	” ”
25	+	4.43	NS	1.20	” ”
26	+	2.69	RN	1.19	” ”
27	+	2.42	RN	0.99	” ”
28	+	3.16	NS	0.60	” ”
29	+	5.23	NS	1.06	” ”
30	+	5.36	NS	0.76	” ”

S = specific; ? = inconclusive; NS = non-specific; RN = repeat-negative in confirmation.

capacity, binds only a fraction of the total quantity of HB_sAg offered to it in the first incubation. The remaining unbound HB_sAg is being discarded. The added anti-HB_s (in relatively higher quantity per well than in the 'dilution' method) has, therefore, an 'easier' task in neutralising (blocking) the HB_sAg bound to the solid phase.

Both 'dilution' and 'blocking' methods gave similar results by eye reading only when the concentration of HB_sAg in the samples was low (below 0.2 µg HB_sAg/ml).

Economy of time and reagent is also important. At least 2 × 6 dilutions of the sample and extra undiluted sample are needed for the 'dilution' method, thus occupying 13 reagent-wells per sample. Strong positives might not reach an endpoint so that even further dilution and retesting are necessary.

The 'blocking' method is performed entirely in the antibody-coated microtitre plate and the number of wells per sample is only three.¹ While in both

¹Instead of two separate wells for antibodies with different subspecificities, as described here, only one well is used in the routine work.

methods about the same quantity of neutralising antibody per sample is used, a saving factor of about four with regard to the remaining reagents is obtained by the 'blocking' method.

The data presented in Table 1 show that the anti-ay was slightly more effective in neutralising the ay subtype than ad and vice versa. However, it is evident that both antisera studied here can be used equally well for neutralisation of both subtypes in the 'blocking' method.

Most of the specificity control results obtained with the 'blocking' method agreed with the RIA results (Table 2). Disagreements were encountered in four cases (Nos. 2, 4, 5, and 13 in Table 2). There is no practical possibility of finding the correct answer since at the moment no method superior in terms of sensitivity and specificity to both EIA and RIA exists.

For a multicentre clinical trial of the EIA method for HB_sAg, which has just been completed, we have chosen the 'blocking' method for specificity control. The results of this trial will be published separately, but it is of interest to note here that, out of over 1100

cases in which presumptive positivity was found in screening, the 'blocking' method gave only 27 inconclusive results.

We conclude that the 'blocking' method is superior to the 'dilution' method for specificity control of the enzyme-immunoassay for HB_sAg for the following reasons:

- the neutralisation procedure is more efficient;
- it is less laborious;
- it has a reagent-saving factor of about four.

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An improved method for handling tissues for immunofluorescence

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One of the major problems in handling tissues for immunofluorescence is that they must be quick-frozen (Pearse, 1968; Nairn, 1969; Feltkamp-Vroom, 1975). This can be accomplished in a number of ways, but each method has its drawbacks. Liquid nitrogen is commonly used, but this is expensive, not always available, and also hazardous to work with. A special container or thermos flask has to be used to contain it, and this may rupture because of the extremely low temperatures. The liquid nitrogen also evaporates rapidly, producing a concomitant build-up of pressure so that the container cannot be tightly stoppered. An alternative method of obtaining low temperatures is to use dry ice. Again, depending on the supplier, it may not always be available and it, too, is somewhat expensive. If the dry ice is used by itself water may condense on the surface, thus insulating it and resulting in a warmer surface temperature. Another method of using dry ice is to make a slurry with acetone and then freeze the tissue

either in a tube or wrapped in foil. This method is somewhat more efficient but there is always the problem of losing the specimen in the slurry, or not having good contact with the chilling mixture with concomitant bad preservation due to slow freezing. Carbon dioxide may also dissolve in the tissues or serum samples, resulting in a lowered pH, and this may have an adverse effect on antibodies.

We have devised a method for overcoming most of these problems that is easy, economical, and very efficient.

The principle of our procedure involves partially filling a one-litre thermos flask with the material¹ used by a number of companies to form refrigerator or ice packs. The nature of this material is a trade secret but apparently it is some sort of plastic and it is stated to be non-toxic and non-corrosive. In the centre of the flask a closely fitting chamber is created, using a template (Fig. 1). For the template, a standard liquid scintillation counting vial (Wheaton Glass Co, Brampton, Ontario) wrapped several times with wax paper is used. A one-inch layer of the refrigerant is poured around this and is allowed to solidify in the freezer, thus allowing for expansion before subsequent layers are put on top of this. As can be seen, the top of the vial is left protruding above the surface so that it can easily be removed from the flask. The chamber can also be readily bored with a

¹The trade name of the material is Ice Pak, manufactured by Stanbel Ltd Braided Products Division, 11600 Albert Hudon Blvd, Montreal, Quebec H1G 3K2, Canada.