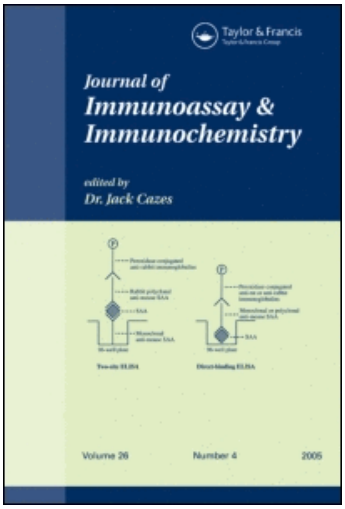

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Dissociation of Hepatitis B Surface Antigen (HBsAg) from Immobilised Anti—HBsAg Antibody in a Two Site Immunoradiometric Assay: Its Relevance to Hook Effect and Recycled Assay

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DISSOCIATION OF HEPATITIS B SURFACE ANTIGEN (HBsAg) FROM IMMOBILISED ANTI-HBsAg ANTIBODY IN A TWO SITE IMMUNORADIOMETRIC ASSAY: ITS RELEVANCE TO HOOK EFFECT AND RECYCLED ASSAY

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ABSTRACT

The mechanism of the hook effect in a two site immunometric assay of hepatitis B surface antigen (HBsAg) was studied using a novel method. The results showed that HBsAg dissociated from immobilized anti-HBsAg at low as well as high doses of antigen (Ag). This probably was not due to lack of immobilized antibody (Ab) or dissociation of HBsAg non-specifically bound to the immobilized Ab. The dissociated Ag sequestered the labelled Ab in the second reaction and contributed to the decrease in percent binding (B %) of the assay and thus the hook effect. The dissociated Ag could contaminate the recovered pooled labelled Ab in recycled assay and give rise to high non-specific binding. Prolonged washing decreased the side reaction but also the B % of the assay.

(KEY WORDS: Antibody Dissociation. Hook Effect. Recycled Assay)

INTRODUCTION

The high dose hook effect has been shown to occur in two-site immunoradiometric assays (IRMA)(1,2) using polyclonal or monoclonal antibodies (Ab) (3,4). This effect has been attributed to heterogeneity of immobilized Ab, inadequate washing (4) and insufficient concentration of labelled Ab in the second reaction of the assays (5) based on computer simulation models . From experimental work on ferritin using dual labels, Perera (6) concluded that sequestration of labelled Ab by dissociation of ferritin non-specifically bound

to coated tubes is the cause of the hook effect. Casey (7) showed that repeated washings did not eliminate the high dose hook effect but did increase the plateau area. In this paper direct experimental evidence for the dissociation of hepatitis B surface antigen (HBsAg) from the immobilized Ab in a two-site IRMA is presented and its relevance to the hook effect is discussed.

MATERIALS AND METHODS

Dissociation of HBsAg from Immobilised Antibody

Sera from six HBsAg positive patients were pooled to give a mean percent binding (B%) of labelled Ab of 5.1 % after 700 fold dilution in a modified IRMA assay as described below, using ^{125}I Ausria II kits (Abbott Laboratory, North Chicago, IL 60064 U.S.A.). The high pool was diluted with a pool of 50 negative sera, non-reactive for HBsAg and anti-HBsAg Ab, to give a B % between 5 and 8.

The modified ^{125}I Ausria II kit procedure was carried out as follows. Assays for all pools were set up in quintuplicate. Two hundred microlitres of the two pools and of negative serum were incubated overnight with anti-HBsAg coated polystyrene beads (the first reaction). These were then subjected to 4 thirty-second washes with deionized water (using Pentawash, Abbott Laboratory). The beads were allowed to react overnight with 200 ul of ^{125}I labelled anti-HBsAg antibody (the second reaction). The supernates were then recovered and pooled. The beads were further washed 4 times and the bound radioactivities were counted in an Abbott Autologic gamma counter (Abbott Laboratory). The dissociation of HBsAg in the second reaction was demonstrated directly by overnight reaction between 200ul of the recovered pooled labelled Ab with unused anti-HBsAg coated beads (n=4 for each pool). Owing

to loss of labelled Ab in the recovery procedure the number of beads used (n) were decreased from 5 to 4. The B% of the beads reflected the amount of labelled Ab linked to dissociated HBsAg. The residual binding capacity of the original Ab-coated beads after exposure to the high pool in the above reaction was assessed by reusing these beads to assay positive serum (with mean B% of 7.7 ±0.19 % n=5) using new labelled Ab and the modified kit procedure. All the experiments were carried out at room temperature.

Non-specific Binding of HBsAg by Protein-coated Beads

Ten anti-thyroid-stimulating-hormone (TSH) antibody coated beads (obtained from Abbott Laboratory) were substituted for anti-HBsAg coated beads in the assay of the high pool (n=5) and the negative serum (n=5) using the modified procedure. The dissociation of HBsAg (non-specifically bound to protein-coated beads) in the second reaction was estimated as above using supernates from the pooled reaction with fresh beads coated with anti-HBsAg.

Effect of Washing on the Dissociation of HBsAg from the Immobilized Antibody

Three sets of unused beads A, B, and C (n = 5 for each set) were used to assay the high pool using the modified kit procedure described above. The degree of dissociation of HBsAg from each set of beads was estimated as described above. The patterns of washes were different for the three sets of beads. Set A beads were washed 20 times (30 seconds per wash), and soaked overnight in 2 ml of deionized water after the first reaction, and were further washed 20 times after the second reaction. The set B beads were subjected to the same pattern of washes as Set A beads except that the beads were not soaked overnight in water between the first and second reactions. Set C beads were washed 4 times after the first and second reactions. The bound radioactivities of all washed beads were counted.

Effect of Dissociation of HBsAg from the Immobilized Antibody on the Recycling of Labelled Antibody

Sera from three sero-positive patients showing B %)15 were assayed along with 40 negative sera using the modified kit procedure. The labelled Ab was pooled from all tubes after the second reaction and was used for the reassay of the negative sera using new anti-HBsAg coated beads.

The possibility of adsorbing HBsAg - ^{125}I labelled anti-HBsAg complexes in the presence of labelled anti-HBsAg antibody by unused anti-HBsAg coated beads was examined as follows. Complexes were generated by mixing the positive pool with labelled antibody to give a mean B % of 2.5 $\pm$0.4 % (n=5) for the labelled Ab by unused anti-HBsAg coated beads. Three aliquots of 1 ml of the Ag-labelled Ab complexes were incubated overnight at room temperature with 1, 2 , and 3 fresh anti-HBsAg coated beads respectively to adsorb the complexes. The three sets of the supernates (containing labelled antibody and Ag-labelled Ab complexes) were recovered from the reaction mixtures. Two hundred microlitre of each set of recovered supernates were allowed to react with fresh anti-HBsAg coated beads. The bound radioactivities of the beads were counted.

RESULTS

The reaction between the recovered labelled antibody and new anti-HBsAg coated beads showed the formation of immune complex between the labelled antibody and dissociated HBsAg during the second reaction of the modified kit procedure for low and high pools (table). The B% for the recovered labelled antibody from the low and high pool tubes were respectively 7.5 and 300 times that of the negative tubes. This indicated that dissociation of HBsAg from immobilizedAb occured at high as well as low Ag concentration. Re-utilization of beads from the high pool tubes

TABLE

	Percent Binding (%) of Fresh Labelled Antibody by Anti-HBsAg Coated Beads in the Second Reaction of IRMA (n=5)	Percent Binding (%) of Recovered Labelled Antibody after the Second Reaction of IRMA by Fresh Anti-HBsAg Coated Beads (n=4)
Negative Pool	0.13±0.02	0.04±0.01
Low Pool	5.10±0.30	0.30±0.01
High Pool	12.60±0.10	12.00±0.04

(with B % = 12.6 ± 0.1 %) in the assay of positive serum (with B % =7.7 ±0.2 %) gave a net increase of B% of 6.8 ±0.9 % . This showed that there was sufficient residual immobilized Ab remaining on the bead, after initial reaction with high concentration of HBsAg to further bind the Ag in the positive serum.

Incubation of immunoglobulin (anti- TSH) coated beads with high and negative pools resulted in a slight increase in B % of labelled anti-HBsAg to 0.7±0.02 % and 0.5±0.03 % respectively for the two pools. The mean B % of the recovered labelled Ab from the positive and negative tubes by fresh anti-HBsAg coated beads were 0.2±0.01 % and 0.17±0.07 % respectively. The extent of dissociation was less than that of anti-HBsAg coated beads exposed to the low pool (see table).

The effect of prolonged washing on the dissociation of HBsAg from the immobilized Ab were outlined in figure . Prolonged washing with overnight soaking of set A beads after the first reaction was more effective than other two patterns of washes as shown by the lowest level of B % reached by this method. The dissociation of HBsAg from the immobilized antibody in the second

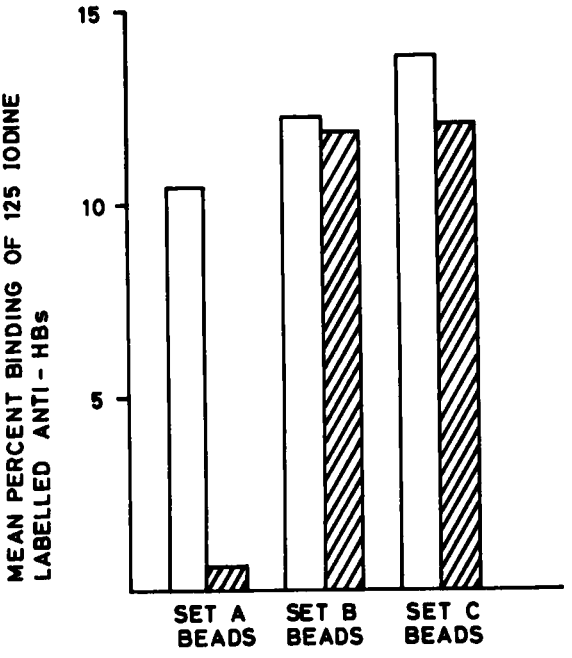

Figure showing the percent binding in opened bars of set A, B, and C beads after different methods of washes (see text for explanation). The percent binding in hatched bars indicated the degree of dissociation of HBsAg from immobilized anti-HBs in the second reaction after different methods of washes.

reaction after the wash was the lowest of the three patterns of washes as shown by the low percent binding of the recovered labelled antibody by fresh anti-HBsAg coated beads. Prolonged first wash without overnight soaking (set B beads) did not decrease the dissociation of HBsAg from the antibody-coated beads in the second reaction.

Assay using recycled pooled labelled antibody from 3 sero-positive and 40 sero-negative tubes resulted in an mean overall 10 fold increase in nonspecific binding of all antibody coated beads exposed to negative sera from B% = 0.1 ± 0.01 % to 1.0 ± 0.02 % (n=40) indi-

cating contamination of the recovered pooled labelled antibody by dissociated HBsAg. The adsorption experiment of HBsAg - ^{125}I anti-HBsAg complexes by the fresh beads showed a decrease in B % for the labelled antibody, adsorbed by 1, 2, and 3 new beads, from 2.5±0.4 % (for unadsorbed labelled Ab) to 2.0+0.2 %, 1.7±0.2 %, and 1.4±0.2 % respectively (for adsorbed labelled antibody).

DISCUSSION

Using the method described above, it is possible to demonstrate directly the dissociation of HBsAg from the immobilized antibody during the second reaction. This dissociation occurred both at high and at low doses of Ag as postulated by Rodbard (4).The excessive dissociation of Ag from immobilized antibody exposed to high dose of HBsAg probably accounted for the hook effect observed in immunoradiometric assay. There was no evidence of lack of immobilized antibody as shown by assay of positive serum using recycled beads which have been exposed to the high pool. The incremental increase in binding was close to the expected B % value of the positive serum.

Assay of the high pool using anti-TSH coated beads and ^{125}I labelled anti-HBsAg showed proportionately less dissociation of HBsAg from the immobilizedantibody in the second reaction than assay of the low pool. This suggested that the dissociation of HBsAg non-specifically bound to the immobilized antibody probably was not an important cause of sequestration of labelled antibody in the second reaction leading to the hook effect.

The washing experiment indicated that certain HBsAg were loosely bound to the immobilized antibody and could be readily removed by prolonged first washes. This was shown by the marked decrease in B % of the new beads exposed to the recovered labelled antibody from A beads as compared with those exposed to recovered labelled

antibody from B, and C beads. Prolonged washing of unused anti-HBsAg coated beads can be shown not to decrease the B % in the subsequent assay of positive serum.

The dissociation of HBsAg from the immobilized Ab in the second reaction in a routine HBsAg assay of 3 positve and 40 negative tubes significantly contaminated the recovered pooled labelled antibody from the assay and precluded unselective pooling of labelled antibody for use in a recycled assay (8). The result also indicated that adsorption of Ag-labelled Ab complexes in the recovered labelled Ab is ineffective for reducing the non-specific binding of recycled labelled Ab. Further work is required to show whether the mechanism of the reaction described above for viral antigen also hold true for other polypeptides.

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