Platelet Aggregation by Hepatitis B Surface Antigen-Antibody Complexes
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Platelet aggregation indicating antigen-antibody complex formation was observed when hepatitis B surface (HB) antigen and antibody were mixed. Platelet aggregation titers were determined for serum specimens found positive by radioimmunoassay for either HB, antigen or HB, antibody. From these determinations, incidence of HB, antigen-antibody complexes was found to be higher in HB, antigen sera than in HB, antibody sera. There was an inverse correlation between platelet aggregation titers and radioimmunoassay values that was statistically significant for HB, antigen sera but not for HB, antibody sera. The incidence of anti-complementary activity was twice as high for platelet aggregation-positive HB, antigen and antibody sera as for platelet aggregation-negative sera. HB, antigen sera that were positive by platelet aggregation exhibited nearly three times the incidence of anti-complementary activity as did HB, antibody sera. However, the low incidence of anti-complementary activity was distributed about equally between HB, antigen and antibody sera that were negative by platelet aggregation. Additional HB, antigen preincubated with HB, antigen-positive sera effectively inhibited platelet aggregation, whereas additional HB, antibody was somewhat less effective. On the other hand, preincubation of HB, antigen sera with anti-IgG serum effectively enhanced platelet aggregation, whereas preincubation of HB, antigen sera with HB, antibody did not.

The role of immune complexes in immunologic reactions is recognized as an important factor in the immune response. Antigen complexed with antibody can interact with lymphoid cells to alter both cellular and humoral immune mechanisms in vivo. Complexes were demonstrated by Perlmann et al. (22) to inhibit antibody-dependent lymphocyte destruction of target cells bearing antigenic determinants. In addition, humoral immune responses to weakly antigenic doses of viral (10) and to nonviral (18) proteins were enhanced by homologous antibody. Formation and deposition of viral antigens in complex with antibody has been implicated in the persistence of Aleutian mink disease virus (11), mouse leukemia virus (19), and, more recently, hepatitis B surface (HB) antigen in our own study (7).

The platelet aggregation test developed by Penttinen et al. (21) has been used successfully to detect the presence of antigen-antibody complexes in serum. In this test, human platelets aggregate in the presence of antigen-antibody complexes to form a characteristic sedimentation pattern. Platelet aggregation tests have demonstrated antigen-antibody complexes in sera from patients with Mycoplasma pneumoniae infections (2), sarcoidosis (9), varicella virus (16), and rubella virus (17). In post-rubella infections, the highest platelet aggregation titers coincided with clinical symptoms of thrombocytopenia purpura, which is thought to be due to deposition of viral antigen-antibody complexes. Likewise, the serum sickness and arthritic symptoms associated with HB (1) are coincident with the occurrence of HB, antigen antibody complexes. In chronic active hepatitis, it is most likely that too little antibody is produced for antigen clearance, but that adequate antibody is produced for continuous formation of immune complexes. Consequently, HB, antigen in these complexes may remain undetected by conventional serological procedures.

The possible occurrence of double-antibody complexes (HB, antigen-antibody–anti-immunoglobulin G [IgG]) in HB was previously postulated from data by Daugharty and Gogel (6). We have further detected a high incidence of HB, antigen-antibody complexes in HB, antigen- and antibody-positive sera by using a platelet aggregation test that is known to be more sensitive than immunodiffusion or complement fixation (CF) (14). Preincubation of HB, sera with excess antigen or antibody al-
tered platelet aggregation titers in most cases. The anti-complementary activity of these sera in CF was indicative of the presence of HB, antigen-antibody complexes.

MATERIALS AND METHODS

Specimens and reagents. The HB antigen- and antibody-positive sera were selected from diagnostic specimens received from the Viral Diseases Branch, Bureau of Epidemiology, Center for Disease Control, Atlanta, Ga. Sera positive for HBs antigen were selected by a competitive radioimmunoassay (RIA) in which unlabeled HB, antigen inhibited precipitation of $^{131}$I-labeled HB, antigen by standard anti-HB. HB antibodies in specimens were measured by the same RIA procedure; however, instead of inhibiting precipitation of $^{131}$I-labeled HB, antigen, HB, antibodies enhanced precipitation of $^{131}$I-labeled HB, antigen in the standard assay system. The HB, antigen-negative control specimens were from a National Institutes of Health group of sera having RIA results of a statistically determined random distribution (12).

A known positive lot of HB, antibody-containing serum was purchased from Medical Sciences International, Inc., Medical Services Division, Stoneham, Mass. The antisera had been obtained from a hemophiliac who had received multiple therapeutic transfusions. In our RIA, this lot of anti-HB, serum precipitated twofold greater amounts of $^{131}$I-labeled HB, antigen than did serum specimens obtained from patients with HB. The HB antigen-positive specimens used in preincubation experiments was obtained from the Atlanta Chapter of the American Red Cross. It was positive for HB, antigen by immunizing goats with IgG H chain antigen (end-point dilution, 1:2), and by CF (end-point dilution, 1:125).

Antiserum specific for the IgG H chain was made by immunizing goats with IgG H-chain antigen preparations (5). Human IgG H chains were prepared by sodium sulfate precipitation, diethylaminoethylcellulose chromatography, and mercaptoethanol reduction of intact IgG. Animals were immunized with an initial intradermal injection of 20 to 25 mg of IgG H chain antigen and with two subsequent Intravenous booster doses 3 weeks apart.

Platelet aggregation test (15). (i) Platelet preparation. Either platelet-rich plasma or a platelet concentrate was obtained from the American Red Cross and was used in the test on the day it was delivered. In preparation for the procedure, the erythrocytes were sedimented at 275 x g for 15 min at room temperature. The supernatant plasma was removed, and platelets were further sedimented at 1,000 x g. The platelet pellet was resuspended in Dulbecco phosphate buffer (0.15 M NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8.0 mM Na$_2$HPO$_4$, 1.0 mM glucose, pH 7.8) without Ca and Mg. The platelets were sedimented and washed by two additional cycles of sedimentation and resuspension. Finally they were suspended in a volume of Dulbecco buffer to give a final standard concentration of 1.34 x 10$^9$ platelets/µl, as determined by a Coulter counter.

(ii) Platelet assay for complexes. Standard platelet suspension was added in 0.05-ml aliquots to each of the wells of a microtiter plate containing twofold dilutions of serum specimens diluted in Dulbecco buffer. After the reagents were mixed, microtiter plates were incubated at 4°C for 15 h and read with indirect light against a black background. Endpoint dilutions of specimens were determined as the maximum dilution of specimen at which a positive agglutination settling pattern was observed.

(iii) Determination of the effect of excess antigen or antibody upon platelet aggregation. In microtiter plates, specimens were diluted twofold in 0.025-ml aliquots of buffer. To one of three replicates was added 0.25 ml of diluent as control. To the second was added 0.025 ml of HB, antigen, and to the third was added 0.25 ml of HB, antibody (at the lowest dilution of antigen or antibody which gave negative platelet aggregation results when tested previously, usually a 1:4 or 1:8 dilution). After the reagents were mixed and the plates preincubated at 37°C for 2 h, 0.05 ml of the standard platelet suspension was added to each well. The plates were incubated for 15 h at 4°C, and the aggregation end points were determined.

(iv) Block titration. All dilutions were made in the wells of microtiter plates. Eleven twofold serial dilutions (1:2 through 1:2,048) of 0.025 ml of antigen were made in the horizontal rows of the plates. As a control, only diluent was added to the last vertical row. Seven twofold serial dilutions of the antibody were made in tubes with sufficient volume per tube for each of the antigen dilutions in horizontal rows. The antibody was then added to wells of each horizontal row in 0.025-ml aliquots except for the bottom horizontal row, which was used for antigen controls. Diluent (0.025 ml) was added to each well of the antibody and antibody control rows. After the reagents were mixed and the plates preincubated at 37°C for 2 h, 0.05 ml of standard platelet suspension was added to each well of the plates. The plates were further incubated at 4°C for 15 h and read for agglutination.

RIA. A liquid-phase RIA was used for measuring both HB, antigen and antibody (8). HB, antigen was assayed by its competitive inhibition of precipitation of $^{131}$I-labeled HB, antigen by a standard anti-HB, dilution which maximally precipitated $^{131}$I-labeled HB, in preliminary determinations. HB, antibody was assayed by its enhanced precipitation of $^{131}$I-labeled HB, when the standard anti-HB, was omitted. Double-antibody complexes were formed with anti-IgG in either the HB, antigen or antibody assay. The method of ammonium sulfate precipitation described by Coller et al. (4) was used for separating bound $^{131}$I-labeled HB, antigen from the unbound $^{131}$I-labeled HB, antigen.

CF. CF reactivity of HB, antigen- and antibody-positive specimens was determined by M. H. Hatch, Virology Division, Center for Disease Control. The micromethod of CF (3) was used for block titration of antigen and antibody.

RESULTS

Platelet aggregation in block titration of HB, antigen and antibody. Sedimentation of
aggregated platelets in a positive agglutination pattern was demonstrated with various mixtures of HB, antigen and antibody. In the block titration of Fig. 1, dilutions of a plasma that was RIA positive for HB, antigen were mixed with dilutions of a plasma positive for HB, antibody. The mixtures were preincubated for 2 h at 37°C, and a suspension of washed human platelets at the standard concentration was added to each well of the microtiter plate. After incubation for 15 h at 4°C, the sedimentation patterns were read. Positive agglutination is indicated. Platelet aggregation was obtained with HB, antigen dilutions as great as 1:2,048 when the antigen was incubated with a HB, antibody dilution of 1:16. At lower dilutions of HB, antigen (higher concentrations), platelet aggregation was not enhanced. In fact, as the concentration of HB, antibody was reduced from a dilution of 1:16 to 1:32, platelet aggregation was obtained only with HB, antigen dilutions of 1:8 or less. Platelet aggregation was not observed for control dilutions of the standard HB, antibody at ≥1:2 or antigen at >1:4 when diluted alone was added without additional HB, antigen or antibody. Likewise, the standard platelet suspension alone did not form aggregates.

Incidence of platelet aggregation in HB, antigen and antibody specimens. Sera that were RIA positive for either HB, antigen or HB, antibody were tested by platelet aggregation for the presence of antigen-antibody complexes. The frequency distributions of positive platelet aggregation by these specimens are presented in Fig. 2. The incidence of positive agglutination titer greater than 8 was 50% higher for the HB, antigen-positive specimens (10/30) than for the HB, antibody-positive specimens (7/32). An even greater difference (62%) was observed between HB, antigen and antibody specimens with agglutination titers greater than 4. Sixty percent of the HB, antigen specimens had agglutination titer greater than 4, whereas only 37% of the HB, antibody specimens had titers in that range. The geometric mean platelet titers for HB, antigen (31.6) and antibody (41.2) specimens did not vary as widely. Sera with agglutination titers less than 8 demonstrated an approximately normal bell-shaped distribution, with median agglutination titers of 4 for HB, antigen sera and 2 for HB, antibody sera. Another group of normal sera tested to confirm the incidence of nonspecific agglutination reactions had median agglutination titers of 2 and 4 on two different assays. These titers represent the normal distribution of nonspecific platelet agglutination.

Statistical correlations were estimated between agglutination titers by platelet aggregation and HB, antigen and antibody values measured by RIA. The observed association between platelet aggregation titers and RIA values for HB, antigen (Fig. 3) had a negative correlation coefficient, indicating an inverse relationship. This negative correlation between platelet aggregation for complexes and RIA for HB, antigen was statistically significant (P = 0.04), whereas platelet aggregation with HB,
Platelet aggregation and anti-complementary activity. Data obtained from platelet aggregation tests for complexes in HB, antigen and antibody sera were examined to determine what role, if any, the complexes may have in CF. HB, antigen and antibody sera that were platelet aggregation positive (Table 1) had nearly twice the incidence of anti-complementary activity as did platelet aggregation-negative sera. Only 14% (6 of 44) of the platelet aggregation-negative sera were anti-complementary in CF, whereas 29% (5 of 17) of platelet aggregation-positive sera were anti-complementary.

An even greater difference in anti-complementary activity was noted for HB, antigen sera that were positive by platelet aggregation when compared with HB, antibody sera positive by platelet aggregation. The incidence of anti-complementary specimens was three times greater in the HB, antigen-positive sera than in the antibody-positive sera. Four of ten HB, antigen sera positive by platelet aggregation were anti-complementary in CF, whereas only one of the seven HB, antibody sera positive by platelet aggregation was anti-complementary. Likewise, platelet aggregation-negative sera had an equally low incidence of anti-complementary activity, whether they were HB, antigen or antibody positive by RIA.

Effect of excess HB, antigen or antibody or of added anti-IgG upon platelet aggregation. The addition of either excess HB, antigen or HB, antibody to sera containing antigen-antibody complexes would be expected to cause alterations of enhancement or inhibition of platelet aggregation. The effects of adding either HB, antigen or HB, antibody to complex-containing sera were investigated. The results in Table 2 show that the platelet aggregation titers were changed most by the addition of HB, antigen. Addition of excess HB, antigen to sera positive by RIA for HB, antigen caused a decreased platelet aggregation titer in 90% of the specimens. On the other hand, addition of excess HB, antigen to sera positive for HB, antibody caused alteration of the titers in only 57% of the specimens. In similar determinations, it was observed that the addition of excess HB, antibody was much less effective in altering the platelet aggregation titers.

The influence of anti-IgG upon platelet aggregation titers was also investigated. Anti-IgG was preincubated with HB, antigen and HB, antibody specimens known to contain antigen-antibody complexes. We observed (Table 3) that added anti-IgG enhanced platelet aggregation.

**Table 1. Incidence of anti-complementary (AC) activity in platelet aggregation-positive and -negative sera containing HB, antigen and antibody**

<table>
<thead>
<tr>
<th>Positive by RIA for:</th>
<th>Non-AC*</th>
<th>AC*</th>
<th>Total no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet aggregation positive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB, Ag</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>HB, Ab</td>
<td>6</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td><strong>Platelet aggregation negative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB, Ag</td>
<td>17</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>HB, Ab</td>
<td>21</td>
<td>3</td>
<td>24</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are percentages.
gation by HB, antigen sera. On the other hand, anti-IgG reduced platelet aggregation by HB, antibody sera. With the HB, antigen sera, preincubation with anti-IgG caused enhanced platelet aggregation in six of nine (67%) specimens; there was no reduction of platelet aggregation in these specimens. With HB, antibody specimens, preincubation with anti-IgG caused enhancement of aggregation in two of seven, inhibition of aggregation in three of seven, and no change in two.

Table 2. Effect of excess HB, antigen or antibody upon platelet aggregation

<table>
<thead>
<tr>
<th>Specimens positive by RIA for:</th>
<th>Effect upon platelet aggregation when preincubated with:</th>
<th>Total no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HB,Ab Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>HB, Ag</td>
<td>0</td>
<td>1 (10)</td>
</tr>
<tr>
<td>HB, Ab</td>
<td>0</td>
<td>2 (28)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are percentages.

* Only one specimen not affected by either HB, antigen or antibody.

Table 3. Effect of preincubating with anti-IgG upon platelet aggregation

<table>
<thead>
<tr>
<th>Specimen positive by RIA for:</th>
<th>Effect upon platelet aggregation when preincubated with anti-IgG</th>
<th>Total no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>HB, Ag</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>(67)</td>
<td>(33)</td>
<td></td>
</tr>
<tr>
<td>HB, Ab</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>(28.5)</td>
<td>(43)</td>
<td>(28.5)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are percentages.

The platelet aggregation test has been used in attempts at determining whether HB, antigen-antibody complexes have a more common occurrence in the antigen or antibody phase of hepatitis. It was indicated that complexes are present more often in the earlier antigenic phase of the disease compared with their occurrence during the later antibody phase. Consequently, complexes may be expected to exert certain effects that could modify the course of the disease.

We have demonstrated platelet aggregation reactions with HB, antigen and antibody plasma. In these reactions, platelets in the presence of preformed HB, antigen-antibody complexes formed positive agglutination sedimentation patterns. Preincubation of the HB, antigen and antibody at various ratios in the block titration (Fig. 1) resulted in detection of complexes up to a 1:2,048 dilution of the HB, antigen and a 1:16 dilution of the HB, antibody. A positive platelet aggregation was less readily obtained with the higher dilutions of HB, antibody in the block titration with HB, antigen. This relatively greater sensitivity of platelet aggregation to antibody concentration has been noted by Penttinen et al. (21). It is theorized that the degree of saturation of the polyvalent antigen is the limiting factor in determining the platelet aggregation titer.

Our data indicated that sera with HB, antigen excess by RIA had a higher incidence of complexes and larger correlation (Fig. 2) with HB, antigen levels than did sera with HB, antibody excess. Again, the greater reactivity of the polyvalent HB, antigen in sera with antigen excess may account for the higher incidence of immune complex formation in these sera. Alternatively, the kinetics of specific antibody formation in cases of hepatitis may preclude the possibility of complexes forming as frequently in sera that are positive for HB, antibody. HB, antibody formation at a later time and persistence of the antibody over a longer time period without the presence of the antigen provides less opportunity for complexes to form in sera that are positive for HB, antibody.

During the course of a disease like hepatitis B, it is probable that when complexes are formed early, even before peak antibody levels are reached, there would be evidence of antigen-antibody formation as indicated by platelet aggregation. However, with progressive formation of HB, antigen-antibody complexes and subsequent elimination of these complexes from circulation, the concentration of circulating HB, antigen detected by RIA would consistently diminish. In contrast, however, with malignancies that perpetually produce carcinoembryonic antigen, positive associations have been observed between the titers of complexes by platelet aggregation and the level of carcinoembryonic antigen by RIA (Daugharty et al., manuscript in preparation).

Nevertheless, the fact that HB, antigen-antibody complexes occur more frequently in sera with HB, antigen excess than in sera with HB, antibody excess is an essential finding suggest-
ing that the complexes affect the persistence or the waning of circulating antigen as well as the development of an immune response. Our previous study suggested that HB, antigen persisted longer in guinea pigs receiving inocula containing HB, immune complexes in antibody excess, whereas an upsurging immune response occurred after inoculation with immune complexes in antigen excess (7). Consequently, complexes of HB, antigen and antibody formed in vivo would be expected to modulate the persistence of HB, antigen and development of antibody. The presence of complexes during the antigen phase of hepatitis probably marks the onset of the immune response, which in turn holds the antigen level in check. Prospective studies should help to show a more precise relationship between the immune complexes and the overlap of the antigen and antibody phases of hepatitis. It may well be that this relationship regulates the antigen and antibody phases and determines the balance between acute and chronic hepatitis.

The normal sera in our test controls consistently gave low levels of agglutination (usually less than 1:8). The positive agglutination titers for these sera, however, exhibited a normal bell-shaped frequency distribution that was evident for titers below 1:8. The greatest significance could be attached to the platelet aggregation results when agglutination titers were above 1:8. Titers less than 1:8 most likely resulted from nonspecific reactions involving agglutination of antigen-antibody systems other than HB antigen-antibody. That is, perhaps a part of the nonspecific agglutination was caused by immune complexes formed at very low concentrations by the unlimited variety of antigens with which our bodies constantly come into contact. Nevertheless, the low levels of agglutination of test sera do help contribute to the inverse correlation shown between platelet aggregation titer and HB antigen concentration of the serum (Fig. 3). Consequently, many of these low-titered complexes detected by platelet aggregation would be expected to consist of HB antigen-antibody. This postulation would be true especially when a serum panel was obtained from a group that had been exposed to HB virus during a recent outbreak.

Anti-complementary activity like elevated platelet aggregation titers was more closely associated with HB, antigen sera than with HB, antibody sera. This again indicates that once HB, antigen levels have diminished, the circulating complexes are likewise diminished, which indicates that the presence of HB, antigen is a requisite in the formation of complexes. A specific charge redistribution in the Fc fragment of IgG molecules bound in immune complexes is responsible for attachment to receptor sites on platelets (13, 24). Similarly, the Fc is the principal portion of the IgG molecule involved in the mechanism of complement fixation. Consequently, it is understandable that a high incidence of anti-complementary activity would be observed in sera with platelet aggregation activity (Table 1). Anti-complementary activity in HB, sera has been shown by Schulman and Barker (23) to be reversible by the addition of excess antigen or antibody.

The platelet aggregation titers of all but one of the HB, antigen sera tested were altered by addition and preincubation of the specimen with excess antigen or antibody (Table 2). The inhibitory effect of the added HB, antigen was not surprising, because large excesses were demonstrated in each of the sera by RIA. The large excess of HB, antigen in the immune reaction mixture during preincubation may shift the equilibrium so that complexes dissociate. Decreased immune complex concentrations then result in lowered platelet aggregation titers. The fact that the addition of excess antigen to several HB, antibody sera caused diminution of the platelet aggregation titers is not readily explained.

It was observed that anti-IgG significantly enhanced platelet aggregation of HB, antigen sera (Table 3). The mechanism of the enhancement has not been determined; however, rheumatoid factor is not known to affect platelet aggregation through interaction with unbound autologous globulin (24). A likely mechanism is the reaction of anti-IgG with the IgG of the immune complex. This reaction would be facilitated by distortion of the IgG in its combination with HB, antigen, which would allow anti-IgG to react with complexes of specific (or even nonspecific) IgG bound to HB, antigen in the formation of double-antibody complexes. Several of our observations supported this explanation. HB, antigen sera had a higher incidence of immune complexes than did HB, antibody sera, and their platelet aggregation titers were more readily altered by the addition of excess HB, antigen. Finally, the incidence of anti-complementary activity was higher in HB, antigen sera than in HB, antibody sera.

LITERATURE CITED


