Whole-Blood Hemagglutination Inhibition Test for Venereal Disease Research Laboratory (VDRL) Antibodies

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Received 8 October 1999/Returned for modification 5 December 1999/Accepted 11 June 2000

Serological tests for syphilis comprise both nontreponemal and treponemal (confirmatory) tests (5). The nontreponemal or screening tests for syphilis involve detection of reaginic serum antibody (2). A variety of methods is employed for determining the presence of these antibodies in serum. The standard Venereal Disease Research Laboratory (VDRL) test is a tube flocculation test which makes use of the VDRL antigen. Detection of flocculation may be enhanced using carbon particles, e.g., the VDRL or rapid plasma reagin (RPR) card tests.

Other methods have been used to detect and enhance agglutination in serological tests. These include the use of red blood cells (RBCs) and liposomes (6). The aim of this study was to use VDRL liposomes and the patient’s own RBCs to develop an agglutination test in which whole blood could be used. It was proposed that antibody to human RBC membranes be conjugated to VDRL liposomes. We expected that VDRL antibody would bind to the liposomes and enhance RBC agglutination by means of cross-linking. However, we discovered that hemaggglutination was actually inhibited in the presence of VDRL antibodies, and the new test was consequently a hemagglutination inhibition (HAI) test.

MATERIALS AND METHODS

Preparation of liposomes. A previously published method was used with minor modification to prepare the VDRL liposomes (4). In addition, phosphatidylethanolamine (PE) was incorporated into the liposomes to provide a site for protein conjugation (7). Cardiolipin (3 mg/ml; Sigma, St. Louis, Mo.), cholesterol (5 mg/ml; Sigma), phosphatidylcholine type VE (2 mg/ml; Sigma), and PE type III (0.5 mg/ml; Sigma) in solution in methanol-chloroform (1:2) were evaporated to dryness under a nitrogen stream. Mecles were prepared by adding 1 ml of 0.15 M NaCl with vortex mixing.

Cholesterol concentrations lower than those reported by Harris et al. (4) were used in the preparation of VDRL liposomes. This was because of difficulty in maintaining a stable suspension.

Reactivity of modified VDRL liposomes with nontreponemal antibody. The ability of the modified VDRL liposomes to adsorb to nontreponemal antibody was tested to ensure that the altered liposome composition did not affect antibody binding. VDRL liposomes were subjected to a reaction with four sera from patients with syphilis, and the RPR test was done on the supernatant before and after reaction with the liposomes (Table 1). Equal volumes of VDRL liposomes and sera from the patients with syphilis were incubated at room temperature for 1 h. Following centrifugation (27,000 × g for 5 min), the supernatant was subjected to a reaction with the RPR card test (Becton Dickinson), and the titers as determined by the RPR test before and after immunosorption were compared.

Attachment of RBC antibodies to liposomes. The rabbit anti-human RBC antibody (Cappel, Durham, N.C.) was conjugated to the PE group incorporated into the liposomes by creating a third-reactive phospholipid derivative (7). To 1 ml of liposomes suspended in borate-buffered saline, pH 9.0, 25 µl of 20 mM N-hydroxysuccinimidy 3-(2-pyridyldithio)propioniate (SPDP) (Pierce Chemical Co., Rockford, Ill.) was added, and the reaction was carried out for 30 min. The mixture was passed over a gel filtration column (Sephadex G-25 M) equilibrated with phosphate-buffered saline, pH 7.2. The liposome-containing fractions were reconcentrated in a microcentrifuge. Twelve milligrams of dithiothreitol was added for 30 min at room temperature. The antibody-containing fraction was subjected to gel filtration (with phosphate-buffered saline, pH 7.2), and the reaction was carried out with the liposome-containing fraction for 18 h. The liposomes were separated by centrifugation. The effect of adding RBC antibodies on the reactivity of the liposomes with nontreponemal antibody was determined by antibody absorption tests (see above and Table 1).

RBC agglutination determination of titer. To determine the least quantity of liposomes required to produce hemagglutination in the absence of nontreponemal antibodies, 4% RBC suspensions from an RPR-negative patient were prepared in 0.15 M saline and mixed with serial dilutions of liposomes. The end titer giving definite agglutination after incubation for 2 h at 37°C was used in subsequent experiments.

HAI test. The working dilution of the liposome-anti-RBC conjugate was determined as described above. EDTA-blood samples were diluted 1:10 to provide an approximately 3 to 4% suspension of RBCs (the patient’s hematocrit was not determined before dilution). Diluted blood (100 µl) was subjected to a reaction with 100 µl of the liposome-anti-RBC conjugate for 2 h at 37°C. Following centrifugation at 800 rpm for 1 min, the presence of macroscopic agglutination in the samples was determined.

Sensitivity of HAI test. Titters obtained with by the liposome method and the RPR test were compared. To carry out the HAI test, the RBC suspension was maintained at 4°C and the plasma was diluted appropriately.

Patients. Routine antenatal care in the Peninsula Maternal and Neonatal Services in Cape Town, South Africa, involves blood grouping (performed on blood collected into EDTA-containing tubes) and the VDRL tube test for syphilis. Samples with positive VDRL tests (with any titer of antibody) are routinely subjected to Treponema pallidum hemagglutination (TPHA) testing (3).

For the study, we used blood left over from the EDTA tube sample after blood grouping had been performed. Samples were stored at 4°C following collection. Grossly hemolyzed samples and those more than 48 h old were discarded. The
TABLE 1. Effect of varying VDRL liposome composition on immunoabsorption of nontreponemal antibody

<table>
<thead>
<tr>
<th>Test serum no.</th>
<th>RPR titer following absorption of sera with VDRL liposomes of various compositiona</th>
<th>RPR titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol (9 mg/ml)b</td>
<td>Cholesterol (5 mg/ml)c</td>
</tr>
<tr>
<td>1</td>
<td>1:16</td>
<td>NTf</td>
</tr>
<tr>
<td>2</td>
<td>1:16</td>
<td>1:1</td>
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<tr>
<td>3</td>
<td>1:32</td>
<td>1:1</td>
</tr>
<tr>
<td>4</td>
<td>1:32</td>
<td>1:1</td>
</tr>
</tbody>
</table>

a See text for method.
b Cardiolipin, 3 mg/ml; cholesterol, 9 mg/ml; phosphatidylcholine, 2 mg/ml.
c Cardiolipin, 3 mg/ml; cholesterol, 5 mg/ml; phosphatidylcholine, 2 mg/ml.
d Cardiolipin, 3 mg/ml; cholesterol, 5 mg/ml; phosphatidylcholine, 2 mg/ml; PE, 0.5 mg/ml.
e Cardiolipin, 3 mg/ml; cholesterol, 5 mg/ml; phosphatidylcholine, 2 mg/ml; PE, 0.5 mg/ml; RBC antibody.
f NT, not tested.
g NEG, negative.

hemagglutination inhibition test was performed on 951 sequential samples without knowledge of the VDRL test results. Approval for the study was obtained from the Ethics Committee of the University of Cape Town.

RESULTS

Effect of liposome composition and RBC antibody conjugation on reactivity with nontreponemal antibody. Table 1 shows the results of comparisons made between VDRL liposomes prepared by the method described above (4) (these liposomes contained 9 mg of cholesterol per ml), liposomes with less cholesterol (5 mg/ml), liposomes with 5 mg of cholesterol per ml and PE, and liposomes with RBC antibodies attached. It can be seen that altering the liposome composition did not have any significant effect on the ability of the VDRL liposomes to adsorb nontreponemal antibody.

Sensitivity. Known antibody-positive specimens with RPR test-determined titers of 1:1, 1:2, and 1:32 were evaluated by the HAI test using 4% erythrocyte suspensions from patients known to be negative for non-treponemal antibodies. HAI test-determined titers were consistently 16 times higher (i.e., 1:16, 1:32, and 1:512, respectively). The liposome method was therefore found to be 16 times more sensitive than the RPR test. However, when the HAI test is performed as a clinical test, the whole-blood sample is diluted 1:10. Therefore, in terms of the working dilutions used for patient samples, the liposome method is calculated to be 1.6 times more sensitive than the RPR test.

Patient samples. Of 951 tests, 50 showed HAI. Of the 50, 49 were VDRL test and TPHA positive. The remaining sample was negative by the VDRL test; no TPHA was done. (A total of 901 samples were negative by the VDRL test and showed no HAI.) Compared to the VDRL test, the sensitivity of HAI was 100%, the specificity was 99.9%, the positive predictive value was 98%, and the negative predictive value was 100%.

DISCUSSION

Essentially, the test described in this work makes use of the VDRL antigen but provides a new way of reading the result. We originally hypothesized that enhanced agglutination would be found in the reagin-containing blood samples. However, we discovered the reverse. Presumably, binding of the reaginic antibody to the liposomes induces steric hindrance, preventing RBCs from binding to the anti-human RBC antibody.

The results presented here indicate that the VDRL HAI test has similar reactivity to the VDRL tube test. There was one discordant result (i.e., HAI positive and negative VDRL). Unfortunately, the TPHA (or other treponemal) test was not done in this case, and patient tracing proved unsuccessful. At the dilutions selected, the HAI test would be slightly (1.6 times) more sensitive than the VDRL test. While this may allow more true positives to be detected, it is possible that more biologic false positives would also be found.

What potential advantages does the new test have? The main advantage is that it uses whole, anticoagulated blood. Tubes can be prepared containing anticoagulant, diluent, and liposomes. Following addition of whole blood (100 μl) the test can be performed. There is no need for additional tubes or removal of serum, reducing sample handling. Because no expensive equipment is needed and tests can be macroscopically read, they could be carried out at point-of-care centers. For a number of reasons, this may be useful at prenatal clinics in developing countries. Firstly, the prevalence of untreated maternal syphilis is relatively high (e.g., 10 to 15% in many parts of sub-Saharan Africa), and congenital syphilis is a significant cause of perinatal mortality (8). Secondly, a significant number of pregnant women attend a prenatal clinic and have a serological test for syphilis performed, but the result is not received or acted upon before delivery (1, 9). This occurred in 49% of VDRL test-positive pregnant women in one recent study and was partly due to centralized laboratory testing (9). These factors make testing at peripheral units popular, allowing immediate identification of patients requiring further testing (e.g., with confirmatory treponemal tests) and treatment.

The hemagglutination test has potential for use in peripheral point-of-care or near-patient testing. Before more widespread introduction, however, greater numbers of samples require testing. Potential ways of prolonging the shelf-life of the liposomes need to be explored, and the costs thereof must be ascertained.

REFERENCES