Rapid Detection of Hepatitis B Virus Surface Antigen by an Agglutination Assay Mediated by a Bispecific Diabody against Both Human Erythrocytes and Hepatitis B Virus Surface Antigen^V

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Bispecific antibodies have immense potential for use in clinical applications. In the present study, a bispecific diabody against human red blood cells (RBCs) and hepatitis B virus surface antigen (HBsAg) was used to detect HBsAg in blood specimens. The bispecific diabody was constructed by crossing over the variable region of the heavy chains and the light chains of anti-RBC and anti-HBsAg antibodies with a short linker, SRGGGS. In enzyme-linked immunosorbent assays, this bispecific diabody showed specific binding to both RBCs and HBsAg. When this bispecific diabody was mixed with human blood specimens in the presence of HBsAg, the dual binding sites of the diabody caused agglutination of human RBCs. This diabody-mediated agglutination assay was then used to test 712 clinical blood specimens and showed 97.7% sensitivity and 100% specificity when the results were compared with those of the conventional immunoassay, which was used as a reference. This autologous RBC agglutination assay provides a simple approach for rapid screening for HBsAg in blood specimens.

Bivalent and bispecific antibodies have many potential applications, including immunodiagnosis and immunotherapy of cancer, autoimmune diseases, and infectious diseases. Bivalency can allow antibodies to bind to antigens with great avidity (24). Bispecificity can allow the cross-linking of two antigens, for example, through the recruitment of cytotoxic T cells to mediate the killing of tumor cells (20, 25, 26, 28). Although a bispecific antibody can be produced by chemical linking or by fusion of hybridomas (17), significant difficulties with the production and purification of bispecific antibodies have limited their use in clinical applications. The progress that has been made in molecular biology and protein engineering now allows scientists to design bispecific antibodies with new formats, which are based on the manipulation of antibody fragments such as Fab, the variable region (Fv), and single-chain Fv (ScFv). These new generations of bispecific antibodies can be expressed, in recombinant form, as the main product and can be purified to homogeneity by the use of practical purification procedures. Using molecular biology techniques, scientists can clone the genes encoding antibody variable domains from hybridomas (21) or by panning phage-displayed antibody libraries (14). However, Fab, Fv, and ScFv fragments each carry a single antigen-binding site. Recombinant antibody fragments with two binding sites (two of the same sites or two different sites) have been made in several ways; for example, bispecific F(ab)2 fragments have been created either by chemical coupling of Fab fragments (27) or by heterodimerization through leucine zippers (5, 11). Even smaller bispecific antibody fragments have been constructed based on ScFv: the linking strategies include the introduction of cysteine residues into an ScFv monomer to form a disulfide linkage between two ScFv fragments (1, 3, 4, 6, 10, 15) and linkage via a third polypeptide linker (8, 13, 19). Bispecific or bivalent ScFv dimers have also been formed by using the dimerization properties of the kappa light chain constant domain (16) and other domains, such as leucine zippers and four helix bundles (22, 23). An alternative form of bispecific antibody is diabody (9). When a linker is too short to allow pairing between the light-chain variable region (VL) domain and the heavy-chain variable region (VH) domain on the same chain, the two domains are forced to pair with the complementary domains from another VH-VL peptide and create two antigen-binding sites. The two antigen-binding domains are shown by crystallographic analysis on opposite sides of the complex, such that they are able to simultaneously bind to two antigens.

In the present study, we isolated different antibody genes by panning phage-displayed antibody libraries against each particular antigen. Starting with an ScFv fragment against human red blood cells (RBCs) (30) and an ScFv fragment against hepatitis B virus surface antigen (HBsAg) (29), we constructed and produced a bispecific diabody against both RBCs and HBsAg. When HBsAg is present in human blood specimens, this diabody could agglutinate autologous RBCs and the agglutination could be visualized with the naked eye. On the basis of this observation, we developed a novel assay for the rapid detection of serum HBsAg. This assay is simple, the results can be obtained quickly, and no special equipment or training is required.

MATERIALS AND METHODS

Materials. The soluble ScFv expression vector was modified from the vector pCOMB3H, a gift from C. F. Barbas (2), by adding tag coding sequences (see below) at the 3’ end of the VH domain and replacing the sequence between the kappa chain and the Fd domain with an ScFv linker, RS(GGGGS)3 (Fig. 1, upper panels). The tags used in the vector were a c-Myc decapeptide, which
could be detected by a monoclonal antibody against Myc (18), and a hexahistidine tag, which could be used to facilitate purification by immobilized metal chelation chromatography (IMAC). The human anti-HBsAg and mouse anti-human RBC ScFv genes were previously cloned from two phage-displayed antibody libraries in our laboratory (29, 30). HBsAg-associated ~20-nm envelope particles, the small spherical particles without a viral core, were purified from HBsAg-positive blood by a modified polyethylene glycol precipitation and gradient centrifugation method (7) and were then quantified at the Peking University Hepatology Institute. The human blood specimens were collected from the Peking University People’s Hospital, the No. 302 Hospital, and the Navy General Hospital.

Construction of expression vectors for diabodies. The ScFvs of anti-RBCs and anti-HBsAg were originally on separate expression vectors (see “Materials” above). The ScFv-coding region was under the control of the lacZ promoter; shaded bars, Escherichia coli OmpA signal peptides; rectangles, VL domains; ovals, VH domains; VLrbc and VHrbc, VL and VH of anti-RBCs, respectively; VLhbs and VHhbs, VL and VH of anti-HBsAg, respectively; shaded rectangles (Tags), c-Myc and hexahistidine tags; octagons, stop codons.

FIG. 1. Scheme used to construct the expression vector consisting of ScFv and diabody. The genes for anti-HBsAg ScFv and anti-RBC ScFv were isolated from phage-displayed antibody libraries and then placed in the ScFv expression vector. When the linker of ScFv was shortened from 17 amino acid residues [SR(GGGGS)3] to 6 amino acid residues (SRGGGS), the vectors expressed bivalent diabodies. The VH of anti-RBC ScFv was then cross-linked with the VL of anti-HBsAg ScFv and vice versa to form two VL–VH hybrids. These two VL–VH hybrids, with a second internal ribosome entry site placed between them, were inserted downstream of a lacZ promoter to form the expression vector for a bispecific diabody. Arrows, lacZ promoters; shaded bars, Escherichia coli OmpA signal peptides; rectangles, VL domains; ovals, VH domains; VLrbc and VHrbc, VL and VH of anti-RBCs, respectively; VLhbs and VHhbs, VL and VH of anti-HBsAg, respectively; shaded rectangles (Tags), c-Myc and hexahistidine tags; octagons, stop codons.

Expression and purification of ScFvs and diabodies. XLI-Blue cells harboring ScFv or diabody expression vectors were shaken at 200 rpm and 37°C overnight in Luria-Bertani medium containing ampicillin and glucose. The cultures were then diluted 1:50 in fresh glucose-free superbroth medium and were further grown at 37°C until the absorbance at 600 nm was between 0.8 and 1.0. Isopropyl-β-D-galactopyranoside was then added to a final concentration of 1 mM, and the cultures were allowed to continue to grow at 30°C for 10 h. After the cultures were clarified by centrifuge and filtration, antibody fragments were purified on an IMAC Sepharose column (GE Healthcare), with an approximately 90% recovery rate, and, if necessary, were further purified by size-exclusion chromatography (see below).

A Sephacryl S-200 SF column (GE Healthcare) was calibrated by the separation of molecular mass markers of 67 kDa, 43 kDa, 25 kDa, and 13.7 kDa in phosphate-buffered saline (PBS) eluted with PBS at flow rate of 1 ml/min. The diabody purified by affinity chromatography was applied to this column, and the proteins were separated by using the same conditions used for column calibration. The fractions were collected and tested by an RBC agglutination assay. The diabody fraction was purified by this approach, with an approximately 50% recovery rate.

Antigen-binding ELISA. For the HBsAg-binding enzyme-linked immunosorbent assay (ELISA), 96-well plates were coated with 0.1 µg per well purified HBsAg overnight at 4°C. For the RBC-binding ELISA, 96-well plates were coated with 2 × 10⁵ per well fresh human RBCs for 1 h, followed by fixation with 0.5% glutaraldehyde for 10 min and then quenching of the endogenous peroxidase with 3% hydrogen peroxide in methyl alcohol for 1 h. The antigen-coated plates were washed with PBS and blocked with 250 µl per well of 1% bovine serum albumin (BSA) in PBS at 37°C for 1 h. Two hundred nanograms per well ScFvs or diabodies was added to the plates, and the plates were then incubated at 37°C for another 1 h, following by washing three times with PBS containing 0.05% Tween 20. Then, 50 µl per well of 1 µg/ml anti-Myc monoclonal antibody was added and the plates were incubated at 37°C for 1 h. The plates were then washed again as described above. The bound anti-Myc antibody was detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (50 µl per well at 50 ng/ml) in PBS containing 1% BSA, followed by...
HBsAg at levels as low as 0.20 ng/ml in blood specimens with 99.9% specificity.

The reference ELISA used in these studies was performed with the Hepanostika HBsAg Uni-Form II kit (BioMerieux, The Netherlands), which can detect the number of true-negative samples.

RBC agglutination assay. In the RBC agglutination test, the indicated amount of purified HBsAg was mixed with 20 μl whole blood (collected from HBsAg-negative healthy donors and placed in tubes containing heparin) and added to the wells of a 96-well plate. A volume of 50 μl of 200 ng/ml ScFv, monospecific diabody, or different fractions of bispecific diabody from size-exclusion chromatography was added. After 10 min of incubation at room temperature, the mixture was scored visually for agglutination.

When developing the RBC agglutination assay in the research laboratory, we used 96-well plates. As slide agglutination assays are more commonly used in clinics, we adapted this 96-well plate assay format to the slide agglutination assay format. Except for the holding vehicle, the other experimental settings were unchanged, and no difference in RBC agglutination was observed between these two assays. To confirm RBC agglutination, we observed the results for some samples under a microscope.

Tests with clinical specimens and the bispecific diabody in agglutination assay. To determine the sensitivity and the specificity of the agglutination assay mediated by the bispecific diabody against both RBC and HBsAg, we compared the agglutination assay with a standard ELISA in tests with 712 clinical specimens. The 512 specimens from the No. 302 Hospital and the General Navy Hospital were randomly selected from people visiting the hospitals for HBsAg testing. The 200 specimens from Peking University People's Hospital were randomly selected from people visiting the hospitals for HBsAg testing. The 200 specimens from Peking University People's Hospital were randomly selected from people visiting the hospitals for HBsAg testing. The 200 specimens from Peking University People's Hospital were randomly selected from people visiting the hospitals for HBsAg testing.

RESULTS

Construction and expression of anti-RBC diabody. Anti-RBC ScFv was isolated from a phage-displayed antibody library, which was constructed by using RNA isolated from peripheral blood monocytes of an HBsAg antibody-positive patient (29). In the expression vector of the bispecific diabody against both RBC and HBsAg, the N terminus of the anti-RBC V<sub>1</sub> domain was connected to the C terminus of the anti-HBsAg V<sub>L</sub> domain by using a 6-amino-acid linker, SRGGGS, to restrict the intrachain pairing of V<sub>L</sub> and V<sub>H</sub>. A second V<sub>L</sub>-V<sub>H</sub> crossover hybrid was constructed by connecting the N terminus of the anti-HBsAg V<sub>H</sub> domain to the C terminus of the anti-RBC V<sub>L</sub> domain by using the same linker. These two V<sub>L</sub>-V<sub>H</sub> hybrids were cloned in tandem into one expression vector, resulting in only 6 residues (SRGGGS) in the anti-RBC ScFv, we constructed an anti-RBC diabody expression vector (Fig. 1, upper panel) and transformed it into <i>Escherichia coli</i> for production. As a hexahistidine tag was attached on the V<sub>H</sub> domain of the diabody, the diabody could be purified on an IMAC affinity column. To verify the existence of bivalent dimers, the affinity-purified diabody was further purified by size-exclusion chromatography. Two major fractions from size-exclusion chromatography were collected and tested by the RBC agglutination assay. The 52-kDa fraction (fraction B in Fig. 2) was the major fraction (approximately 75%) and contained 0.8 mg/ml protein, which could cause direct agglutination at a concentration of 100 ng/ml, indicating the bivalency of the anti-RBC diabody. As the Myc tag was attached on the diabody V<sub>L</sub> domain, the anti-Myc antibody could cross-link the anti-RBC diabody and enhance the diabody-mediated RBC agglutination. When the anti-Myc antibody was present, the agglutination observed was more pronounced and the agglutination formed more quickly (table on the right in Fig. 2). In contrast, the 27-kDa fraction (fraction A in Fig. 2) was the minor fraction (approximately 25%) and contained 0.25 mg/ml protein, which at a concentration of 200 ng/ml did not agglutinate RBCs when it was used alone or in combination with the anti-Myc antibody, suggesting that this fraction was unassembled V<sub>L</sub>-V<sub>H</sub>. The anti-RBC ScFv caused RBC agglutination only when it was bridged by the secondary anti-Myc antibody (Fig. 2). These results demonstrate the successful construction of the expression vector and presence of anti-RBC bivalent dimers in the diabody produced.

Construction and expression of bispecific diabody for agglutination assay. The anti-HBsAg ScFv was screened from another phage-displayed antibody library, which was constructed by using RNA isolated from peripheral blood monocytes of an HBsAg antibody-positive patient (29). In the expression vector of the bispecific diabody against both RBC and HBsAg, the N terminus of the anti-RBC V<sub>1</sub> domain was connected to the C terminus of the anti-HBsAg V<sub>L</sub> domain by using a 6-amino-acid linker, SRGGGS, to restrict the intrachain pairing of V<sub>L</sub> and V<sub>H</sub>. A second V<sub>L</sub>-V<sub>H</sub> crossover hybrid was constructed by connecting the N terminus of the anti-HBsAg V<sub>H</sub> domain to the C terminus of the anti-RBC V<sub>L</sub> domain by using the same linker. These two V<sub>L</sub>-V<sub>H</sub> hybrids were cloned in tandem into one expression vector, resulting in
a bispecific diabody expression vector (Fig. 1, lower panel). In this vector, each crossover chain was preceded by an OmpA signal sequence to direct the secretion of diabody into the Escherichia coli culture medium. The purified bispecific diabody was tested for its activity of binding to RBC and HBsAg by ELISA. As shown in Fig. 3, both anti-RBC and anti-HBsAg activities were detected from the bispecific diabody, whereas the parental ScFvs could bind to only one of the antigens.

To prove that both the anti-RBC- and the anti-HBsAg-binding sites were located on the same molecule of the bispecific diabody, we did an RBC agglutination test by incubating the diabody with RBCs in the absence of HBsAg or in the presence of different amounts of HBsAg. An HBsAg envelope particle containing many copies of HBsAg can act as a bridge between two or more bispecific diabodies and can agglutinate RBCs. As shown in Fig. 4, in the presence of HBsAg, the bispecific diabody did cause agglutination (wells 4 and 5), whereas no agglutination was observed in the well of monospecific ScFv against RBCs alone (well 2) or in combination with ScFv against HBsAg (well 1), indicating the bispecific nature of the diabody. The bispecific diabody could bind to both RBCs and HBsAg, whereas ScFv or monospecific diabody bound to only one of the antigens. In the absence of HBsAg, the bispecific diabody alone did not cause RBC agglutination (well 3), which ruled out the possibility that a small amount of monospecific bivalent anti-RBC diabody agglutinated RBCs.

On the basis of the observation that the agglutination of RBCs could occur only in the wells to which bispecific diabody and HBsAg were added, we developed an agglutination assay for the rapid detection of HBsAg in clinical blood specimens. The purified HBsAg was serially diluted into HBsAg-negative human blood for the agglutination assay, and the detection limit of the assay was 2 ng/ml HBsAg (data not shown), which was higher than that of the reference ELISA (0.2 ng/ml).

Detection of HBsAg in clinical blood specimens by the bispecific diabody-mediated agglutination assay. In clinics, especially in rural areas of China, glass slides are more commonly used than 96-well plates for agglutination assays, so we adapted the agglutination assay from 96-well plates to glass slides. On glass slides, the bispecific diabody did cause RBC agglutination in HBsAg-positive specimens but not in HBsAg-negative specimens, as observed with the naked eye and under a microscope (Fig. 5). The detection limit of the slide agglutination assay was the same as that of the assay performed in a 96-well plate (data not shown). Using the slide agglutination assay mediated by the bispecific diabody, we did two independent studies to detect HBsAg in clinical specimens. In the first study, 200 specimens were used: 100 HBsAg-positive specimens and 100 HBsAg-negative specimens, preidentified by ELISA in the clinical laboratories of the Peking University People’s Hospital. Among the 100 positive specimens, 98 specimens were positive by the agglutination assay (i.e., sensitivity of the agglutination assay, 98%), and 2 of the specimens that were not positive by the agglutination assay were weakly positive by our
ELISA (optical density at 490 nm, <0.3). All of the 100 ELISA-negative specimens were negative by the agglutination assay (Table 1).

In another study, 512 blood specimens were randomly selected from patients visiting the No. 302 Hospital (n = 312) and the Navy General Hospital (n = 200) for an HBsAg test. The results of the ELISA and the agglutination assay for the detection of HBsAg in these specimens were compared side by side. Among the 165 positive specimens identified by ELISA, 161 were positive by the agglutination assay (i.e., sensitivity, 97.6%), and all 347 ELISA-negative specimens were also negative by the agglutination assay. Again, those ELISA-positive and agglutination-negative specimens showed a weak signal in the ELISA.

In these two independent studies, the sensitivity and the specificity of the RBC agglutination assay were comparable. By combining the results of these two studies, the overall sensitivity of the agglutination assay is 97.7% and the specificity is 100% (Table 1).

**DISCUSSION**

The bispecific diabody against both RBC and HBsAg was constructed by using ScFvs panned from two phase-displayed antibody libraries. As many copies of HBsAg are present on a viral envelope particle, when one molecule of the bispecific diabody binds to both HBsAg and RBCs, the HBsAg-diabody complex cross-links many RBCs, and therefore, RBC agglutination can take place. On contrast, if the two binding sites of HBsAg and RBCs locate on separate molecules of antibody fragments, the agglutination would not occur. As shown in Fig. 4, the bispecific diabody could cause RBC agglutination by itself, whereas the combination of anti-RBC ScFv and anti-HBsAg ScFv did not cause agglutination, which demonstrated that the bispecific diabody bound to RBC and HBsAg simultaneously. This property of bispecific diabody was applied to the development of an agglutination assay for HBsAg detection.

The spread of hepatitis B is a serious health care problem worldwide, especially in Asian countries. The most commonly used diagnostic and blood screening marker sought is HBsAg. An individual positive for HBsAg is considered infected with hepatitis B virus and is therefore potentially infectious. Current routine diagnostic tests for HBsAg, such as ELISAs, are usually complex. ELISA procedures require sample preparation and several incubation and washing steps and is therefore time-consuming. Using the bispecific diabody against HBsAg and RBCs, we developed an autologous RBC agglutination assay to detect HBsAg in whole-blood specimens. In the study in which 712 clinical specimens were tested, the observed sensitivity was 97.7% and the specificity was 100%. Additionally, we found that the degree of agglutination was correlated to the titer of HBsAg present, and the time that agglutination occurred was dependent upon the concentration of HBsAg (data not shown). In the presence of the bispecific diabody, the specimens containing high concentrations of HBsAg agglutinated in as soon as 1 to 2 min and then stabilized, whereas those that contained low titers of HBsAg took longer to agglutinate, usually 5 to 10 min (data not shown). These findings make it possible to develop a semiquantitative agglutination assay if the necessary validation steps can be achieved.

Although the specificity of this bispecific diabody-mediated agglutination assay was comparable to that of ELISA, the detection limit of this test (2 ng/ml) was 10-fold higher than that of ELISA (0.2 ng/ml), which makes this agglutination assay less sensitive. In most patients with HBV infection, serum HBsAg levels range from 5 ng/ml to 600 μg/ml (12), which are above the detection limit of the agglutination assay. However, in patients with chronic hepatitis B virus infection, the serum HBsAg level could be too low to be detected by the agglutination assay. Therefore, this whole-blood agglutination test is suitable only for primary screening. The agglutination assay requires no special equipment and only a small volume of blood specimen (20 to 50 μl). The simple drop-and-mix steps usually allow the results to be read in 10 min. For these reasons it is considered an ideal assay for use in areas that lack trained technicians or complex equipment, such as remote areas in developing countries.

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