Short Communication

Antibodies to Bovine Serum Albumin in Human Sera: Problems and Solutions with Casein-Based ELISA in the Detection of Natural Japanese Encephalitis Virus Infections

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SUMMARY: An ELISA system for measuring antibodies to nonstructural protein 1 (NS1) of Japanese encephalitis virus has already been established. This system uses an ELISA diluent containing casein, instead of bovine serum albumin (BSA). During a survey, we found that 21 (21%) of 102 children aged 1–5 years, who had no history of Japanese encephalitis vaccination and were without detectable neutralizing antibodies, showed positive results with this ELISA system. Western blotting analysis showed that sera from 19 (91%) of these 21 subjects had antibodies to BSA, but not NS1. These sera reacted with BSA antigen remaining in immunoaffinity-purified NS1 antigen. One solution to this problem was to reduce the BSA level to ≤1% of the NS1 amount. Another was to use a control well sensitized with BSA with the same amount as that contained in the NS1 antigen preparation.

Japanese encephalitis virus (JEV) of the genus Flavivirus in the family Flaviviridae, is the leading cause of viral encephalitis in Asia (1). While Japanese encephalitis (JE) is vaccine preventable (2), the recommendation for JE vaccination in Japan was suspended in 2005 (3,4). The significance of a vaccination program is a topic that has generated much debate. This is because the current low JE incidence may also be attributable to the decreased number of vector mosquitoes along with the relocation of pig farms further away from residential areas. However, the natural infection rate in humans is a critical factor for evaluating any need for continuous vaccination.

Antibodies to JEV nonstructural protein 1 (NS1) constitute a marker of natural infection among populations vaccinated with an inactivated JE vaccine (5). Although ELISA is a serological method suited for antibody surveys, nonspecific reactions occurring in human sera have hampered the reliable measurement of low-level NS1 antibodies induced in subclinically infected humans. We have found that the nonspecific reactions occurring in a system using an ELISA diluent containing bovine serum albumin (BSA) were minimized when casein-based diluent was used (6).

The ELISA protocol has been described (6). The only characteristic feature compared with those of a conventional ELISA scheme was the ELISA diluent, which was composed of 0.05 M Tris-HCl (pH 8.0) containing 0.2% casein, 0.05% Tween 20, 1 mM EDTA, and 0.15 M NaCl. Briefly, microplates sensitized with purified NS1 antigens at 10 ng/well were incubated serially with test sera and alkaline phosphatase-conjugated goat anti-human IgG. Both were prepared in the ELISA diluent. The NS1 antigen used for sensitization was immunoaffinity-purified from culture fluids of NS1-expressing cells using a monoclonal antibody specific for NS1. Nonsensitized control wells were run in parallel and differences in absorbance from antigen-sensitized wells were regarded as NS1-specific reactions.

During an NS1 antibody survey using sera collected in Kumamoto Prefecture from 2004 to 2008 (7), we noticed that a large population (21%, 21 of 102) of children aged 1–5 years, who had no vaccination history and no detectable neutralizing antibodies, showed positive results in our ELISA for measuring NS1 antibodies. Although NS1 antibodies detected by our ELISA do not necessarily correlate with E antibodies detected by the neutralization test, we could find no specific reasons to explain this high prevalence of NS1 antibodies.

These 21 samples were then analyzed on Western blots. Since the antigen lot used for the ELISA that produced the above results (positive for NS1 antibodies) had run out by the time of the presentation study, a different lot was used as the antigen for this analysis. Of these, 19 (91%) showed a visible band at the molecular weight equivalent to that of BSA but did not show any bands corresponding to NS1. Three examples are shown in Fig. 1A. To confirm the reaction with BSA, we examined these samples for reactivity against varying amounts of BSA sensitized on an ELISA plate (Fig. 1B). The reaction was found to depend on the amount of BSA. These results indicated that some sera contained antibodies to BSA that may have caused false-positive results in the ELISA measuring NS1 antibodies. BSA antibodies and their effects on serological tests have

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been reported in healthy human sera, particularly from infants, and are probably induced by dietary proteins (8,9).

The above analysis also showed the presence of BSA in this lot of the affinity-purified NS1 preparation. A direct ELISA using horseradish peroxidase-conjugated rabbit anti-BSA (Alpha Diagnostic International, San Antonio, Tex., USA) measured 1.25 ng of BSA in 10 ng of this NS1 antigen lot, corresponding to one plate well. It appeared that the immunoaffinity-purification strategy cannot always eliminate the BSA originally contained in the maintenance medium of NS1-expressing cells at a concentration of 0.075% (750 μg/ml). Therefore, minimizing the amount of BSA remaining in the NS1 antigen preparation appears to be a solution to the problem occurring in casein-based ELISA. The above dose-response curve (Fig. 1B) showed that the reaction was undetectable in an amount of BSA of ≤1 ng/ml (≤0.1 ng/well), indicating that the effect of BSA on the present ELISA was minimized at ≤1% of the NS1 antigen (10 ng/well).

Attempts to minimize the relative amount of BSA by repeating immunoaffinity purification steps failed: a high BSA:NS1 ratio remained and there was a considerable loss of NS1 antigen (Fig. 1C). The most effective method to reduce the amount of BSA antigen was absorption with rabbit anti-BSA (Alpha Diagnostic Inter-

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Fig. 1. Reaction between BSA antibodies in human sera and BSA antigen contained in a purified NS1 antigen preparation. (A) Western blotting analyses of sera of children with no history of JE vaccination and with no detectable neutralizing antibodies. Analyses were performed essentially as previously described (6). Briefly, an immunoaffinity-purified NS1 antigen was run on standard Laemmli gels under nonreducing conditions and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with test specimens and alkaline phosphatase-conjugated anti-human IgG. Test sera were collected in Kumamoto Prefecture (7): sera #2 (3-year-old female), #3 (4-year-old female), and #4 (5-year-old female) determined as negative (−) for neutralizing antibodies (Neut Ab) and positive (+) for NS1 antibodies (NS1 Ab) in our ELISA. As a reference, sera were silver-stained or blots were reacted with mouse monoclonal JE-6H4 (MAb) or sera from a JE patient and a subclinically infected human (#1). (B) Dose-dependent ELISA reactivities of human sera against varying concentrations of BSA used for sensitization of the plate. Samples were sera #2 (open circle), #3 (closed circle), and #4 (closed triangle). (C) Purification process and NS1 antigen purity. The antigen concentration of immunoaffinity purification was expressed as an average of the first four eluates. The “Second-round affinity” indicates that the eluates obtained from the “First-round affinity” process were repeatedly affinity-purified by the same procedure. “Absorption” indicates that the “First-round affinity” sample was incubated with rabbit anti-BSA coupled to Sepharose 4B beads at 0°C for 2 h. The latter two procedures were performed with a portion of the “First-round affinity” sample; with the antigen amounts obtained adjusted to those obtained after the “First-round affinity” process. (D) Effect of the use of BSA-sensitized wells on NS1 antibody levels. Using sera with (10 samples; closed circles) or without (6 samples; open circles) a band corresponding to BSA on Western blots, NS1 antibody levels obtained using BSA-sensitized wells were compared with the original NS1 antibody levels obtained using nonsensitized wells.
national) coupled to Sepharose 4B beads (NHS-activated Sepharose 4B Fast Flow; GE Healthcare UK, Buckinghamshire, England). Specifically, the absorption reduced the amount of BSA to \( \leq 1\% \) of NS1 without loss of the NS1 antigen (Fig. 1C).

Another way to resolve the issue was the use of an equivalent amount of BSA in a nonsensitized control. Subtraction of the absorbances obtained with nonsensitized control wells from those obtained with antigensensitized wells is considered in theory, and is often used to reduce nonspecific reactions occurring in ELISA. Thus, we sensitized wells with BSA with the same amount contained in the purified NS1 antigen preparation (1.25 ng per well) and compared the results with those obtained using truly nonsensitized wells prepared by incubation only with ELISA coating buffer. As shown in Fig. 1D, 10 sera that were positive for BSA antibodies on Western blots turned negative for NS1 antibodies in ELISA when BSA-sensitized wells were used, whereas six NS1-positive sera that were negative for BSA antibodies on Western blots remained positive for NS1 antibodies.

In conclusion, the present study showed that BSA antibodies, which were found in a relatively large population of children, were the cause of false-positive results in our ELISA using casein-based diluent. However, the present study was able to minimize the effect of BSA antibodies by reducing the BSA level to \( \leq 1\% \) in the NS1 antigen preparation, and achieved the same by using an equivalent amount of BSA in nonsensitized control wells. Although the use of medium without BSA would be a theoretical solution, some serum samples that were originally negative became positive when NS1 antigens prepared using serum-free or casein-containing medium were used.

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