JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1997, p. 609–613 Vol. 35, No. 3
0095-1137/97/$04.00+0
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Comparison of the Western Blot Assay with the Neutralizing-Antibody and Enzyme-Linked Immunosorbent Assays for Measuring Antibody to Verocytotoxin 1

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Received 20 September 1996/Returned for modification 15 November 1996/Accepted 13 December 1996

A Western blot (immunoblot) assay (WBA) was developed to detect immunoglobulin G (IgG) antibodies against *Escherichia coli* Verocytotoxin 1 (VT1) by using a chemiluminescence detection system. The assay was compared with a VT1-neutralizing-antibody (VT1-NAb) assay and an anti-VT1 IgG enzyme-linked immunosorbent assay (ELISA). When four human serum samples that were known to be positive by VT1-NAb assay and ELISA were titrated to the endpoint by the three assays, the WBA gave endpoint titers that were up to 8-fold higher than those by ELISA and up to 256-fold higher than those by the VT1-NAb assay. Of 32 serum samples that were known to be positive by VT1-NAb assay and ELISA, 31 (97%) were positive by WBA; the one sample with a discrepant result gave borderline results by the VT1-NAb assay and ELISA. Of 52 serum samples that were known to be negative by the VT1-NAb assay and ELISA, 50 (96%) were negative and 2 (4%) were positive by WBA. Of 44 serum samples that gave discrepant results by the VT1-NAb assay and ELISA, neither of the latter correlated with the results of WBA. In an investigation of 19 pairs of acute- and convalescent-phase serum samples from patients with hemolytic-uremic syndrome, 10 pairs that were positive by the VT1-NAb assay were also WBA positive, while 9 pairs that were NAb negative were also WBA negative. The WBA is inherently more specific and sensitive than either the NAb assay or the ELISA and may be used as a "gold standard" to detect IgG antibodies to VT1. Like the NAb assay and the ELISA for detecting antibodies to VT1, the WBA has little to offer in the diagnostic setting but is expected to play an important role in seroepidemiological studies.

Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) (18, 19), also referred to as Shiga-like toxin (SLT)-producing *E. coli* (23, 24), is a major food-borne cause of gastroenteritis (11, 14) that may be complicated by two serious and potentially life-threatening conditions, namely, hemorrhagic colitis (31) and the hemolytic-uremic syndrome (HUS) (15, 17). Investigations to date suggest that the latter result from systemic VT-mediated damage to vascular endothelial cells in the kidneys, gastrointestinal tract, and other organs and tissues (26, 29, 30).

VTs are a family of protein toxins which share a polypeptide subunit structure consisting of an enzymatically active A subunit (~32 kDa) that is linked to a pentamer of B (binding) subunits (~7.5 kDa) (25). The holotoxin binds to the glycolipid globotriaosylceramide (Gb3) (21, 22) on the eukaryotic target cell surface and is internalized by the process of receptor-mediated endocytosis (34). The A subunit is proteolytically nicked to an enzymatically active A_1 fragment (~27.5 kDa) which acts on the 60S ribosomal subunit to inhibit protein synthesis (5, 25).

Human VTEC strains elaborate at least three serologically distinct toxins, either alone or in combination. These three toxins are VT1 [SLT I; type strain C600(H19J)], VT2 [SLT II; type strain C600(933w), and VT2c [SLT IIc; type strains E32511; B2F1; 7279] (5, 23, 25, 38).

Knowledge about the nature and measurement of the antitoxic immune response following VTEC infection and its relationship to protective immunity is limited. The peak age incidence of VTEC infection and of HUS in young children (14, 33) suggests that infection in childhood may be associated with an age-related increase in immunity, possibly to VTs, but this has yet to be demonstrated. However, anti-VT1 seropositivity has been correlated with the absence of symptoms in family outbreaks of VTEC infection (16). Furthermore, immunization of laboratory animals with VT1 or the Shiga toxin B subunit protects them against systemic challenge with VT1 or Shiga toxin (2, 4, 12). If immunization with toxoid prevent human disease is to be considered, assays that accurately measure the presence or absence of antitoxic antibodies in human sera will be essential for identifying immune individuals or susceptible populations who might benefit from immunization. Unfortunately, current approaches for measuring antibodies to VT have limitations. The original approach for detecting anti-VT antibody was the serum VT neutralization assay in cell cultures (15, 17). Up to about 10% of healthy individuals were shown to have VT1-neutralizing antibody (VT1-NAb), and this correlated with the presence of anti-VT1 IgG measured by enzyme-linked immunosorbent assay (ELISA) (16). In contrast, virtually all individuals have serum neutralizing activity against VT2 (7). It was further shown that the VT2-neutralizing activity in serum is due not to specific antibody but rather to a component of the serum high-density lipoprotein fraction whose clinical significance is uncertain (7). The nonspecificity of the NAb assay is emphasized by the fact that it measures only the neutralization of biological activity and not the presence of serum immunoglobulins. Furthermore, it is tedious to perform and difficult to standardize the NAb assay.

To overcome the limitations of the NAb assay, ELISAs to

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detect immunoglobulin G (IgG) antibodies to VT1 have been developed (1, 16). Theoretical limitations in the sensitivity of the NAb assay influence the sensitivity and specificity of the ELISA (16) since the latter was developed by using the VT1-NAb assay as a "gold standard." Furthermore, we have reported and continue to find (unpublished data) that up to 5% of samples tested in parallel by the NAb assay and the anti-VT1 IgG ELISA (ELISA) give discrepant results (16).

A further concern with the VT1-NAb assay and the ELISA is that only a relatively small proportion of patients infected with VTEC isolates that express VT1 develop detectable VT1-NAb or anti-VT1 IgG, as measured by ELISA or by VT1-NAb assay (1, 3, 6, 16). The reason for this is unclear, and several possibilities have been raised to explain it, including immunosuppression arising from the sensitivity of B lymphocytes to VT in vitro (9, 20). Given the very high biological activity of VT1, it may be argued that the disease-eliciting dose of VT is insufficiently antigenic to evoke an easily detectable antibody response by conventional methods (28), a situation analogous to that for tetanus (37). If this were the case, one would expect that populations with higher levels of exposure to VTEC, such as dairy-farm families, would have a higher frequency of seropositivity (due to booster antibody responses) than those with a lower frequency of exposure, such as urban populations. This hypothesis was confirmed by our finding that about 42% of residents of dairy farms have detectable VT1-NAb, in contrast to a frequency of VT1-NAb of about 7% in urban residents (28, 40).

The objective of the present study was to explore the utility of a Western blot (immunoblot) assay (WBA) for enhancing the sensitivity of anti-VT1 IgG detection. This report describes a WBA with chemiluminescence to detect antibodies to VT1 and reports on its performance in comparison to those of the VT1-NAb assay and the ELISA.

MATERIALS AND METHODS

Sera. The sera used in the study comprised (i) sera from two rabbits (one preimmunization and one postimmunization with VT1) to establish the specific reactivity of the WBA; (ii) four human serum samples from our collection that were known to be positive for VT1-NAb and for anti-VT1 IgG by ELISA; these were used in serial twofold dilutions to the titration endpoints to compare the relative sensitivities of the NAb assay, the ELISA, and the WBA; (iii) 128 human serum samples, including those described for groups (ii) (see above) and iv (see below), were tested for NAb endpoint titers and, at a 1:100 dilution, for anti-VT1 IgG by ELISA and by WBA (the sera were divided into the following four groups according to the NAb and ELISA results: group I, 32 serum samples positive by VT1-NAb and ELISA; group II, 52 serum samples negative by NAb assay and ELISA; group III, 38 serum samples positive by the NAb assay but negative by ELISA; and group IV, 6 serum samples negative by the NAb assay but positive by ELISA); and (iv) 19 pairs of serum specimens (acute and convalescent phase) from patients with HUS associated with infection by VTEC expressing VT1.

VT1-NAb assay and ELISA. The VT1-NAb assay and ELISA were conducted as described previously (16). Activity of 1 CD_{50} was defined as the amount of VT activity required to produce a 50% cytopathic effect at 3 days of incubation by using a tissue culture assay system with a Vero cell line (VERO 76; American Type Culture Collection). The endpoint was taken as the highest serum dilution causing inhibition of the cytotoxic effect (1 CD_{50}) at 3 days. VT1-NAb titers of $<$1:4 were considered a negative result (16).

WBA to detect IgG to VT1. The WBA was adapted from the method of Towbin et al. (39). VT1 was purified as described previously (27, 30) from recombinant strain JB28 (kindly provided by J. Brunton). The purified toxin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using 9% stacking and 15% separating gels. The proteins were transferred from the gel onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, Calif.) for 1 h by using a current of 0.15-0.20 A. Each membrane was cut into longitudinal strips. One strip was stained with Coomassie blue to confirm protein transfer. All steps of the WBA were conducted with shaking at room temperature, with Tris-buffered saline (TBS; Tris, 50 mM [pH 7.4]; Bio-Rad Laboratories) used as the wash solution. Strips were blocked for nonspecific binding by incubation with a solution consisting of TBS–5% skim milk and 10% goat serum for 1.5 h; they were then washed three times with TBS and incubated for 1 h with test sera diluted 1:100 in TBS–1% skim milk and 2% goat serum. After washing three times, the strips were incubated with a 1:10,000 solution of horseradish peroxidase-labelled goat anti-human IgG (heavy- and light-chain specificity; Bio-Rad Laboratories). For detection, the enhanced chemiluminescence technique (Amersham, Little Chalfont, England), which is based on the oxidation of luminal by the horseradish peroxidase in the presence of a chemical enhancer (phenols), was performed with a commercially available system. The light emission was detected by autoradiography (X-OMAT AR film; Eastman Kodak Company, Rochester, N.Y.). Sera that demonstrated specific antibody binding to at least one of the A, A_1, or B subunits were considered to be positive, whereas sera that showed a complete absence of binding were considered negative. The results of the WBA were read in a blinded fashion.

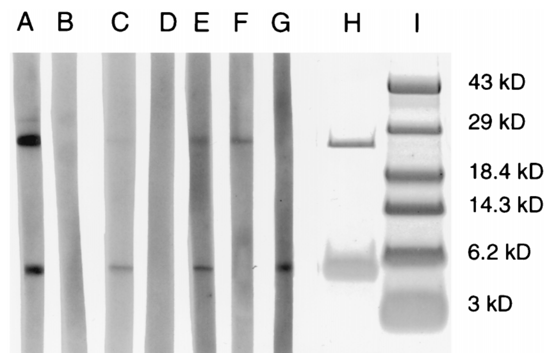

FIG. 1. Western immunoblots of rabbit and human sera against VT1. Lanes A to G, anti-VT1 Western immunoblots with immune rabbit serum (Lane A; sera reactive against the VT1 A, A_1, and B subunits), preimmune rabbit serum (lane B; nonreactive), positive control human serum (lane C; reactive against VT1 A1 [weak] and B subunits), negative control human serum (lane D; nonreactive); positive human sera (lane E, reactive to the A_1 and B subunits; lane F, reactive to the A_1 subunit only; lane G, reactive to the B subunit only); lane H, SDS-PAGE of VT1 showing the A_1 and B bands visualized after Coomassie blue staining. Note that whereas the predicted mass of the B subunit, on the basis of sequencing data, is 7.7 kDa (35), the mass of the B subunit, by SDS-PAGE either as part of the VT1 holotoxin or as the isolated peptide (13), by SDS-PAGE has been lower than 6 kDa; lane I, low-molecular-weight protein standards (GIBCO BRL).

RESULTS

The purified VT1 that we used resolved into bands by SDS-PAGE consistent with the A_1 fragment and the B subunit (Fig. 1, lane H); the A band was usually not visualized on the SDS-polyacrylamide gel. After transfer, the A_1 and B bands were detectable by Coomassie blue staining and by chemiluminescence after immunoblotting. Reactive human sera showed antibody binding to either the A_1 subunit alone (Fig. 1, lane F), the B subunit alone (Fig. 1, lane G), or to both subunits (Fig. 1, lanes C [weak reactivity] and E). Even though it was not visualized by SDS-PAGE, the A fragment was detectable after immunoblotting with the immune rabbit sera (Fig. 1, lane A) but not after immunoblotting with the human sera (Fig. 1, lanes C, E, and F). The specific reactivity of the WBA was confirmed with rabbit sera obtained preimmunization with VT1 (Fig. 1, lane B; no signal) and postimmunization (Fig. 1, lane A; positive signals for A, A_1, and B subunits against VT1).

Relative sensitivities of the VT1-NAb assay, ELISA, and anti-VT1 WBA by using endpoint titration. Four human serum samples (known to be positive by NAb assay and ELISA) were titrated to the endpoint by the three assays by using serial twofold dilutions (Table 1). The WBA gave endpoint titers that were from zero to 8-fold higher than the ELISA titers and between 64- and 256-fold higher than the NAb assay titers. The ELISA gave titers that were from 8- to 256-fold higher than the NAb assay titers.

Detection of anti-VT1 IgG by WBA in different groups of human sera selected on the basis of their reactivities by the VT1-NAb assay and ELISA. Thirty-one (97%) of 32 serum samples that were positive by the NAb assay and ELISA

TABLE 1. Endpoint titers of four human serum samples by VT1-NAb, ELISA, and anti-VT1 IgG WBAs

Serum sample no.	NAb assay titer	ELISA titer	WBA titer
1	4	1,024	1,024
2	16	256	1,024
3	32	512	4,096
4	64	512	4,096

(group I) were positive by WBA (Table 2). The one discrepant serum sample had a VT1-NAb titer of 8 and an ELISA value of 0.22, which is barely above our breakpoint ELISA value of 0.21. Of the 52 serum samples that were negative by the NAb assay and ELISA (group II), 50 (96%) were negative by WBA. Thus, for sera which gave positive or negative results by both the NAb assay and the ELISA, there was an excellent correlation between the results of either of these tests and the WBA. On the other hand when the results of the NAb assay and the ELISA did not agree (groups III and IV; Table 2), the correlation of the results of either test with the result of WBA was poor. This was most notable for group IV serum specimens, where only one of six ELISA-positive specimens correlated with a positive WBA result.

In total, 51 serum samples were positive by WBA, of which 26 (51%) were positive for antibody to both the A_1 and B subunits, 15 (29%) were positive only for antibody to the A_1 fragment, and 10 (20%) were positive only for antibody to the B subunit.

Use of WBA to detect VT1 IgG in acute- and convalescent-phase sera of patients with HUS. We tested acute- and convalescent-phase sera from 19 patients with HUS associated with VTEC strains that produced either VT1 alone or VT1 and VT2. All 10 pairs of serum samples that were positive by the NAb assay were also positive by WBA, and all nine pairs that were negative by the NAb assay were also negative by the WBA (100% concordance). The ELISA results correlated with the results of the WBA and the NAb assay for all except one serum samples, which was positive by the WBA and the NAb assay but negative by ELISA.

DISCUSSION

Our findings indicate that the WBA is more sensitive and specific than the NAb assay and the ELISA. It should be noted that the antibodies measured by the different assays may not be the same. Whereas the WBA and ELISA detect specific antibodies that react with epitopes on VT1, the NAb assay only measures neutralization of biological activity. This makes the NAb assay inherently less specific than the other two assays.

Western blotting has been used empirically by some investigators to look for antibodies to VT1 and VT2, but it has not been compared directly to the NAb assay and ELISA for sensitivity. Greatorex and Thorne (10) developed an anti-VT1 and anti-VT2 WBA using a serum dilution of 1:1,000 and 4-chloro-1-naphthol solution as the substrate. They tested 24 serum samples from patients with HUS for VT1 IgG antibodies: 5 were positive by WBA and ELISA and 2 were positive only by ELISA. Chart et al. (8) developed a VT1 and VT2 WBA using ^{125}I-labelled IgG and IgM. They tested 60 serum samples (from 30 patients with proven O157-associated HUS and from 30 healthy controls), but none of them was found to be positive for either anti-VT1 or anti-VT2 antibodies.

We used four human serum samples to obtain titration endpoints by the three assays and showed that the WBA was significantly more sensitive for antibody detection than both the NAb assay and the ELISA. The WBA gave endpoint titers that were up to 8-fold higher than the ELISA titers and up to 256-fold than the NAb titers. Thus, the WBA may be used legitimately as the gold standard for assaying serum antibodies to VT.

When the performance of our WBA was compared to those of our NAb assay and ELISA with different groups of sera, we found that the WBA correlated very well with the last two assays when both gave concordant results (Table 2, groups I and II). In practice, 95% or more sera give a concordant result in the ELISA and the NAb test (16). Sera with discrepant results, which usually account for less than 5% of sera, were overrepresented in this study (Table 2, groups III and IV) in order to better understand the basis for the discrepancies. Thus, with sera in groups III and IV (Table 2), which represented instances in which the ELISA and the NAb test results did not agree, the correlation of either test with the WBA was poor. For 38 serum samples that were positive by the NAb assay and negative by ELISA (Table 2, group III), only 17 (45%) were positive by WBA. This implies (assuming WBA to be the gold standard) that as many as 21 (55%) serum samples in group III gave a false-positive result in the NAb assay, possibly due to the presence of nonspecific neutralization factors (7). On the other hand, in group IV, among 6 serum samples that were negative by the NAb assay but positive by ELISA, five were WBA negative, implying that the ELISA results were false positives.

The criterion for WBA positivity used in this study was serum reactivity to either the A_1 or the B subunit, or both. Of the 51 samples that were positive by WBA, 26 (51%) were positive for antibody to both the A_1 and the B subunits, 15 (29%) were positive only for antibody to the A_1 fragment, and 10 (20%) were positive only for antibody to the B subunit. The significance of reactivity to both subunits as opposed to reactivity to either the A_1 or the B subunit is not known and requires further study. Neutralizing activity in sera from experimental animals immunized with VT1 is generally directed toward the B subunit (14, 25). In analysis of sera in groups I and II (Table 2), in which a correlation between the presence of NAb and anti VT1-IgG reactivity to the B subunit would be expected on theoretical grounds, of 31 NAb-positive samples, only 23 (74%) showed reactivity to the B subunit, while of 52 NAb-negative samples, 50 (96%) were negative for reactivity to the B subunit. This indicates that in human sera a negative test for NAb correlates well with a negative anti-VT1 IgG reactivity for the B subunit, whereas a positive test for NAb correlates with anti-VT1-IgG reactivity for the B subunit in about three-quarters of the samples. The basis for this discrepancy is not known and requires further study. It may reflect nonspecific neutralizing activity or the neutralizing activity of another antibody such as IgA.

Given the increased sensitivity of the WBA over those of the

TABLE 2. Anti-VT1 IgG WBA results for 128 human serum samples by groups

Group	No. of serum samples	Selection criteria		WBA results (no. of samples)	
		VT1-NAb	VT1-ELISA	Positive	Negative
I	32	+	+	31	1
II	52	−	−	2	50
III	38	+	−	17	21
IV	6	−	+	1	5

NAb assay and the ELISA, we speculated that the WBA should allow for the detection of antibody in some samples that were nonreactive by the NAb assay and the ELISA (Table 2, group II). This hypothesis was confirmed in that the WBA detected antibody in 2 (4%) of 52 serum samples that were negative by both the NAb assay and the ELISA.

Acute- and convalescent-phase sera from 19 patients with known VTEC infections (expressing either VT1 alone or VT1 plus VT2) were investigated to determine whether the WBA could detect antibody in sera that were negative by the NAb assay and ELISA. Sera from 9 of these 19 patients which were negative for antibody by the NAb assay and ELISA remained negative by the WBA. Thus, despite its demonstrated higher sensitivity over those of the NAb assay and the ELISA, the WBA was unable to detect a putative primary response to VT in sera that were negative by the other two assays. The significance of this observation in relationship to immunity remains unclear. However, there are at least two reports of patients with a second VTEC O157-associated illness (32, 36). This indicates that a primary VTEC infection may not evoke sufficient protective antitoxic immunity against a subsequent VTEC infection. This is analogous to tetanus, in which a primary infection does not usually lead to protection against a subsequent challenge (37). Thus, further studies may show that a negative WBA result for anti-VT antibodies is a valid indicator of susceptibility to a primary or a secondary VT-associated illness.

In conclusion, this study has demonstrated that the anti-VT1-WBA is inherently more sensitive and specific than the VT1-NAb assay and ELISA. It should thus be considered the gold standard for the detection of antibodies to VT1. The WBA may be expected to be very useful in seroepidemiological studies to identify populations that are susceptible to systemic VT illness and that would thus be candidates for vaccination with toxoid if such a vaccine were available. On the other hand, like the NAb assay and the ELISA, the WBA has not proven to be a suitable assay for diagnosing a recent VTEC-associated illness.

ACKNOWLEDGMENTS

This work was supported by a Medical Research Council of Canada grant (grant PG11123) and a grant from McDonald's Restaurants of Canada.

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