Detection of Breda Virus Antigen and Antibody in Humans and Animals by Enzyme Immunoassay

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Enzyme immunoassays were developed for the detection of Breda virus antibody and antigen. Cattle sera collected in the United Kingdom were found to have a high prevalence of antibody (55%) to Breda virus when examined in a competitive enzyme-linked immunosorbent assay. A low prevalence of antibody was found in pigs (2.2%), and no antibody was found in sheep or goat sera. No antibody to either Breda virus or Berne virus was detected in human sera collected from veterinarians and farm workers. Only 1 of 430 human fecal specimens (0.2%) contained Breda virus antigen detectable by enzyme-linked immunosorbent assay.

Breda virus was first isolated from diarrheic calves in the United States in 1982 (9). It was shown to be infectious by mouth, causing diarrhea in gnotobiotic and conventionally reared calves. Two serogroups of Breda virus have been proposed, although they have yet to be grown in tissue culture. Berne virus is antigenically and morphologically related to Breda virus, which was isolated from a rectal swab taken from a horse in Switzerland in 1973 and which can be grown in tissue culture. In 1984, Horzinek et al. proposed that Berne virus should be the type strain of a new family of viruses called the Toroviridae, which are characterized by their unique morphology by electron microscopy (EM), polypeptide profile on sodium dodecyl sulfate-acrylamide gels, and genome organization (4, 5).

We have reported previously the existence of Breda viruslike particles in stool samples from children (1, 2). We were able to show morphological similarities to Breda virus and a serological cross-reaction by immune EM with a hyperimmune serum raised against Breda II. However, since direct EM has not proved to be a sensitive technique for the detection of these viruses in humans, we have developed an enzyme-linked immunosorbent assay (ELISA) for Breda virus antigen by using sera raised against Breda II virus to look for further evidence of torovirus infection in humans.

Antibodies to toroviruses are widespread in animals. A high prevalence of neutralizing antibody to Berne virus has been found in cattle and horses, and a much lower prevalence has been found in rabbits and rodents (7). A high prevalence of antibody to Breda virus has been found in cattle in the United States by hemagglutination (HA) inhibition (HAI) (9) and indirect ELISA (8, 10). We have developed a competitive ELISA to investigate the prevalence of antibody to Breda virus in animals and humans.

MATERIALS AND METHODS

Virus. The Breda virus preparations were a gift from G. Woode and were received as samples of calf feces. The virus was purified for use in the competitive ELISA as previously described (1). Briefly, clarified 10% (vol/vol) fecal extracts were precipitated with an equal volume of saturated ammonium sulfate. After centrifugation, the precipitate was suspended in and dialyzed against 0.01 M Tris hydrochloride, pH 7.2, with 0.001 M EDTA and 0.15 M NaCl (TES buffer) for 18 h. The virus was further purified by centrifugation at 100,000 × g for 2 h through a preformed linear sucrose gradient consisting of 15 to 60% (wt/wt) solutions of sucrose in TES. The peak fractions by ELISA were pooled, dialyzed as before against TES, and diluted in phosphate-buffered saline (PBS), pH 7.2, containing 0.2% Tween 20 (PBST) with 1% bovine serum albumin for use in the competitive ELISA.

Antiserum. A hyperimmune antiserum (GC76) to Breda II virus was provided by G. Woode. It was produced by intramuscular inoculation of a previously infected gnotobiotic calf with Breda II virus purified from the feces of the calf. The hyperimmune guinea pig serum (GP475) was produced in this laboratory by intramuscular inoculation with the virus-containing gradient fractions from the purification procedure described above. The peak fractions were mixed with an equal volume of Freund incomplete adjuvant. Two inoculations were given 1 month apart. The calf immunoglobulin was purified by precipitation in 40% ammonium sulfate and biotinylated for use in the ELISA by a method previously described (11).

HAI tests. HA and HAI tests were performed as previously described (1).

Berne neutralization test. Sera were tested for neutralizing activity against Berne virus grown in embryonic mule skin (EMS) cells grown in microtiter plates. Serial dilutions of serum were incubated with 100 TCD50 of Berne virus for 1 h at 37°C before the addition of 50 μl of EMS cells to each well. The cells were observed for cytopathic effect for 1 to 5 days.

ELISAs. Breda virus antigens were detected in a simple sandwich assay. Irradiated polystyrene microtiter plates (Nunc model 239454) were coated with a 1/500 dilution of GP475 for 2 h at 37°C in 0.1 M carbonate-bicarbonate buffer, pH 9.6. After being washed in PBST, the test specimen (usually a 10% fecal suspension in PBS) was added and kept overnight at 4°C. The presence of virus was determined by developing the test with a 1/300 dilution of biotinylated detecting serum (GC76), followed by a 1/200 dilution of avidin peroxidase at 37°C for 1 h and then tetramethyl benzidine in citrate-acetate buffer, pH 6.2.

Sera were tested for the presence of antibody to Breda virus by a competitive enzyme immunoassay; this was a

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modification of the standard enzyme immunoassay method (12). The wells of an irradiated polystyrene microtiter plate (Nunc model 239454) were coated with a 1/1,000 dilution of Gp475 in 0.1 M carbonate-bicarbonate buffer, pH 9.6. After incubation at 37°C for 2 h, unbound antibody was removed by washing the plates in PBST. The serum to be tested was then added to the plate; 20 μl of a 1/32 dilution of serum was used as the screening dilution. A 1/65 dilution of purified Breda II virus (80 μl) was then added to the serum in the plate, and this serum-antigen mixture was kept for 18 h at 4°C. The optimum dilutions of coating serum, antigen, and test serum were determined by chessboard titration. The Breda virus antigen used in the test was a dilution of the sucrose density gradient-purified virus made up in PBST–1% bovine serum albumin. After the plates were washed, the amount of virus binding was measured by the reaction of the wells with a 1/300 dilution of biotinylated calf serum (GC76), followed by a 1/200 dilution of avidin peroxidase and then tetramethyl benzidine substrate. With this method, the presence of Breda virus-specific antibody was demonstrated by the inhibition of binding of the biotinylated anti-Breda virus antiserum. For the purpose of this study, a serum was considered to contain Breda virus-specific antibody if it produced a greater than 60% inhibition of binding, when compared with a known positive convalescent-calf serum (GC21) and a known negative serum (GC5). Sera generating inhibitions between 40 and 60% were considered equivocal, and those producing less than 40% inhibition were considered negative. Serum titrations were done by testing doubling dilutions of serum in the test. The titer of a serum was considered to be the highest dilution giving greater than 50% inhibition of binding. All results were read spectrophotometrically at 450 nm.

**Human fecal specimens.** A total of 400 10% fecal extracts were examined. These were routine specimens received at the Regional Virus Laboratory for the investigation of diarrhea between January and April 1986. Most of these samples were from children younger than 5 years. Thirty human fecal specimens were sent to the Regional Virus Laboratory because they contained rotavirus-like particles on examination by EM.

**Sera examined.** The specificity of this ELISA for Breda virus-specific antibody was assessed by examining well-documented gnotobiotic calf sera in the test. Sera from convalescent gnotobiotic calves infected with Breda I virus (SB219), Breda II virus (GC21), and rotavirus (GC5) were given by G. Woode.

A total of 209 sera from five groups were tested for antibodies to Breda virus: (i) 92 sera collected in the United Kingdom in 1983 and 1984 from veterinarians, veterinary students, and farm workers; (ii) 10 sera collected from 6 herds of goats, all less than 2 years old; (iii) 11 sera collected from 6 herds of sheep between 1 and 4 years old; (iv) 51 sera collected in 1983 from 12 herds of dairy cattle, aged between 7 months and 12 years; and (v) 45 sera collected in 1983 from 20 herds of pigs.

**RESULTS**

**Specificity and sensitivity of ELISA for antigen detection.** The specificity and sensitivity of the ELISA for antigen detection were assessed by examining Breda I and Breda II virus preparations in the assay and by comparing the sensitivity of the ELISA with that of HA. The results are shown in Fig. 1. The assay was more sensitive for Breda II virus than for Breda I virus; 8 HA U of Breda II virus and 16 HA U of Breda I virus gave an optical density at 450 nm (OD450) of >0.1.

The specificity of the assay was assessed by examining fractions from a sucrose density purification of Breda II virus preparation. The results are shown in Fig. 2. In the isopycnic sucrose gradient centrifugation of GC62, a gnotobiotic calf fecal preparation, a single peak of ELISA activity was detected in fractions with a density of 1.14 g/ml. This corresponded to the peak virus fractions by EM and HA. Figure 3 shows the result of an isopycnic sucrose gradient centrifugation of GC46, a gnotobiotic calf fecal preparation. Peaks of ELISA activity were detected in the fractions with densities of 1.16 and 1.07 g/ml. Virus particles without peplomers were detected by EM in the denser peak; no particles were seen in the 1.07 g/ml peak. In addition, several fecal specimens from gnotobiotic calf studies were examined. Only those specimens shown to contain Breda virus by HAI and EM reacted.

**Specificity of ELISA for antibody detection.** The results of testing the four well-defined sera in ELISA and HAI are
shown in Table 1. GC5, the negative control serum, showed a low titer in the assay. In order to assess this apparent nonspecific competition, sera from 10 colostrum-deprived calves, five guinea pigs, and five rabbits (which had been isolated from birth) were titrated in the test. A screening dilution of 1/32 was chosen because these sera showed significant competition up to a dilution of 1/16.

**Investigation of human fecal samples.** We looked for evidence of Breda-like viruses in humans. Specimens of feces \((n = 400)\) were examined, and 15 gave reactions of \(>0.1\) OD\(_{450}\) in the Breda virus antigen ELISA. These 15 specimens were investigated further by testing them in parallel in the antigen test by coating the plate with pre- and postimmune sera (GP475). All 15 reacted equally in both tests, and none of these specimens contained any Breda virus-specific hemagglutinin. A further 30 human specimens, which contained toroviruslike particles by EM, were examined. One of these specimens (designated 1690) gave a positive reaction \((>0.100\) OD\(_{450}\)) in the test. Fractions from a sucrose density gradient purification of this specimen were examined in the ELISA; the results are shown in Fig. 3. A single peak of reactivity was detected in the fractions at a density of 1.14 g/ml; this peak contained hemagglutinin which was inhibited by serum from a convalescent calf infected with Breda II virus (1).

**Antibody prevalence.** Sera \((n = 209)\) from animal species were tested for evidence of Breda virus-specific antibody. The results are shown in Table 2. A high prevalence of antibody was found in cattle (55%). All cattle sera giving inhibitions of \(>40\%\) (42 of 51) were also tested by HA, and all 42 had titers of \(>20\). Of 45 pig sera examined, 1 gave an inhibition of \(>60\%\) in the ELISA, and this serum had an HA titer of 320 to Breda II virus. Of 11 sheep sera, 1 gave \(>60\%\) inhibition in the ELISA, but the serum had an HA titer of \(<20\). No antibody to Breda virus was detected in sera collected from goats. No Breda virus antibody or Berne virus-neutralizing antibody was detected in the 92 sera collected from veterinarians, veterinary students, and farm workers.

**DISCUSSION**

Previous studies have used either HA or EM to identify Breda-like viruses in clinical material (8, 9). For human samples, the value of HA was limited by the widespread distribution of rat cell hemagglutinins in human feces (1). Similarly, EM is of limited use because Breda viruses disintegrate rapidly on storage (9). Thus, the ELISA for antigen detection should prove valuable as a diagnostic tool in the investigation of clinical material for both serotypes of Breda virus.

The specificity of this assay was confirmed for Breda viruses by testing fractions separated from Breda virus suspensions by centrifugation through a sucrose density gradient. The finding of ELISA activity in fractions at three different densities \((1.14\text{ g/ml} \text{ [whole virus]}, 1.16\text{ g/ml} \text{ [whole virus]})\) probably indicates that the ELISA detected at least two viral antigens. Studies of Berne virus with radioimmuno precipitation showed a peak of activity at a density of 1.07 g/ml, which was associated mainly with the 22-kilodalton polypeptide and to a lesser extent with the 37-kilodalton polypeptide (3). Therefore, it is likely that in addition to the virus polypeptide, the Breda virus ELISA test also recognized a polypeptide similar to that of Berne virus and associated with the virus envelope. We found evidence of one Breda virus infection in humans; the virus was identified by ELISA, EM, and HA. However, apart from this (in our study of diarrhea in children in the United Kingdom by ELISA), we have been unable to demonstrate the presence of other human toroviruses. Perhaps the cross-reactive antibodies observed by immune EM between Breda virus antisera and human particles had insufficient avidity to be useful in ELISA.

A competitive ELISA was chosen for detecting Breda virus-specific antibody because our only source of viral and control antigens was calf feces. Attempts to develop an indirect ELISA for antibody had revealed extensive reactions between human and animal sera and the negative control preparations. In addition, because Breda viruses have a host-derived envelope, bovine proteins may be incorporated into the virus envelope, and these could give rise to false positive results in an indirect ELISA. The sensitivity of the ELISA was broadly similar to that of HA, and antibodies were detected in calf sera at similar titers without peplomers, and 1.07 g/ml [viral membrane] probably indicates that the ELISA detected at least two viral antigens.

The serological activity of the ELISA was confirmed by the presence of specific antibodies in sera from cattle, sheep, goats, and humans. The ELISA gave a single peak of activity in sera from cattle, with a titer of 1/16, and in sera from sheep, with a titer of 1/32. In sera from goats, the ELISA gave a titer of 1/16, and in sera from humans, it gave a titer of 1/16. These results are in agreement with the findings of previous studies, which have shown that Breda viruses are prevalent in cattle and are rare in sheep and goats.

**TABLE 2.** Distribution of Breda virus antibodies in different animal species and humans, measured by competitive ELISA

<table>
<thead>
<tr>
<th>Species</th>
<th>Positive ((&gt;60%))</th>
<th>Equivocal ((40-60%))</th>
<th>Negative ((&lt;40%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>28</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Pigs</td>
<td>1</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>Sheep</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Goats</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Humans</td>
<td>0</td>
<td>1</td>
<td>91</td>
</tr>
</tbody>
</table>

\* Percent inhibition of ELISA.

\* Veterinarians, veterinary students, and farm workers.

**FIG. 3.** Fractions (0.2 ml) from a sucrose density gradient centrifugation (10 to 50%, w/v) of two toroviruses assayed by ELISA. Symbols: ●, GC46 (Breda II fecal preparation); ○, 1690 (human fecal specimen); ---, density.
after both Breda I and Breda II virus infections. However, the ELISA does not suffer from the problem of interpreting low titers caused by nonspecific inhibitors.

We have been able to show a high prevalence of antibody to Breda virus in cattle in the United Kingdom, as has already been reported in the United States (9, 10). There is good evidence that one of the pig sera examined contained antibody to Breda or a closely related virus, but we have found no evidence of infection in sheep or goats in this study.

It has been previously reported that sera from Swiss blood donors contained no Berne virus-neutralizing activity (7). We extended this study by examining sera from veterinarians and farm workers as a possible “sentinel group” for evidence of a zoonotic torovirus infection. We also examined the same sera for antibodies to Breda virus, since the serological relationship between Berne and Breda viruses is not yet clear. Because no antibody was found to either virus in these sera, these viruses are unlikely to be circulating widely in humans, although similar viruses are clearly present in many different species. This does not exclude the possibility that a serologically unrelated torovirus may be circulating in humans.

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LITERATURE CITED