Surface proteins of Breda virus

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SUMMARY

The serotypes 1 and 2 of Breda virus from feces of experimentally infected gnotobiotic calves were studied with respect to their sedimentation and density properties in sucrose gradients and their structural polypeptides; Berne virus, the proposed prototype of the new family Toroviridae, was included for comparison. After Breda-1 virus had been stored at 4 C for a prolonged period, it showed a heterogeneous sedimentation behavior (480 to 520 Svedberg units [S]) and density (1.18 to 1.21 g/ml) indicative of its poor state of preservation. In contrast, freshly prepared Breda-2 virus sedimented at 350 S and showed a buoyant density of 1.18 g/ml; these values compare well with those of Berne virus (400 S and 1.16 g/ml, respectively). Efficient purification of the Breda viruses could be achieved by a 2-step method, involving pelleting by ultracentrifugation followed by isokinetic and isopyknic sucrose gradient centrifugation. Radioiodinated purified virus showed polypeptides with apparent molecular weights of 105,000, 85,000 37,000, and 20,000; another labeled protein of 65,000 D is of doubtful virus specificity. Mouse immune serum raised against Breda-2 virus recognized the polypeptides of the homologous virus and the 2 highest molecular weight proteins of Breda 1 virus in radioimmune precipitation. The same serum inhibited hemagglutination of the heterologous serotype to a low, but significant, degree and efficiently neutralized the infectivity of Berne virus. These observations are taken as indications that the 105,000- and 85,000-D polypeptides represent surface structures of torovirions, probably peplomeric proteins.

The high frequency of diarrhea in young calves is a major problem in calf rearing. Many epizootics are due to a combination of viral and bacterial infections. The role of rota- and coronaviruses as major viral pathogens in calf diarrhea has been established, and the importance of calicivirus-like agents and adeno-, astro-, and parvoviruses has been recognized.[1-7]

During an enzootic of calf scours in Iowa in 1982, virus particles which differed in morphology from known bovine viruses were detected in fecal material. This Breda agent (Iowa 1 isolate) was antigenically unrelated to bovine corona-, parainfluenza-, rota-, parvo-, and bovine pestiviruses (bovine viral diarrhea/mucosal disease).[8] Experimentally infected gnotobiotic calves developed severe diarrhea with virus replication in the jejunal and duodenal mucosa of the small intestine and in the spiral colon, as evidenced by immunofluorescence.[9,10] Two further isolations of Breda virus (BRV) were reported—the Ohio 1 strain from 5-month-old diarrheic calves[a] and the Iowa 2 isolate from a 2-day-old experimental calf.[11] On the basis of hemagglutination inhibition tests, enzyme-linked immunosorbent assay (ELISA), and immune electron microscopy, the 3 isolates were assigned to 2 serotypes: serotype-1 (BRV-1) being represented by the first Iowa isolate and serotype-2 (BRV-2) by the Ohio 1 and Iowa 2 isolates.[11]

The BRV are morphologically and antigenically related to a virus isolated from a horse in Berne, Switzerland.[12,13] The Berne virus (BEV) strain P138/72 has been adapted to growth in cell culture and has been partially characterized.[12,14,15] On the basis of its unique morphology, antigenic properties, protein composition, and replication, BEV has been proposed as the prototype of a new family named Toroviridae[13] along with BRV, Lyon virus (isolated from cattle in France),[16] and particles of similar morphology in stool specimens of children and adults with gastroenteritis.[17]

The purposes in the present study were to obtain data on the buoyant density and sedimentation behavior of BRV-1 and BRV-2 preparations and to examine their protein composition. It was demonstrated that the antigenic relatedness of the BRV serotypes with BEV[12] is reflected by a close similarity in the physicochemical properties.

Materials and Methods

Virus purification—Feces suspensions from gnotobiotic calves 18 and 62 experimentally infected with BRV-1 and BRV-2, respectively, were used for virus purification. Breda virus 1 had been passaged in gnotobiotic calves, which were found free from other enteric viruses by electron microscopy. A rotavirus contamination of the BRV-2 isolate was eliminated by passage in rotavirus-immunized gnotobiotic calves. Approximately 25% suspensions (w/v) in TES buffer (0.02*M* Tris-HCl, pH 7.2, 1.0 m*M* sodium-EDTA, 0.1*M* NaCl) were clarified by low-speed sedimentation (6,000 × g for 45 minutes), and virus from the supernatant was pelleted through a 15% (w/w) sucrose cushion by ultracentrifugation (100,000 × g for 4 hours). Pelleted supernatants of BEV-infected embryonic mule skin cells[b] served as a reference; alternatively, BEV was purified as described.[12]

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[a] Saif LJ, Redman DR, Theil KW. Studies on an enteric 'Breda' virus in calves (abstr). *62nd Annu Conf Res Workers Anim Dis* 1981;236.

[b] Provided by Dr. E. C. Pirtle, National Animal Disease Center, Ames, Iowa.

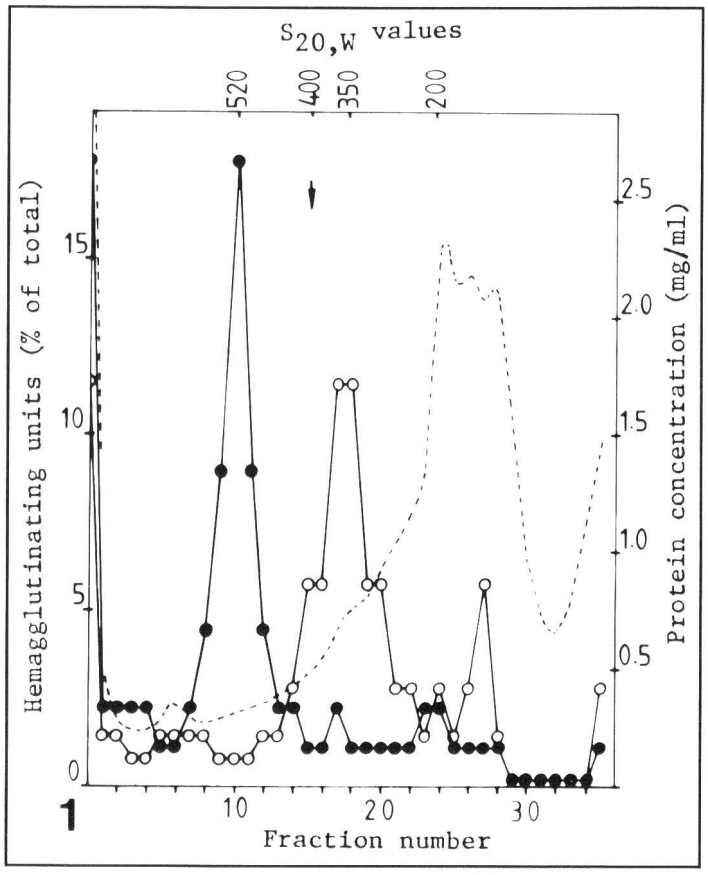

Fig 1—Distribution of the hemagglutinating activity of Breda-1 virus (—●—) and Breda-2 virus (—○—) preparations in an isokinetic sucrose gradient (10% top concentration). The interrupted line without symbols indicates the protein concentration. Centrifugation was done for 40 minutes at 41,000 rpm in a Spinco SW41 rotor. The infectivity peak of Berne virus is indicated by the arrow. The sedimentation coefficients ($S_{20,W}$) for the conspicuous peaks of activities are given across the top. Direction of sedimentation is from right to left.

The pelleted materials were layered on top of an isokinetic sucrose gradient (10% to 25.5% w/w) in phosphate-buffered saline solution (PBSS), calculated for an assumed particle density of 1.2 g/ml for centrifugation at 5 C, and centrifuged at 41,000 rpm in a Spinco SW41 rotor for 40 minutes.[18] After bottom fractionation was done, using a peristaltic pump,[c] the protein contents were determined by measuring the optical density (OD) spectrophotometrically; the empirical formula of conversion used was as follows: mg/ml = $1.45 \times OD_{280} - (0.75 \times OD_{260})$. The antigen distribution was monitored, using hemagglutination (HA)[8] for BRV and an infectivity test for BEV. Fractions with maximal HA activity were pooled and layered on top of a linear 10% to 50% (w/w) sucrose gradient and centrifuged to equilibrium in a Spinco SW27.1 rotor at 50,000 × g for 16 hours (isopyknic gradient).

Metabolic labeling of BEV was done as described.[14,15]

Hemagglutination assay of BRV—Serial 2-fold dilutions of the gradient fractions were made in 10-µl volumes of PBSS in V-bottom microtitration plates.[d] Bovine fetal serum[e] was diluted 1:5 and adsorbed with packed rat erythrocytes until all spontaneous agglutination was lost, and 10-µl volumes of a 1:200 dilution in PBSS were added to each well. After 10 µl of a 1% suspension of the rat erythrocytes was added to PBSS containing 0.1% ovalbumin, the plates were incubated for 2 hours at room temperature and read.

[c] Peristaltic pump P-3, Pharmacia Fine Chemicals, Uppsala, Sweden.
[d] Greiner, Nürtingen, West Germany.
[e] Flow Laboratories, Irvine, Scotland.

TABLE 1—Purification balance of Breda virus preparations from bovine feces, using a two-step procedure

Virus material	HAU (× 10^5)	Protein concentration (mg/ml)	HAU/mg of protein (× 10^5)	PF
Breda-1 pellet from ultracentrifugation	16	7.8	2.1	...
Isokinetic gradient	0.3	0.06	5.0	2.4
Breda-2 pellet from ultracentrifugation	16	26.9	0.6	...
Isokinetic gradient	1.2	0.7	1.7	2.8

HAU = hemagglutinating units; PF = purification factor.

Infectivity assay of BEV—Titrations of BEV containing gradient fractions were done in tissue-quality Terasaki plates[d] as described.[14]

Protein analysis—Purified virus was radioiodinated, using the Iodogen[f] (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) reagent.[19] The reaction was started by pipetting 75 µl of gradient peak material, 10 µl of 1*M* K_2PO_4, 12.8 µl of H_2O, and 2.2 µl of [^{125}I]NaI (200 µCi, sp act 13.6 mCi/µg)[g] into Eppendorf cups coated with 2 µg of Iodogen (dissolved in 50 µl of chloroform and evaporated). After incubation was done for 5 minutes at 4 C, 25 µl of 10 m*M* tyrosine in 0.1*M* NaOH was added to stop iodination. The labeled materials (0.5 µCi/µl) were treated with 2% sodium dodecyl sulfate (SDS) for 30 minutes at room temperature and analyzed on 15% polyacrylamide gels, using a modified Laemmli procedure.[20]

Immunization—Male Balb/c mice were immunized by injecting (by the subcutaneous and intraperitoneal routes) 100 µg (120 µl) of a BRV-2 preparation (isokinetic gradient peak) emulsified with an equal volume of Freund's complete adjuvant. Four weeks later, an intraperitoneal booster injection was given, using 80 µg of BRV-2 in aluminum hydroxide after one cycle of freezing (−20 C) and thawing was completed. Blood samples were obtained from the mice via puncture of the coccygeal veins 4 days after the booster injection had been given.

Hemagglutination-inhibition (HI) assay—Serial 2-fold dilutions of the serum to be tested were made in 10 µl of PBSS and mixed with equal volumes of BRV gradient peak material diluted to contain 8 hemagglutinating units. After incubation for 30 minutes at room temperature was completed, 10-µl volumes of a 1% suspension of mouse erythrocytes in PBSS containing 0.1% ovalbumin were added. Mouse erythrocytes were used to make the test homologous; differences in HA and HI titers had not been observed in comparative assays between rat and mouse erythrocytes. The plates were further incubated at room temperature for 2 hours before they were read. The HI titers are given as the reciprocal of the highest dilution of serum showing inhibition.

Enzyme-linked immunosorbent assay—Pooled fractions from isokinetic gradients were diluted 1:100 in a 0.1*M* Na_2CO_3/$NaHCO_3$ buffer (pH 9.6; coating buffer), and 50 µl/well was adsorbed to flat-bottom microtitration plates. After overnight incubation at 37 C was completed, the plates were washed 3 times with PBSS containing 0.5% Tween 80 (ELISA buffer); 100-µl volumes of 1% ovalbumin in coating buffer were added, and the plates were incubated for 30 minutes at room temperature. After 6 cycles of washing were completed, 50 µl of serial 2-fold serum dilutions was added to the wells followed by a 30 minute incubation at room temperature. The plates were washed again, and 50 µl of peroxidase-labeled rabbit anti-mouse immunoglob-

[f] Pierce Chemical Corp, Rotterdam, The Netherlands.
[g] The Radiochemical Center, Amersham, United Kingdom.

ulin G[h] diluted 1:2000 in ELISA buffer was added to the wells. After an incubation of 30 minutes at room temperature and additional rinsing were done, 100 µl of the substrate solution was added, which consisted of citric acid (0.05*M*, pH 4.0) containing 0.008% H_2O_2 and 0.11 mg of 2,2′-azinodi-(3-ethylbenzthiazoline sulfonic acid)/ml. The reactions were recorded, using an ELISA reader (Titertek Multiscan),[c] at 405 nm after incubation for 1 hour at room temperature was completed. The ELISA titer is given as the reciprocal of the highest serum dilution which resulted in a more than 2-fold increase of absorbance at 405 nm over the values obtained with the preimmune serum.

Radioimmune precipitation (RIP)—Radioiodinated BRV (10 µl) and mouse serum (2 µl) were incubated overnight at 4 C in 290 µl of TES buffer containing 0.1% Triton X-100; 50 µl of a 10% suspension of *Staphylococcus aureus* cells in the same buffer and 70 µl of 3*M* KCl were added. After incubation for 30 minutes at 4 C was completed, the complexes were pelleted at 10,000 × g, washed 3 times in TES buffer containing 0.1% Triton X-100, and suspended in Laemmli sample buffer (60 m*M* Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue). Details of the procedure have been given.[20]

Results

Sedimentation behavior—The sedimentation properties of BRV-1 and BRV-2 were studied in isokinetic sucrose gradients, including BEV for comparison (Fig 1). Sedimentation coefficients calculated for BRV-1 at 20 C in water varied between the experiments and ranged between 480 and 520 Svedberg units (S; 519 ± 36 S; n = 3), in contrast to those of BRV-2 which were reproducibly found at 350 S (353 ± 4 S; n = 3). Berne virus had an estimated sedimentation coefficient of 400 S. All 3 viruses showed a small, but reproducible, peak of virus-specific activity near the top of the gradients (estimated size, 50 to 200 S). A purification balance of BRV from fecal suspensions is shown in Table 1. Although significant loss was encountered when comparing the HA activity of the starting material (ultracentrifuge pellet) and that of the peak fraction, the protein concentration was decreased approximately 100-fold, resulting in a satisfactory degree of purification, comparable for both serotypes.

Buoyant density—Distributions of the HA activity of BRV-1 and BRV-2 in linear sucrose gradients are shown in Figure 2. Estimates of BRV-1 virion buoyant densities ranged from 1.18 to > 1.21 g/ml (1.19 ± 0.012 g/ml; n = 4), whereas BRV-2 again banded reproducibly at 1.17 g/ml (1.17 ± 0.018 g/ml; n = 3). Berne virus included for comparison showed a density of 1.16 g/ml.

Virion polypeptides—In autoradiographs of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) slabs on which purified, radioiodinated BRV-1 and BRV-2 preparations had been analyzed, distinct protein bands were detected; ether extraction of the preparations was done before they were analysed to obtain indications of the relative hydrophobicity of the polypeptides. As demonstrated in Figure 3A, nearly identical patterns were obtained for both non-treated virus preparations (lanes 1 and 3); the calculated molecular weights were 105,000, 85,000, 65,000, 37,000, and about 20,000. Ether treatment resulted in weaker 105,000-, 85,000-, and 37,000-D bands (the 65,000-D species being hardly affected) and in a shift of the band in

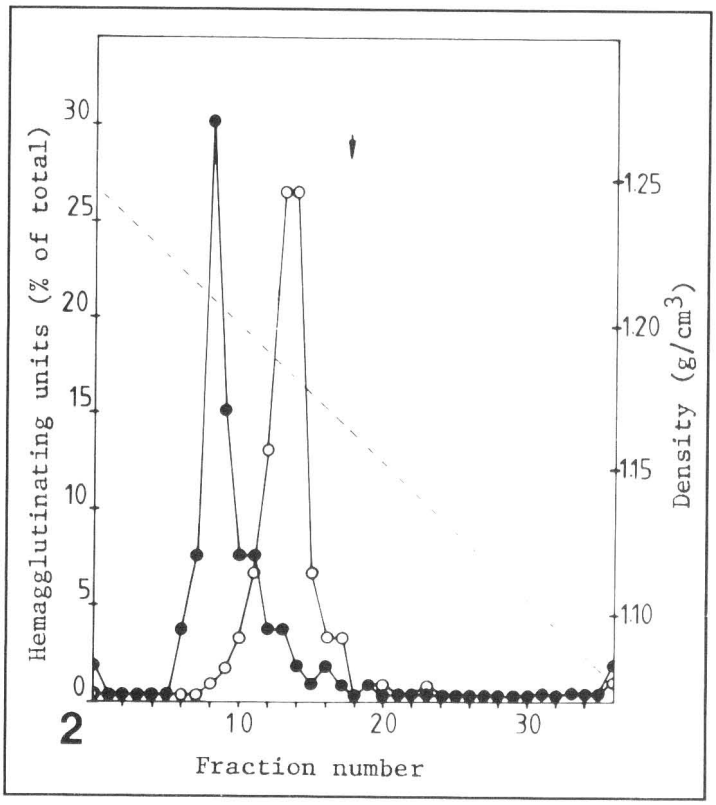

Fig 2—Distribution of the hemagglutinating activity of Breda-1 virus (—●—) and Breda-2 virus (—○—) preparations in a linear 15% to 50% (w/w) sucrose gradient; the density gradient is represented by the interrupted line. Centrifugation was done for 16 hours at 50,000 × g in a Spinco SW27.1 rotor. The arrow indicates the peak of Berne virus infectivity. Direction of sedimentation is from right to left.

the 20,000-D region to slightly higher molecular weight. Similar molecular weights were found after running 7.5%, 10%, and 12.5% polyacrylamide gels (not shown). For comparison of the BRV polypeptides with those of another torovirus, we have included metabolically labeled extracts from BEV-infected (Fig 3B, lanes 1 and 3) and non-infected embryonic mule skin cells (lanes 2 and 4) in the analysis after immune precipitation was done, using a rabbit hyperimmune serum and *S aureus* protein A (lanes 3 and 4). Another control without serum was included (lanes 1 and 2), since protein A selectively recognizes the 20,000-D nucleocapsid protein of BEV.[i] It can be seen that the 85,000-D band of BRV is in the same position as the most prominent [^{35}S]methionine-labeled protein in the high molecular weight range of BEV. A BEV signal above the background could not be detected in the position of the 105,000-D polypeptide, which reproducibly showed a double band in BRV-2 (Fig 3A, lane 3; Fig 3B, lane 6). The only protein of BRV-2 in the low molecular weight range clearly has a size larger than does the BEV nucleoprotein.

For use in homologous RIP, 3 mice were immunized with purified BRV-2. One serum was tested which resulted in a homologous ELISA titer of > 400 and in a HI titer of 320 when BRV-2 was used and of 40 when BRV-1 was used as antigen. Also, this serum neutralized BEV (titer of 320 against 100 median tissue infective doses, calculated using the Spearmann-Kaerber formula). In RIP tests, the

[h] Nordic Immunologic Laboratories, Tilburg, The Netherlands.

[i] Horzinek MC, Ederveen J, Institute of Virology, Veterinary Faculty, State University of Utrecht, The Netherlands: Unpublished data, 1984.

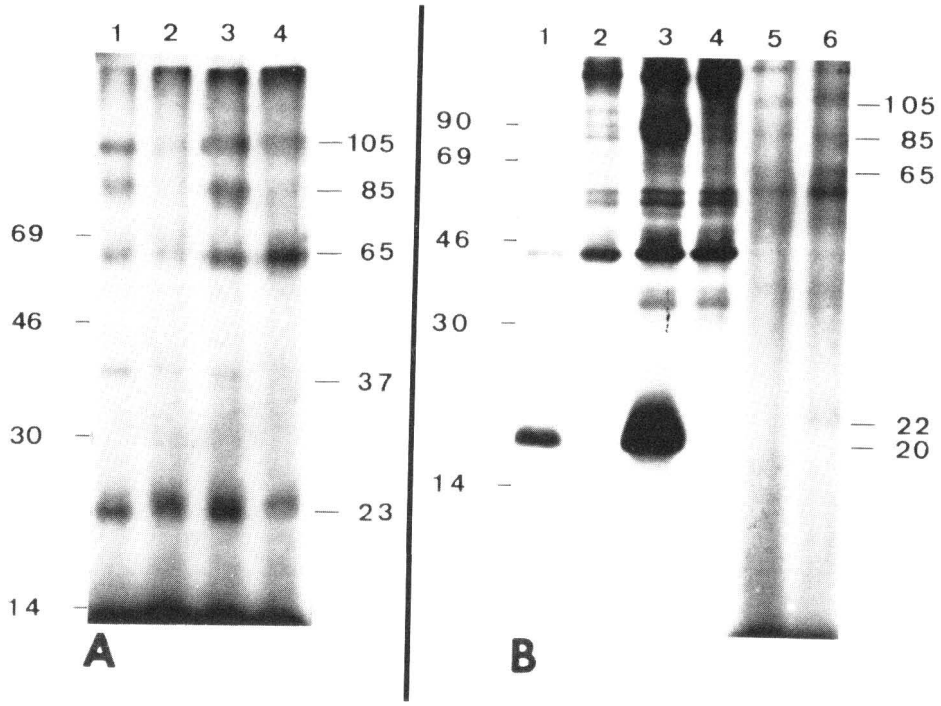

Fig 3A—Polyacrylamide-gel electrophoresis analysis of radioiodinated preparations of purified Breda-1 virus (lanes 1 and 2) and Breda-2 virus (lanes 3 and 4) before (lanes 1 and 3) and after (lanes 2 and 4) extraction with diethyl ether was done. Molecular weights are expressed in thousands.

B—^{35}S methionine-labeled Berne virus proteins from infected (lanes 1 and 3) and noninfected embryonic mule skin cells (lanes 2 and 4) after radioimmune precipitation was done with a homologous rabbit hyperimmune serum and *Staphylococcus aureus* protein A (lanes 3 and 4) and protein A alone (lanes 1 and 2). The preparation of Berne virus has been included to allow reliable comparative estimates of the Breda-1 virus (lane 5) and Breda-2 virus (lane 6) polypeptides.

The numbers to the left of the fluorographs indicate the molecular weights of the following ^{14}C marker proteins coelectrophoresed in the same gel: phosphorylase (90,000) bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,000). The calculated molecular weights (in thousands) of the viral proteins are indicated (right).

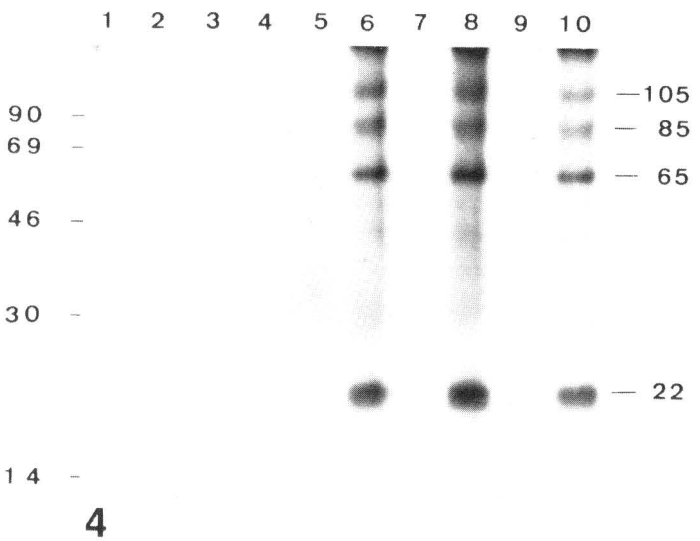

Fig 4—Radioimmune precipitation followed by polyacrylamide-gel electrophoresis, using 3 mouse immune sera raised against Breda-2 virus (even numbers) and the respective preimmunization sera (odd numbers); Breda-1 virus (lanes 1-4) and Breda-2 virus (lanes 5-10) served as antigen. Molecular weights are expressed in thousands. Note weak, but distinct, heterologous reactions in the 105,000-D and 85,000-D range (lane 2). Sera were from mouse 1 (lanes 1, 2, 7, and 8), mouse 2 (lanes 3, 4, 9, and 10), and mouse 3 (lanes 5 and 6).

sera reproducibly recognized the 105,000-, 85,000-, and 65,000-D proteins of BRV-2, as well as a polypeptide in the 20,000-D range (Fig 4). One of the mouse serum preparations recognized also the 105,000- and 85,000-D polypeptides of radioiodinated BRV-1 (lane 2). Background in lane 9 of Figure 4 was probably caused by leakage from the adjacent slot.

Discussion

In the present report, evidence is presented for the close physical and chemical relationship between BRV-1, BRV-2, and BEV, supporting other observations of an antigenic cross-reactivity.[12] The buoyant density and sedimentation coefficient of BRV-2 were reproducibly close to those of BEV. Breda virus 1 values, in contrast, showed variation between experiments. Conceivably, this variation reflects the poor state of preservation of virions, rather than an intrinsic physical virion property. The BRV-1 preparation (gnotobiotic calf 18) had been stored as diarrheic feces for more than 4 years at 4 C before it was used in these experiments; its infectiveness for calves was lost after it had been stored for 3 weeks at 4 C and deteriorated even at −70 C. In contrast, BRV-2 was from a fresh sample (gnotobiotic calf 62, July 1984) and was well preserved at −70 C. Another indication for physical differences between the BRV-1 and BRV-2 preparations came from electron micrographs. As described by Woode et al,[11] peplomers of BRV-1 were shorter than those of BRV-2; this observation, however, may reflect the effect of degradation rather than a property of the native virion. Consequently, we consider the density and sedimentation data obtained for BRV-1 as not representative for toroviruses. Berne virus was found to be exceptionally stable to proteolytic enzymes and extreme pH values and to lose 1.7 log units of infectivity after it had been stored at 4 C for 185 days.[21]

In isokinetic sucrose gradients, both BRV-1 and BRV-2 showed additional peaks of hemagglutinating activity at 200 S and 90 S, respectively. This corresponds with observations of a 50 S peak of virus-specific activities in BEV which is under study at present. So far, BRV has not

been propagated in cell culture, which has hampered thorough investigation of these subviral particles in the Breda viruses.

The protein profiles of both BRV serotypes confirm their close relationship with BEV (Fig 3); molecular species of 105,000, 85,000, 65,000, and about 20,000 D have been demonstrated. The specificity of these proteins is shown by the RIP-PAGE results. We cannot exclude, however, that the 65,000-D band represents bovine serum albumin which is notoriously sticky and may have induced an antibody response in mice (Fig 4); this issue should be clarified by RIP-PAGE, using bovine immune serum. The 200,000-D band detectable in analyses of BEV-infected cells[14] is due to a nonstructural protein. The high molecular weight band appearing in lane 5 of Figure 3B was probably caused by nonspecific aggregates, since it could not be detected in gels with lower degrees of cross-linking (not shown).

When comparing the electrophoretic behavior of the low molecular weight polypeptide of BRV with that of BEV (Fig 3B), it was obvious that it does not comigrate with the 20,000-D nucleocapsid protein of BEV. Identification of the latter is facilitated by its selective adsorption to protein A in the absence of serum.[i] Being larger than the capsid protein, it is a candidate for the 22,000-D envelope protein which is second in abundance in BEV.[15] Association with membrane lipids is indicated by the effect of ether extraction on its migration (Fig 3A).

The 37,000-D protein could not be precipitated, using our mouse anti-BRV-2 sera. As has been discussed for BEV, it is probably a matrix protein[15] of poor immunogenicity.

Cross-reactivity was observed between BRV-1 and BRV-2 in HI tests. Recently, a hemagglutinating activity has been discovered also for BEV.[j] Radioimmunoprecipitation followed by PAGE revealed the presence of high molecular weight proteins (85,000 and 105,000) which were recognized by the heterotypic serum (anti-BRV-2 serum reacted with BRV-1 proteins; Fig 4, lane 2). It is therefore assumed that hemagglutinating determinants are localized on these proteins and that they represent peplomeric subunits. Further support comes from the observations that anti-BRV-2 serum neutralized BEV, for which binding to a virion surface structure would be a prerogative. In the 105,000-D region, 2 bands were visible in the BRV-2 preparations. Experiments are under way to further study the hemagglutinating principle of BRV-2.

It has been found recently in the Utrecht laboratory that the 100,000/75,000-D protein of BEV is *N*-glycosylated[22] and that neutralizing monoclonal antibodies recognize these proteins in radioimmunoprecipitation.[k]

[j] Weiss M, Virology Department, Institute of Bacteriology, Veterinary Faculty, University of Berne, Switzerland: Personal communication, 1986.

[k] Kaeffer B, van Kooten P, Ederveen J, et al. Institute of Virology and Department of Immunology, Veterinary Faculty, State University of Utrecht, The Netherlands: Unpublished data, 1986.

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