Properties of monoclonal antibodies against Berne virus (Toroviridae)

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SUMMARY

Seven hybridomas that secreted monoclonal antibodies (MAB) against the peplomer protein and one that secreted MAB against the nucleocapsid protein of Berne virus (proposed family Toroviridae) were isolated. All MAB directed against the peplomer protein neutralized virus infectivity and, with the exception of MAB 6A7, inhibited each other's binding in competition assays. Neutralization of Berne virus infectivity was potentiated when some MAB were used in pairs. The antibodies have been used to localize toroviral proteins in infected cells; use of antipeplomer MAB 6B10 yielded a diffuse intracytoplasmic immunofluorescence, whereas the antinucleocapsid MAB 1F1 detected antigen in the intra- and perinuclear compartments. By use of radioimmune precipitation, protein A of *Staphylococcus aureus* was found to bind directly to the nucleocapsid polypeptide, without the requirement for specific antibody. Using fluorescein isothiocyanate-conjugated protein A, the intranuclear accumulation of the nucleoprotein of Berne virus was confirmed by results of immunofluorescence.

Berne virus (BEV) is the prototype of a new group of positive-stranded enveloped RNA viruses for which the family designation Toroviridae has been proposed.[1,2] Structural analysis has led to the identification of 4 major virion polypeptides: a phosphoprotein with apparent molecular weight of 20,000 (20 kD) representing the nucleocapsid protein,[3] 2 envelope-associated polypeptides (22 kD and 37 kD) and a set of glycosylated polypeptides in the 75- to 100-kD range constituting the peplomeric surface projections.[4] Berne virus was isolated in 1972 from a rectal swab specimen obtained from a horse and adapted to growth in equine cell culture.[5] Several other toroviruses have been identified, but none has been grown in vitro; the Breda viruses that were isolated from diarrheal calves[6,a] had to be propagated to gnotobiotic calves.[7] Torovirus-like particles were also encountered in the feces of human beings with gastroenteritis.[8,9]

The objectives of the study reported here were to investigate properties of monoclonal antibodies (MAB) directed against the nucleocapsid and peplomer proteins of BEV and to develop a MAB ELISA for the detection of toroviruses and toroviral antibody in swine. Apart from this latter objective, we wanted to confirm the presence of the nucleocapsid protein in the nuclei of infected cells, a localization that is unusual in positive-stranded RNA viruses.[10,11]

Materials and Methods

Viruses and cells—The BEV isolate P138/72, which was rendered mycoplasma-free,[12] was used. Embryonic mule skin (EMS) and equine dermis (ED; ATCC, CCL 57) cellmonolayers were maintained in Dulbecco modified minimal essential medium with Earle salts supplemented with 2% fetal bovine serum, nonessential amino acids (1%), L-glutamine (200 m*M*), sodium bicarbonate, and 0.1% penicillin and streptomycin. The fetal bovine serum had been pretested by use of a neutralization assay for lack of antibody to BEV.[5] Infectivity titration was performed in EMS cells grown in Terasaki microplates[b]; titer was expressed as 50% tissue culture infective dose ($TCID_{50}$). Immunofluorescence was performed, using the ED cell line that, in contrast to EMS cells, is commercially available.

Virus purification—Production of BEV in EMS cells was modified following a procedure described by Weiss et al.[5] Twenty-three hours after infection at a multiplicity of infection of 0.05 to 0.1, supernatants of infected and mock-infected cell cultures, respectively, were pooled and concentrated by addition of a saturated solution of ammonium sulfate followed by centrifugation. The pellets were desalted by passage through a disposable gel filtration column[c] equilibrated with phosphate-buffered saline solution (PBSS). This preparation was designated "semipurified virus." Alternatively, the pellets that were not desalted were subjected to an ultracentrifugation step on a 2-layer sucrose cushion (15 to 50%, w/v). Interphase material was collected and desalted and was designated "purified virus." Purified virus preparations of $10^{7.5}$ to $10^{8.5}$ $TCID_{50}$/ml, with protein concentration of 0.5 to 1 mg/ml (from infected and mock-infected cultures) were obtained. Ammonium sulfate precipitation results were compared with those of a purification step, using 6% polyethylene glycol.[13]

Isolation of hybridomas—Balb/c mice, which had been screened by virus neutralization testing for lack of torovirus antibodies, were inoculated SC with a mixture of semipurified BEV suspension and an equal volume of Freund adjuvant. Twenty days

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[a] Saif LS, Redman DR, Theil KW, et al. Studies on an enteric "Breda" virus in calves (abstr), in *Proceedings*. 62nd Annu Meet Res Workers Anim Dis 1981;236.

[b] Costar Inc, Cambridge, Mass.

[c] Sephadex G-25, Pharmacia Fine Chemicals, Uppsala, Sweden.

later, they were administered booster inoculations IV with purified BEV at days 4 and 3 before fusion. Neutralization titer was about 100 after the first booster inoculation and was >1,000 on the day of fusion. For hybridoma production, splenic lymphocytes were fused with mouse myeloma SP 2/0-Ag14 cells, using essentially the procedure of Köhler and Milstein.[14] Clones were subcloned twice by limiting dilution in the presence of rat thymocytes (normal spleen feeder cells). Between each step, positive clones were selected on the basis of results of neutralization testing and ELISA. Ascitic fluids were obtained from 8 clones, using Balb/c mice preinoculated with pristane.[15]

Screening procedure—Hybridoma supernatants were tested by virus neutralization testing, as described by Weiss et al.[5] An ELISA was performed in disposable 96-well flat-bottom microtitration plates[d] precoated with either purified virus or mock-infected supernatant preparations diluted 1:10 in coating buffer ($Na_2CO_3/NaHCO_3$, 0.1*M*, pH 9.6) and adjusted to the protein concentration that corresponded to 10^6 $TCID_{50}$/well. After overnight incubation at 37 C, the plates were rinsed 5 times with washing buffer (PBSS containing 0.5% Tween 80, v/v). Coating was done by incubating the plates for 30 minutes at 37 C with 50 µl of PBSS/well containing 1% ovalbumin. During the following steps, an avidin-biotin complex[16,e] was added to amplify the signal, with 2, 2′ azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid serving as the chromogen.[f] Optical density at 405 nm (OD_{405}) was measured, using a photometer.[g] Antibody-positive hybridomas were selected after calculation of the ratio between the OD_{405} of virus and mock-coated wells. Wells with ratio ≥ 1.25 were considered to be virus-positive.

Hemagglutination inhibition—Hemagglutination of purified virus was performed as described.[17] In hemagglutination inhibition (HAI) experiments, 8 hemagglutinating units of virus were used.

Indirect immunofluorescence test—Cell monolayers grown on cover slips (5 to 8 × 10^4 cells/cm^2 in 24-well clusters[b]) were collected at different times after infection and fixed for 10 minutes in acetone at 20 C. After 2 washes in calcium- and magnesium-free PBSS, cells were incubated with MAB or polyclonal serum at dilutions ranging from 1:10 to 1:100 in washing buffer supplemented with 10% human serum (50 µl/slip). Three washing cycles were done prior to addition of fluorescein isothiocyanate-conjugated goat anti-mouse IgG[h] diluted 1:40 in washing buffer. After 4 washes, cover slips were mounted in glycerol/PBSS mixture (9:1, containing 1 g of *p*-phenylenediamine/L).[18]

Radioimmune precipitation—This method has been described.[19] Briefly, ascitic fluids (10 µl) or hybridoma supernatant (50 µl) were mixed with 10 µl-volumes of [^{35}S]methionine[i]-labeled cell lysates and diluted to 100 µl with Tris EDTA saline buffer (10 m*M* Tris-HCl pH 7.4, 1 m*M* EDTA, 150 m*M* NaCl), containing 0.1% Triton X-100. After overnight incubation (4 C), KCl was added to the samples to a final concentration of 0.5*M*, and the immune complexes were allowed to precipitate for 30 minutes at 4 C after addition of 25 µl of a 10% suspension of formaldehyde-inactivated *Staphylococcus aureus* cells. After centrifugation at 10,000 × g for 15 min, precipitates were washed 3 times, solubilized in 50 µl of Laemmli buffer, and analyzed by use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting analysis—Purified BEV and mock-infected preparations were subjected to SDS-PAGE for 4 hours at 100 V and 40 mA. The proteins were transferred to a nitrocellulose sheet[j] (pore size, 0.45 µm) for 16 hours at 4 C, using transfer buffer (25 m*M* TRIS, 102 m*M* glycine, 20% methanol, and 0.1% SDS). Antigen detection on the nitrocellulose sheets was performed, using an ELISA with 4-chloro-1 naphthol[k] as a chromogen.[20]

Competition binding assay—Ammonium sulfate precipitates (40%, v/v) from mouse ascitic fluids were biotinylated,[21] and the reactivity of the biotinylated MAB was assayed, using purified virus and mock-infected preparations as coating antigen. The ELISA saturation curves were obtained, using antigen-coated plates (10^6 $TCID_{50}$ of purified virus/well) and serial dilutions (starting with 4.2 µg/well) of biotinylated MAB in calcium- and magnesium-free PBSS containing 0.05% Tween 20. The background was measured for each dilution, using mock infection-coated wells. Because the antigenic mass coated per well could not be determined, virus preparations were pretested against each biotinylated MAB (bio-MAB) to select the optimal dilution for the competition test.[20] Competition results were expressed as a percentage, using the formula:

$$\% \text{ inhibition} = \{[(\text{OD with competitor} - \text{OD background})/(\text{OD without competitor} - \text{OD background})] - 1\} \times 100.$$

Assays were performed in duplicate, with a maximal competitor/probe ratio that ranged from 27 to 60.

Enhancement of neutralization by pairs of MAB—Dilutions of MAB (semipurified by ammonium sulfate precipitation) able to protect ED cell monolayers against 100 $TCID_{50}$/10 µl were determined. Each MAB was then assayed at the respective dilution against tenfold increments of BEV (from $10^{2.5}$ to $10^{5.5}$ $TCID_{50}$). Wells with cytopathic effects were counted at days 3 and 5 after infection; each test was run in sextuplicate. Fifty percent neutralization dose (ND_{50}) was estimated, using the Spearman-Kärber formula, and the square deviation was calculated. Neutralization index (NI) was defined as the difference between logarithms of the titers as follows:

$$NI = \frac{ND_{50}\ (MAB\ 1\ +\ 2)}{(ND_{50}\ (MAB1)\ +\ ND_{50}\ (MAB2)}$$

This index was assumed to be equal to 1 if neutralizing activity of the paired MAB was only additive. The significance of differences between the calculated NI and 1 was estimated at a 5% probability of error. Each MAB diluted in culture medium had a NI equal to 1. A negative control was created by incubating MAB 1F1 (a nonneutralizing MAB) with the other MAB. A positive control was created by incubating MAB 11A1 with polyclonal serum of porcine origin (pig 915, Mei Shan breed; neutralizing titer after experimental immunization, 3,200).

Results

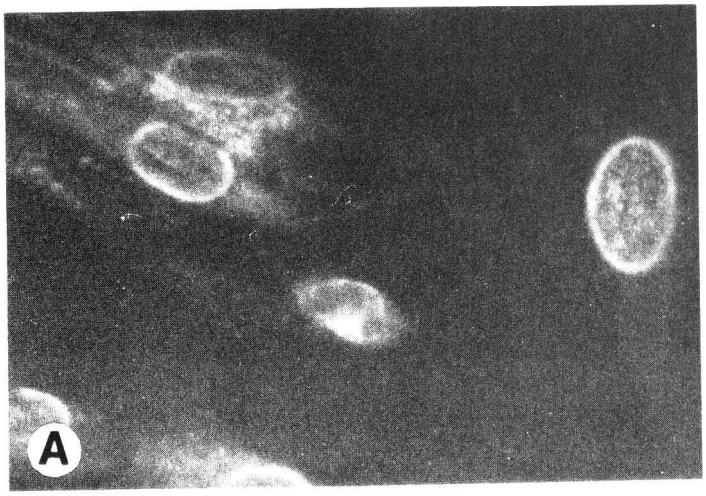
Berne virus growth and purification—Conditions for optimal yield of BEV were determined in EMS cells. Cell density varied between 3.5 × 10^2 and 2.2 × 10^5/cm^2. Highest yields were obtained at density between 4.4 × 10^4 and 2.2 × 10^5 cells/cm^2 (subconfluent and confluent monolayers, respectively). The multiplicity of infection had no influence on the yield 23 hours after infection (2 replicative cycles[5] and appearance of cytopathic effect). In all further experiments, multiplicity of infection between 0.01 and 0.1 was chosen. The passage level of the EMS cells, however, was found to be of importance. Titration of the same virus preparation in EMS cells at passage 25 and 29 re-

[d] Inotech, London, England.
[e] ABC kit, Vector Laboratories Inc, Burlingame, Calif.
[f] 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), Sigma Chemical Co, St Louis, Mo.
[g] Flow Laboratories, Irvine, England.
[h] Nordic Immunological Laboratories, Tilburg, The Netherlands.
[i] The Radiochemical Center, Amersham, England.
[j] Schleicher & Schuell Inc, Dassel, Federal Republic of Germany.
[k] Merck, Darmstadt, Federal Republic of Germany.

TABLE 1—Biologic activities and polypeptide specificities of anti-Berne virus monoclonal antibodies (MAB) directed against the 75- to 100-kD peplomer glycoprotein

MAB	Neutralization titer ($\times 10^3$)	HAI† ($\times 10^2$)	IIF
11A1	41.0	37.5	Weak
8D1G10	14.0	20.0	Weak
8D1G1	1.5	12.0	Weak
6A7 (IgG2b)*	6.0	7.5	Lacking
3B11	2.6	0.5	Weak
6B10	1.3	1.5	Strong

* MAB isotypes were determined, using the Ouchterlony double diffusion technique and were found to be IgG2a in all instances but one. Each MAB was purified and adjusted to 1 mg of protein/ml in phosphate-buffered saline solution. † Native ascitic fluids were used for the hemagglutination-inhibition (HAI) assay.
IIF = indirect immunofluorescence.



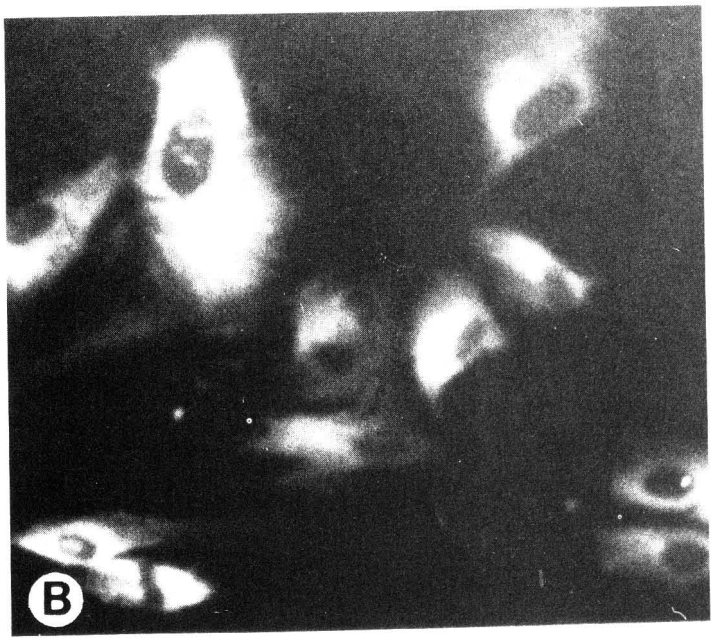


Fig 1—Indirect immunofluorescent staining, using the antinucleocapsid monoclonal antibody (MAB) 1F1 (A) and the antipeplomer MAB 6B10 (B) in equine dermis cells 13 hours after infection with Berne virus (multiplicity of infection approx 2). Notice preferential perinuclear staining with MAB 1F1 and intracytoplasmic staining with MAB 6B10. × 400.

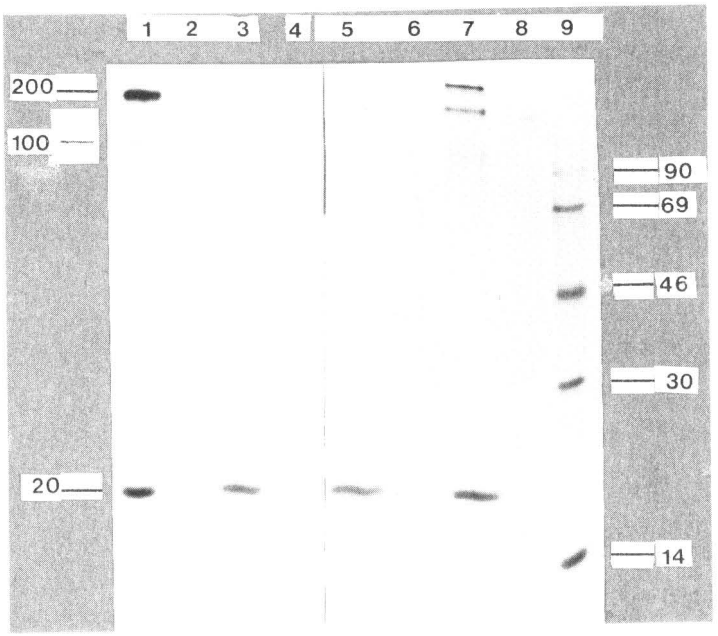


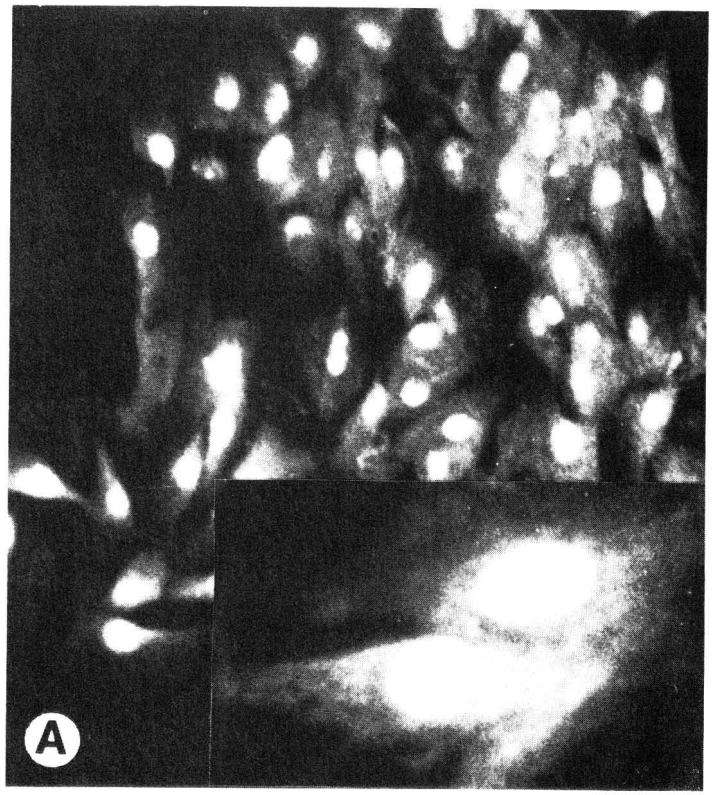
Fig 2—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{35}S-labeled proteins of Berne virus. Infected (odd numbers) and noninfected (even numbers) embryonic mule skin cell extracts were analyzed after immune precipitation, using MAB 3B11 (lanes 1 and 2), 1F1 (lanes 3 and 4) and 8D1G10 (lanes 7 and 8). Controls without MAB are seen in lanes 5 and 6. Molecular weight markers are seen in lane 9.

sulted in titer of 52 and and 4×10^5 $TCID_{50}$/ml, respectively, independent of the cell density (2,000 to 5,000 cells/cm^2). Differences were statistically significant ($P < 0.05$).

For virus concentration, the ammonium sulfate precipitation method was used.[5] Concentration with 6% polyethylene glycol was found to be an unattractive alternative to ammonium sulfate precipitation. Maximal recovery of virus infectivity with a minimum of contaminating protein was found when ammonium sulfate concentration between 25 and 50% saturation was used; precipitation was allowed to proceed for 3 hours. Viral antigen used during the next experiments was prepared following the aforementioned procedure.

Serologic reactivity of the MAB—Seven MAB had BEV-neutralizing properties. Maximal titer was obtained, using MAB 11A1 and 8D1G10 (Table 1), which also had the highest HAI titer. Only low HAI titer was recorded, using MAB 6B10 and 3B11. The MAB 6A7 occupied an intermediate position in both tests. Neither HAI nor neutralization was observed, using MAB 1F1. After infection of ED cells with BEV, antigen was recognized at the periphery of the nucleus and in its immediate vicinity by use of indirect immunofluorescence and MAB 1F1 from the eighth hour after infection onward (Fig 1). Cytoplasmic fluorescence was seen, using several other MAB later in infection (13 hours, before the appearance of cytopathic effect); least distinct fluorescence was found using MAB 6A7.

Polypeptide specificity of anti-BEV MAB—The polypeptide specificity of our MAB was assessed, using radioimmune precipitation (Fig 2). Specific bands were seen in the high molecular weight range (about 90 kD and 200 kD), using MAB 3B11 and 8D1G10. Similar results were obtained with the other MAB and with polyclonal serum (data not



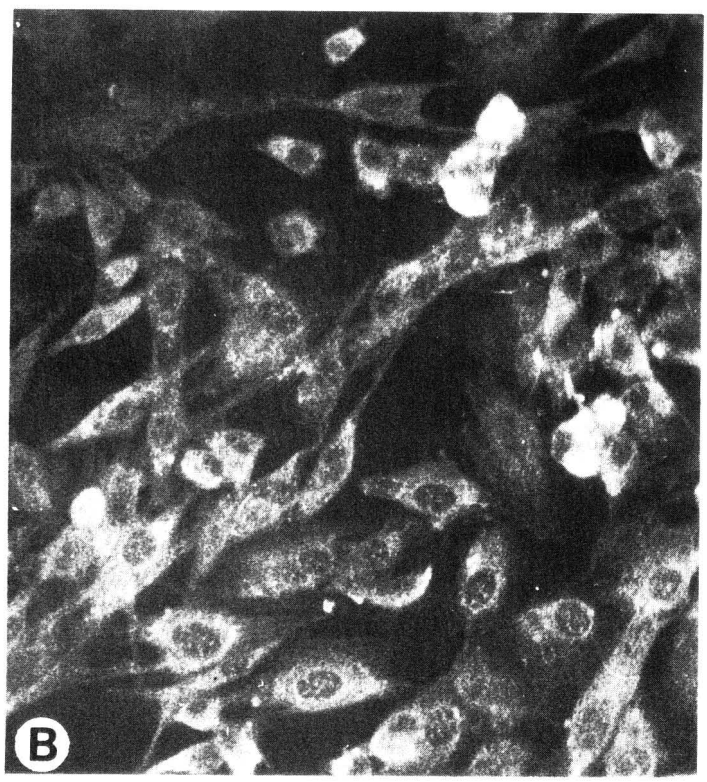


Fig 3—Intranuclear fluorescence, using fluorescein isothiocyanate-labeled protein A conjugate on embryonic mule skin cells 8 hours after infection with Berne virus (A and inset). Mock-infected cells (B) served as a control. × 100. Inset, × 400.

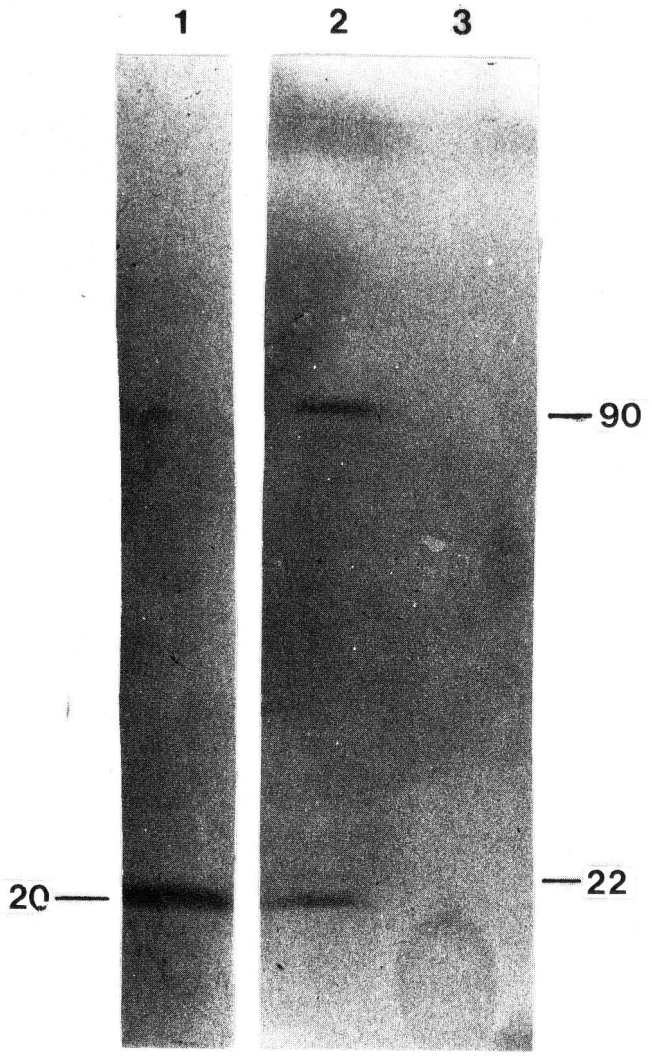


Fig 4—Protein immunoblotting analysis of MAB 1F1 (lane 1) together with a rabbit polyclonal serum (lanes 2 and 3) against Berne virus (lanes 1 and 2) and a mock preparation (lane 3). Note strong binding of MAB 1F1 to the 20-kD polypeptide. Three polypeptides can be identified in lane 2: a 90-kD peplomer protein, a faint line corresponding to the 22-kD envelope polypeptide, and the 20-kD nucleocapsid polypeptide.

shown). Consequently, these MAB were considered to recognize an epitope on the peplomer protein; using pulse-chase experiments, we have reported that the 200-kD glycoprotein represents an intracellular precursor of the heterogeneous (75 to 100 kD) peplomer proteins.[4] It must be concluded from the control (lane 5) that one of the lower molecular weight viral proteins, the 20-kD nucleocapsid protein, is bound to protein A when antibody is lacking.

When mock- and BEV-infected cell monolayers were incubated with a protein A-fluorescein isothiocyanate conjugate, fluorescence was observed inside the nucleus and to a lesser degree in the cytoplasm of most cells; less fluorescence was observed in the mock-infected cells (Fig 3).

Protein immunoblotting analysis—The MAB 1F1, which did not unequivocally react by use of radioimmune precipitation (because of the direct binding of the nucleocapsid protein to protein A; Fig 2), recognized the 20-kD polypeptide in immunoblots; this polypeptide was detected by a polyclonal rabbit serum (Fig 4) that also recognized the 90-kD peplomer protein. Nonspecific binding was observed in the BEV- and mock-infected lanes, whichever antiserum was used, and appeared to be mainly in the 65- to 70-kd range (data not shown). The antipeplomer MAB did not recognize the antigen after BEV prep-

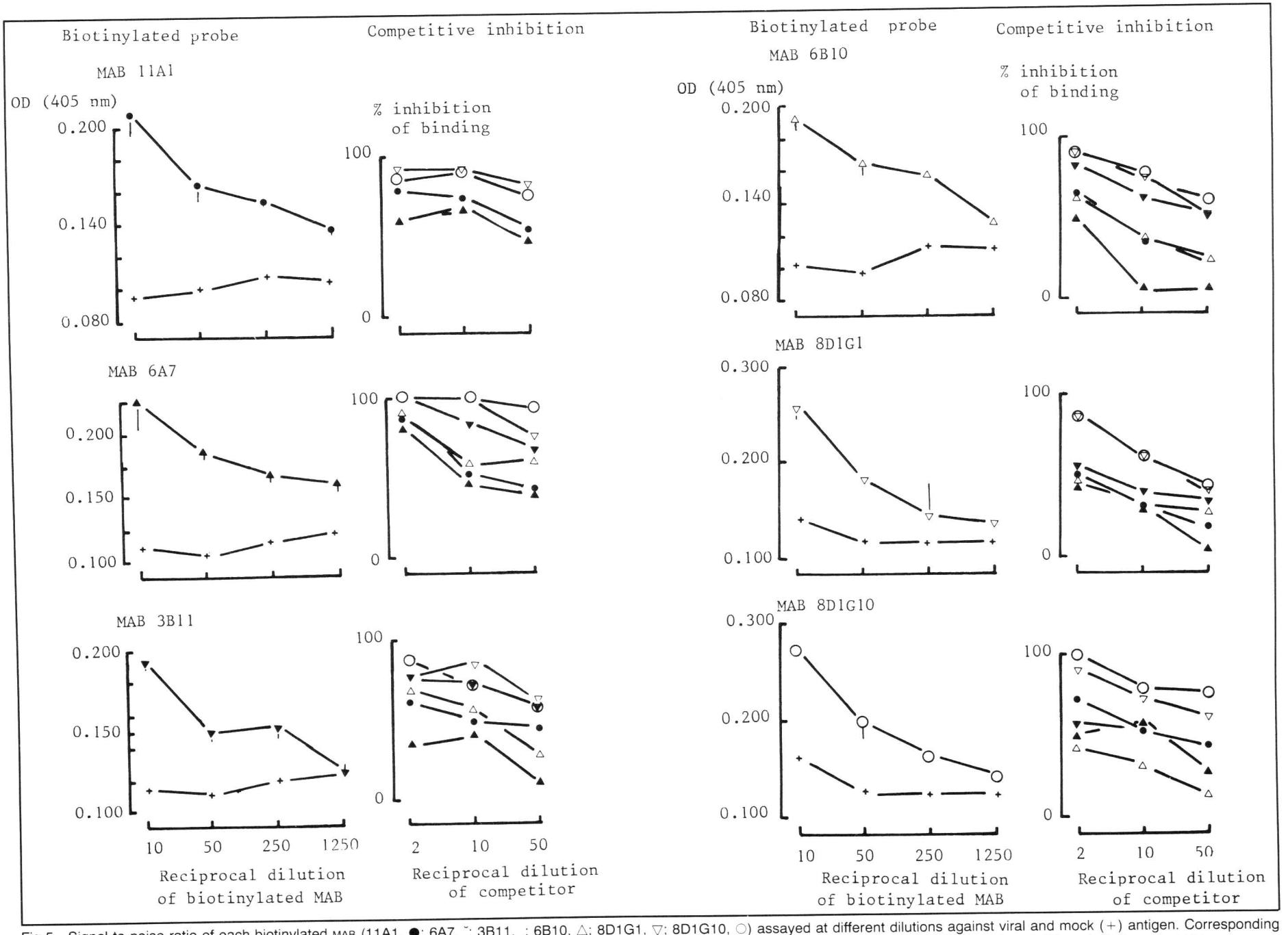


Fig 5—Signal-to-noise ratio of each biotinylated MAB (11A1, ●; 6A7, ˜; 3B11, ¸; 6B10, △; 8D1G1, ▽; 8D1G10, ○) assayed at different dilutions against viral and mock (+) antigen. Corresponding to each biotinylated probe used at a constant concentation (right panel), considerable competitive inhibition, using unlabeled MAB incubated with the probe at different dilutions, is seen.

TABLE 2—Single-step competitive ELISA, using unlabeled (competitor) MAB and their biotinylated counterparts (probe)

Competitor MAB	Ratio of competitor to probe	Biotinylated probes (% inhibition of binding)						Confidence interval*
		11A1	8D1G10	8D1G1	3B11	6B10	6A7	
11A1	30	27.7	72.1	50.4	61.8	62.6	87.8†	[50.4 to 86.8]
8D1G10	38	82.2	100.0	87.6	87.0	87.7	100.0	[80.5 to 100.7]
8D1G1	46	90.0	90.2	87.2	75.9‡	88.2	100.0†	[78.0 to 99.2]
3B11	60	73.1	57.4	57.2	74.1	81.5	100.0†	[51.8 to 96.0]
6B10	27	61.1	51.4	50.3	68.3	67.7	89.9†	[44.8 to 84.7]
6A7	65	58.3	52.4	47.2	33.1	49.7	80.4†	[32.0 to 74.9]

* Confidence interval of the mean is given at a 1% level of probability under the assumption that the competitor MAB has an identical inhibiting effect on each biotinylated MAB. † Above the upper limit of confidence interval. ‡ Below the lower limit of confidence interval.

TABLE 3—Neutralization index (NI)* of monoclonal antibodies (MAB) used alone or in pairs

First MAB	Second MAB						
	11A1	6A7	6B10	3B11	8D1G1	8D1G10	2F11
11A1	1	1.3	1.5	8.3†	1.6	2.3	0.9
6A7	...	1	0.5	1.4	1.0	2.0	1.0
6B10	...	...	1	5.7†	2.8	1.3	5.0†
3B11	...	...	...	1	2.8	4.1†	1.0
8D1G1	...	...	...	...	1	1.1	1.6
8D1G10	...	...	...	...	...	1	2.1
2F11	...	...	...	...	...	...	1

* NI = [(ND_{50} of the paired MAB 1 + 2)/ND_{50} (MAB 1) + ND_{50} (MAB 2)], where ND_{50} is the 50% neutralization dose. † Significantly different at a 5% level of confidence.

arations had been pretreated with SDS and 2-mercaptoethanol (data not shown).

Competition of MAB for epitopes on the peplomer protein—The reactivity of bio-MAB was assayed, using purified virus preparations. Results obtained for all antipeplomer MAB were similar to those obtained with MAB 11A1 (Fig 5). The optimal antigen concentration to be used in competition tests was determined for each bio-MAB and was found to correspond to 10^6 $TCID_{50}$/well. A specific reaction was obtained at virus concentration corresponding to infectivity titer between $10^{7.3}$ and 10^8 $TCID_{50}$/ml.

Competition binding was performed as a single-step test (Table 2). When the inhibition percentage was within the confidence intervals, it was an indication that competitor and probe antibody were physically interfering with each other's binding. With the exception of MAB 6A7, all MAB were able to inhibit the binding of all other MAB.

Enhancement of neutralization—By use of a few combinations (MAB 3B11 paired with MAB 11A1, 6B10, and 8D1G10; and MAB 6B10 paired with MAB 2F11), significant ($P < 0.05$) potentiation was observed (Table 3). Enhancement of neutralization was not recorded with combinations containing the antinucleocapsid MAB 1F1.

Discussion

Eight hybridomas secreting MAB against BEV were established; 7 were directed against the heterogeneous peplomer glycoprotein (75 to 100 kD)[4] and 1 (1F1) was directed against the nucleocapsid protein (20 kD),[3] as evidenced by results of radioimmune precipitation and protein immunoblotting. The system we used to inoculate mice seemed to favor induction of antibodies directed against surface proteins of BEV. The antipeplomer preparations had neutralizing and HAI activities. Incubation of virus preparations with SDS and 2-mercaptoethanol abolished binding of the antipeplomer MAB (data not shown), which suggested conformational rather than linear epitopes. Using a single-step competitive ELISA, a close topologic relationship was detected between 6 MAB; MAB 6A7 did not compete with the other MAB to the same extent. Because this MAB was the only IgG2b isotype in our collection, we hesitate to interpret this finding as an indication of a second antigenic site. Neutralization enhancement was observed in tests, using some MAB pairs, of which one partner was MAB 3B11, and between MAB 6B10 and 2F11; in view of the sensitivity of the epitopes to conformational changes, neutralization in vivo may be the result of cooperative binding of molecules with different specificities, leading to allosteric modifications of the peplomer protein.

Using indirect immunofluorescence, diffuse intracytoplasmic distribution of antigen was observed for the antipeplomer MAB 6B10; in contrast, peri- and intranuclear fluorescence was found for the antinucleocapsid MAB 1F1. Detection of the capsid protein in the nuclei of torovirus-infected cells supplements electron microscopic observation of intranuclear tubular structures in thin sections of infected cells.[7,10,11]

In immunoprecipitation experiments, direct binding of *S aureus* protein A to the 20-kD nucleocapsid polypeptide was detected. We have reported nonantibody-mediated binding of protein A to another viral nucleoprotein, namely to that of equine arteritis virus.[22] The intranuclear localization of the nucleocapsid protein of BEV was confirmed, using fluorescein isothiocyanate-conjugated protein A. So far, toroviruses are the only positive-stranded RNA viruses for which a host cell nuclear function is required for replication.[23]

Three MAB are candidates for use in BEV-specific detection systems. Monoclonal antibodies 1F1 and 6B10 are valuable tools to use in immunofluorescent staining to detect the nucleocapsid and the peplomer polypeptides, respectively, in infected cells. Monoclonal antibody 11A1 has been found suitable as a capture antibody for use in an ELISA. Preliminary experiments yielded satisfactory results with purified and crude antigen and confirmed the presence of torovirus antibodies in different mammals including pigs (data not shown). Earlier, we reported similar results, using neutralization tests.[24] Serum neutralization is highly specific, and antigenically distant toroviruses are unlikely to induce cross-reacting antibody. To document this point, studies with human toroviruses may be quoted; irrespective of their presence in feces and their aggregation by anti-BEV and anti-Breda virus sera,[8,9] neutralizing antibodies were never detected in human beings.[24,25] Therefore, a more comprehensive

test system is required. We developed the ELISA to study epidemiologic features of torovirus infections in swine populations. On the other hand, it may allow prescreening of fecal samples before isolation attempts are made in cell culture. In pigs, electron microscopic evidence of torovirus particles in feces has been obtained.[1,26]

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