Sensitivity of Infectious Bovine Rhinotracheitis Virus to Ether

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The sensitivity of 12 field isolates of infectious bovine rhinotracheitis (IBR) virus and four commercial modified-live infectious bovine rhinotracheitis virus vaccine strains was determined after exposure to 20% ethyl ether (anesthetic) for 16 h at 4 C. The infectivity of five field strains was reduced by varying degrees, whereas seven were found to be resistant. Three vaccine strains were moderately sensitive, and one strain was resistant. Four of the sensitive field strains were isolated from the conjunctiva and the other was isolated from the surface of the epiglottis of natural infective cattle. Strains of virus isolated from fetal tissue and nasal cavity were resistant. All viruses were readily inactivated by chloroform treatment.

The herpesvirus group is composed of relatively large, ether-sensitive deoxyribonucleic acid viruses (1). Infectious bovine rhinotracheitis (IBR) virus, a bovine herpesvirus, has been shown to be ether sensitive by subjecting the virus to ethyl ether treatment under varying laboratory conditions (3, 6, 8, 11). The mechanism of inactivation is by direct action of the solvent on the outer lipid-containing envelope of the virion.

During routine characterization studies of field isolates in this laboratory, many ether-resistant IBR strains were encountered. It was considered that possibly some isolates were of vaccine origin because of the wide use of modified live-virus vaccines. It was postulated that perhaps during the attenuation process some characteristic related to the lipid-containing membrane had been altered, resulting in an increased resistance to the action of ether.

With this hypothesis, the ether sensitivity of vaccine and field strains of IBR virus was determined and related to their passage level in cell culture and animal virulence. In addition, the site of virus isolation was correlated with the viruses reaction to ether. This report describes the results.

MATERIALS AND METHODS

Cell cultures. Madin-Darby bovine kidney (MDBK) cells were grown in tubes and flasks with Eagle minimal essential medium with nonessential amino acids, glutamine 0.5% lactalbumin hydrolysate plus 10% fetal bovine serum, and antibiotics. Minimal essential medium with 2% fetal bovine serum was used as maintenance medium. Primary bovine

embryonic kidney cells in tubes were obtained from a commercial source. Medium 199 plus 2% fetal bovine serum was used for maintenance. All cell culture media contained penicillin (200 U/ml), streptomycin (200 µg/ml), and amphotericin B (2.5 µg/ml).

Specimens. Conjunctival and nasal swabs were collected from feedlot cattle during disease outbreaks and were stored in medium at -70 C until tested. Tissue specimens were obtained at necropsy and were stored at -70 C.

Virus isolation. Small flasks or tubes of MDBK cells were inoculated with clinical materials prepared as previously described (4, 5). Cells and fluid were frozen when approximately 70 to 80% of the cell layer showed a cytopathic effect. Before testing, each virus preparation underwent three freeze-thaw cycles. Virus isolation studies on nasal swabs from vaccinated and inoculated animals were performed by the same procedure.

Viruses. Four different commercial, modified, live-virus vaccines designated as V1, V2, V3, and V4 were used (Table 1). The source and passage histories of field strains are listed in Table 2. Field strain 9565 was terminally diluted three times in MDBK cells and the 10th cell culture passage material was used to inoculate the calves. Control viruses consisted of Colorado 1 strain of IBR, and three strains of bovine para-influenza-3 (PI-3) and bovine enterovirus type 1.

Ether and chloroform studies. Sensitivity to ether was determined by the methods described by Andrews and Horstmann (2). Undiluted virus (2.0 ml) was mixed with 20% (0.5 ml) anesthetic ethyl ether and held for 16 h at 4 C. Ether was removed by evaporation and the virus was assayed for infectivity. The technique employed for the chloroform studies was that of Feldman and Wang (7). One milliliter of undiluted virus was mixed with 0.05 ml

of chloroform, and the mixture was rotated at room temperature for 10 min. The chloroform was removed by centrifugation and the virus suspension was titrated. Untreated virus served as a control and was titrated simultaneously with the treated virus. The PI-3 and bovine enterovirus type I viruses were not included in each day's test but were tested periodically throughout the study.

Virus titrations. Serial 10-fold dilutions of virus were prepared in minimal essential medium and 0.1 ml of each dilution was inoculated into each of four culture tubes. The cells were incubated at 36 C and examined for cytopathic effects. The infectivity titers were determined by the method of Reed and Muench (10) and expressed as mean tissue culture infective dose per 0.1 ml.

Virus identification. The viruses were identified as IBR virus by the microtiter technique employing MDBK cells and known IBR immune serum. The IBR immune serum was prepared in IBR seronegative rabbits by multiple intravenous inoculations of the Colorado I virus which had been terminally diluted three times in MDBK cells.

Animal inoculation. Unvaccinated feeder calves known to be seronegative for IBR antibodies were

used. Two calves were vaccinated intranasally with vaccine 1 (Nasalgen) as directed by the manufacturer (Jensen-Salsbery Laboratories, Kansas City, Mo.). Field virus (9565) was administered intranasally to calves with a gas-powered atomizer. Nasal swabs were obtained before and after virus exposure.

RESULTS

Three of the four vaccine viruses tested exhibited varying degrees of ether sensitivity, and one vaccine virus was resistant to inactivation by ether (Table 1). The percentage of reduction in infectivity of the three sensitive vaccine viruses ranged from 25 to 70%, whereas a slight increase in infectivity was demonstrated with the more resistant virus. After additional cell culture passages, vaccine 1 became more resistant to ether.

The effect of ether on field isolates is shown in Table 2. The IBR isolates from the conjunctiva were more sensitive to ether than were isolates from the other anatomical sites which, with the exception of the epiglottis isolate, were all resistant to inactivation by ether. The conjunctiva isolate from animal number 11316A was sensitive to ether, whereas an isolate from the feces of the same animal was resistant. Both isolates from animal number 11316B were resistant. These four isolates were recovered from two different animals in the same herd during an acute IBR epizootic. Field strain 9565 was also ether resistant at the 10th cell passage level prepared after purification by three terminal dilution passages.

The laboratory strain of IBR virus was ether

TABLE 1. *Effect of 20% ethyl ether on IBR virus in modified live-virus vaccines*

Vaccine	Titer (TCID ₅₀ /0.1 ml ^a)		Difference in titer (Log ₁₀)
	Treated	Control	
V1	4.5	5.0	-0.5
V2	4.5	4.6	-0.1
V3	5.0	4.8	+0.2
V4	4.3	4.5	-0.2

^a TCID₅₀, Mean tissue culture infective dose.

TABLE 2. *Effect of 20% ethyl ether on field strains of IBR virus isolated from various anatomical sites of nonvaccinated cattle*

Virus identification	Isolation site	Cell culture passage number	Titer (TCID ₅₀ /0.1 ml ^a)		Difference in titer (Log ₁₀)
			Treated	Control	
11316-A	Conjunctiva	1	3.5	5.5	-2.0
11316-A	Feces	2	5.0	5.0	0.0
11316-B	Conjunctiva	2	5.4	5.2	+0.2
11316-B	Nasal	2	5.6	5.6	0.0
12882	Fetal liver	1	7.0	7.0	0.0
7898	Epiglottis	2	6.6	7.3	-0.7
3709	Fetal lung	2	6.5	6.5	0.0
8876	Conjunctiva	1	3.5	6.5	-3.0
1683	Conjunctiva	2	4.5	6.0	-1.5
9611	Conjunctiva	3	5.5	6.0	-0.5
9565	Nasal	4	7.0	6.6	+0.4
10266	Nasal	1	5.6	5.0	+0.6
IBR ^b			6.6	6.5	+0.1
PI-3			2.3	7.5	-5.2
BEV type I			6.2	6.2	0.0

^a TCID₅₀, Mean tissue culture infective dose.

^b Colorado I laboratory strain, isolated in 1955. Passage level unknown. BEV, Bovine enterovirus.

resistant. The three strains of PI-3 virus were highly sensitive and the bovine enterovirus was resistant in replicate tests. Although the control viruses were not included in each test, both sensitive and resistant IBR strains were demonstrated in tests in which PI-3 was shown to be ether sensitive.

The two calves vaccinated with vaccine 1 did not develop clinical illness. Field strain 9565 induced a severe upper respiratory disease in both animals, which was manifested by fever, depression, excessive salivation, and a nasal discharge. Viruses identified as IBR were isolated from the nasal secretions of each of the four animals.

The reactions to ether of vaccine 1 virus and field isolate 9565 after one passage in calves are shown in Table 3. Vaccine virus isolates from both calves A and B were resistant to ether. The field isolate (9565) recovered from calf C was highly sensitive to ether, whereas the virus recovered from calf D was resistant.

All strains of IBR virus used in this study were completely inactivated by chloroform.

DISCUSSION

Under the conditions of this study, differences were demonstrated in the ether sensitivity of both field and vaccine strains of IBR virus.

Since the vaccine strains were shown to be relatively resistant to ether, it was thought initially that perhaps some of the resistant field strains recovered from animals were of vaccine origin. However, all viruses reported in Table 1 were from known nonvaccinated animals. Isolates from cattle vaccinated with field strains were later shown to react similarly to the field strains after ether treatment.

Other factors which may have influenced the sensitivity of the virus were examined. The possibility that the passage level of the virus was a factor in resistance to ether was rejected after both sensitive and resistant low passage field strains were found. Further, Armstrong et al. (3) reported a 100-fold reduction in titer of an IBR strain after 100 passages after overnight treatment with 20% ether.

The cell culture type in which the viruses were isolated and propagated was considered as possibly having an influence on the ether resistance of the virus. However, IBR viruses isolated in other cell culture hosts were also found to vary in their sensitivity, as did those shown in Table 1. Replicate tests with different lots of ether and reduced volumes of virus and ether did not influence the results.

The finding that most isolates from the conjunctiva were more sensitive to ether suggests that perhaps their outer membrane differs from

TABLE 3. *Effect of 20% ethyl ether on modified live-virus IBR vaccine and field virus after one calf passage*

Determinants	Titer (TCID ₅₀ /0.1 ml ^a)		Difference in titer (Log ₁₀)
	Treated	Control	
V1 + Calf A	6.5	6.6	+0.1
V1 + Calf B	7.0	7.0	0.0
9565 + Calf C	3.2	6.0	-2.8
9565 + Calf D	5.6	5.5	+0.1

^a TCID₅₀, Mean tissue culture infective dose.

that of viruses recovered from other tissues. The difference in the action of ether on the field virus after animal passage is as yet unexplained. Although the field virus produced severe illness in calves, the nasal isolate from one calf was resistant to ether to the same degree as the virus recovered from animals vaccinated with an attenuated strain. The results suggest that ether-sensitive viruses are not of vaccine origin. Although the vaccine strains were shown to be resistant to ether, it is not proposed that this procedure be used to differentiate vaccine from field isolates.

Since infectivity was reduced by chloroform treatment, it was assumed that the virus preparations did not contain nonenveloped, infectious IBR particles or a resistant viral contaminant. Further, it seems unlikely that a viral contaminant resistant to lipid solvents was mixed with the IBR isolates, since field strain 9565 was shown to be resistant before and after purification by three terminal dilution passages.

In some ether-treated samples the infectivity titers were slightly higher than those of the controls. This observation and the differences demonstrated in the ether sensitivity of the IBR viruses tested are unexplained. However using different techniques, Rouhandeh et al. (11) reported similar or slightly higher infective titers of IBR virus after 2 h of exposure to ether as compared with those obtained after a reaction time of 10 min.

Some members of the poxvirus group are reported to differ in their sensitivity to ether (2, 3). A strain of bovine papular stomatitis virus was recently shown to be resistant to 20% ether but sensitive to chloroform when treated by the technique described in this paper (5).

The treatment of IBR isolates with ether by the methods described in this study was not satisfactory for determining sensitivity to lipid solvents. However, chloroform, known to be a more efficient lipid solvent, inactivated all the viruses. The results dictate caution in the use of ether treatment to determine the presence of

lipid-containing membranes of IBR field viruses.

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