Differentiation of Strains of Infectious Bovine Rhinotracheitis Virus by Neutralization Kinetics with Late 19S Rabbit Antibodies

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Two vaccine, two respiratory (infectious bovine rhinotracheitis [IBR]), and two genital (infectious pustular vulvovaginitis [IPV]) strains of infectious bovine rhinotracheitis virus were compared by neutralization kinetics using late 19S antibody (AB). The two vaccine strains were indistinguishable from one another, but were neutralized far more rapidly than the other four strains when either anti-IBR or anti-IPV 19S AB was used. The two IPV strains were indistinguishable from one another, but were neutralized significantly more rapidly than the two IBR strains when anti-IBR 19S AB was used. The 2 IBR strains were neutralized at a similar rate with the latter globulin preparation. Almost identical results were obtained with anti-IPV 19S AB, except that one IPV strain was neutralized at a rate similar to the IBR strains. However, when early and late rabbit 7S AB were used, IBR strains could not be distinguished from IPV strains by neutralization kinetics. Preliminary experiments indicated that both early and late 19S rabbit antibodies neutralized the homologous strain more rapidly than the heterologous strain, but the difference was more noticeable with late 19S AB. It was also determined that neutralization of IBR-IPV virus by specific early and late 19S rabbit AB and early 7S rabbit AB was markedly enhanced by guinea pig complement. Neutralization of this virus by late 7S AB, however, was only slightly enhanced by complement. These results suggest that vaccine strains of IBR-IPV virus may be distinguished by neutralization kinetics with late 19S rabbit AB, and that genital and respiratory strains may possibly also be distinguishable with some 19S AB preparations.

Infection in cattle with the infectious bovine rhinotracheitis-infected pustular vulvovaginitis (IBR-IPV) virus has been associated with respiratory disease (17), genital disease (13), conjunctivitis (1), encephalitis (5), and abortion (15). The IBR-IPV virus strains originating from cattle with these varied disease syndromes have been shown to be antigenically homogeneous by reciprocal neutralization (1, 5, 6, 15, 18–20, 23, 27), by immunodiffusion (19), and by neutralization kinetics (3) with specific antisera. In one study, both a genital and a respiratory strain produced large and small plaques, and cloned populations of these variants were neutralized equally well by antiserum to mixed and to large plaque variants (20).

Antigenic differences between some IBR-IPV virus strains have been reported, but these differences did not correlate with the origin of the isolates (4, 12). An intestinal strain was related reciprocally to a brain isolate but non-reciprocally to a respiratory isolate when tested by reciprocal serum-virus neutralization (7).

This intestinal strain was found by neutralization kinetics to differ significantly from a respiratory strain, a vaccine strain, and a brain isolate (4). The latter three strains, however, had similar neutralization constants (4). In another neutralization kinetics study, two respiratory strains and one genital strain were closely related, but differed significantly from a second genital strain (12). However, it has been reported that genital and respiratory strains of IBR-IPV virus may be differentiated by carrier-free zone electrophoresis in a glucose density gradient, even though the strains were serologically identical (22, 26).

The purpose of this study was to compare by neutralization kinetics the capacity of various globulin fractions derived from specific rabbit antisera to detect antigenic differences between vaccine, genital, and respiratory strains of IBR-IPV virus.

MATERIALS AND METHODS

Cell cultures. The Madin Darby bovine kidney cell
line (MDBK; 21) and a strain of embryonic bovine tracheal cells (EBT; supplied by P. C. Smith, National Animal Disease Laboratory, Ames, Iowa) were used for the propagation and assay of viruses.

**Cell medium.** Cells were propagated in Eagle minimum essential medium with Earle salts, t-glutamic acid, and nonessential amino acids (MEM) (Grand Island Biological Co., Grand Island, N.Y.). Where a semisolid medium was required for the plating of viruses in cell monolayers, 0.6% Special Agar-Noble (Difco Laboratories, Detroit, Mich.) in Eagle basal medium with Hanks salts and t-glutamic acid (Grand Island Biological Co., Grand Island, N.Y.) was used.

Ten percent fetal calf serum (FCS; Grand Island Biological Co., Grand Island, N.Y.) was included in MEM after the initial seeding of cells, but 4% FCS was used for the maintenance of cells and in agar overlays.

**Viruses.** The virus strains used in this study are listed in Table 1. All the viruses except the vaccine strains were plaque purified three times before commencement of the experiments.

**Virus assay.** Viruses were assayed by the plaque method with EBT cell monolayers propagated in 35-mm petri dishes. Serial 10-fold virus dilutions were made in saline, which consisted of 0.11% glucose, 0.6% NaCl, 0.04% KCl, 0.015% NaHPO₄, 0.015% KH₂PO₄, 0.015% MgSO₄, 7H₂O, 0.1% lactalbumin hydrolysate, 0.0016% CaCl₂, 2H₂O, and 0.00012% phenol red. The medium was removed from the petri dishes containing the EBT cell monolayers, and 1 ml of every serial virus dilution was inoculated into each of two dishes. Virus adsorption was allowed to take place for 1 h at 37°C, after which the virus preparation was replaced with 2 ml of MEM supplemented with 4% FCS and 0.5% specific IBR-IPV virus rabbit antisera. These cells were then incubated for 30 to 48 h for the plaques to develop and then fixed for 10 min by the addition of 2 ml of 20% formalin into each petri dish. The fixed monolayers were rinsed with distilled water and stained by pipetting 1 ml of 5% crystal violet in 20% ethanol into each petri dish. The stain was rinsed out of the petri dishes after 5 min, and the plaques were counted.

**Neutralization kinetics.** Kinetic neutralization of the viruses was carried out as described by McBride (16). Saline, supplemented with 1% heat-inactivated FCS, was used as a diluent in this procedure. Pooled fresh guinea pig serum diluted 1:8 was used as a source of complement. Each batch of guinea pig serum was checked for nonspecific antiviral activity prior to its use in the test. The viruses were diluted to contain 2 × 10⁴ plaque-forming units per ml, while antibody (AB) preparations were diluted so that 0.5 ml neutralized approximately 9 × 10⁴ plaque-forming units (90% of a 0.5-m) preparation) of the homologous virus in approximately 15 min. All the reagents were chilled to 4°C prior to the test. The test was carried out by rapidly mixing 0.5 ml of a virus preparation, 0.5 ml of rabbit antiserum or globulins derived from such serum, and 0.1 ml of complement. A 0.1-ml portion of the mixture was immediately diluted 100-fold by blowing it into 9.5 ml of chilled diluent. The rest of the virus-antiserum mixture was incubated in a water bath at 37°C for 15 min. Portions (0.1 ml) were taken every 5 min from the incubating mixture and diluted as above. The amount of virus present in each diluted portion was then determined by the plaque method. A statistical estimate of the rate of virus neutralization was calculated by linear regression and applying the Student’s t test. The neutralization rate constant (K) for a virus and a particular antiserum was calculated by the method of McBride (16). “Normalized” neutralization rate constants (NK) were calculated for each virus strain, where neutralization rate with the homologous antiserum was arbitrarily set at 100 and neutralization of the other strains by the same antiserum was rated proportionately according to their K values.

**Antiserum production.** Viral preparations used for the immunization of New Zealand albino rabbits were propagated in 16 × 10⁶ MDBK cells, partially purified by two cycles of differential centrifugation, and concentrated to 4 ml by vacuum filtration through dialysis tubing. The MDBK cells were infected at a multiplicity of 5 to 10 PFU per cell.

One ml of the above viral antigen was emulsified in 1 ml of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.,) which was then inoculated intradermally and into the rear footpads of a rabbit. Each footpad received 0.2 ml, and 0.1 ml was inoculated intradermally at 16 sites. Three weeks later a similar inoculation was made, except that incomplete Freund adjuvant was used and the footpad inoculations were not repeated. After a further 3 weeks, 1 ml of the viral antigen was administered intravenously to the rabbits. The rabbits were bled by cardiac puncture 10 days after the first inoculation, and again 10 days after the last inoculation. The serum collected from these blood samples was stored at −20°C and labeled “early serum” and “late serum,” respectively.

**Rabbit globulin purification.** Rabbit globulins were prepared from the rabbit antisera by two cycles of gel filtration through a Sephadex G200 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) column. Each 19S globulin preparation was tested for contamination with 7S globulins by immunoelectrophoresis, using sheep anti-rabbit globulin (Pentex Biochemical, Kankakee, Ill.). The 7S globulin preparation was tested for 19S contamination in the same way. Each globulin fraction was concentrated by vacuum filtration through dialysis tubing to the original volume of the serum from which it was prepared. All the globulin preparations were dialyzed against the saline

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**Table 1. Strains of IBR-IPV virus used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of cell culture passages</th>
<th>Origin</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>650</td>
<td>8</td>
<td>Respiratory tract</td>
<td>C. J. Maré</td>
</tr>
<tr>
<td>1300</td>
<td>9</td>
<td>Respiratory tract</td>
<td>C. J. Maré</td>
</tr>
<tr>
<td>K22</td>
<td>29</td>
<td>Genital tract</td>
<td>D. G. McKercher*</td>
</tr>
<tr>
<td>Steiner</td>
<td>11</td>
<td>Genital tract</td>
<td>D. G. McKercher</td>
</tr>
<tr>
<td>Jensen</td>
<td>111</td>
<td>Vaccine</td>
<td>Jensen*</td>
</tr>
<tr>
<td>Diamond</td>
<td>82</td>
<td>Vaccine</td>
<td>Diamond*</td>
</tr>
</tbody>
</table>

* D. G. McKercher, School of Veterinary Medicine, Davis, Calif.
* Jensen-Salisbury Laboratories, Kansas City, Mo.
* Diamond Laboratories Inc., Des Moines, Iowa.
diluent prior to use in the neutralization kinetics procedure. The globulins were filtered through 450-nm membrane filters (Millipore Corp., Bedford, Mass.) and stored at 4°C for a maximum of 4 weeks. To prevent aggregation of the 19S globulins, an equal volume of FCS was added to the preparations prior to filtration, and then the preparations were subjected to ultrasonic treatment for 30 s with a Bronwill Biosonic sonicator (Will Scientific, Inc., Rochester, N.Y.) set at the 10% position.

Antiserum absorption. In some instances, antisera and globulins derived from such sera were absorbed one to three times with either 12 × 10⁶ EBT cells or 20 × 10⁶ MDBK cells per ml of serum. Prior to the absorption procedure, the cells were subjected to three cycles of rapid freezing and thawing. Absorption was allowed to take place for 2 h at room temperature before the cell debris was removed by centrifugation for 20 min at 800 × g, followed by filtration through a 220-nm filter.

RESULTS

Virus assay procedure. The method of achieving plaque formation with a liquid medium containing specific antiserum (plaquing medium) was found to be very convenient and resulted in clearer plaques than with an agar overlay. The concentration of late rabbit anti-IBR-IPV serum in the plaquing medium was found to be important. The minimal antiserum concentration which still allowed plaque formation was 0.125 to 0.25%. Concentrations of antiserum in liquid overlays below this minimum resulted in indistinct plaques which were difficult to count. Antiserum concentrations greater than 2% were found to be toxic to the cells. However, absorption of antiserum with EBT cells prior to its use in the liquid overlay considerably reduced the toxic effects of the antiserum.

A 0.5% concentration of unabsorbed antiserum was subsequently routinely used in the plaquing medium for the assay of the IBR-IPV virus strains. Plaques first became visible under the liquid plaquing medium after approximately 30 to 36 h of incubation at 37°C, and were usually counted at 48 h postinfection. Such plaquing medium has been stored for up to 11 months at 4°C and was still usable. An example of typical virus plaques which developed under plaquing medium may be seen in Fig. 1.

Neutralization kinetics. Preliminary investigations were conducted with neutralization kinetics to examine the role of complement in the neutralization of IBR-IPV virus by specific early and late rabbit antisera and by purified globulins derived from such sera. The guinea pig sera used in these trials did not affect the titer of IBR-IPV virus significantly in the presence of normal rabbit serum or saline.

Early 19S, late 19S, and early 7S AB required complement for the neutralization of IBR-IPV virus (Fig. 2, 3, and 4). In contrast, late 7S AB efficiently neutralized this virus in the absence of complement, but the rate of neutralization was nevertheless still slightly enhanced by complement (Fig. 5). The complement requirements of whole antiserum resembled that of 7S AB. Early antiserum was complement dependent, whereas late antiserum efficiently neutral-
Differeniation of Strains of IBR Virus

The presence of neutralizing homologous 19S (immunoglobulin M [IgM]) was observed in the absence of complement (c), but rapid neutralization was observed in the presence of complement.

Neutralization of 650 virus (IBR) by late homologous 7S AB (immunoglobulin G [IgG]). The virus was rapidly neutralized only in the presence of complement (c).

Neutralization of 650 virus (IBR) by late homologous 7S AB (immunoglobulin G [IgG]). The virus was rapidly neutralized only in the presence of complement (c).

Neutralization of 650 virus (IBR) by late homologous 7S AB (immunoglobulin G [IgG]). The virus was rapidly neutralized in the absence of complement (c), but complement did seem to slightly enhance the neutralization rate.

The rates at which late anti-650 19S AB neutralized IBR-IPV virus strains is presented in Fig. 6. The K and NK values representing neutralization by this globulin preparation are listed in Table 2. A 1:3 dilution of late anti-650 19S AB was used in these trials. The two genital strains (K22 and Steiner) could not be distinguished from one another, nor could the two respiratory strains (650 and 1309) be separated on the basis of their neutralization rates. Significantly, the genital strains were neutralized more rapidly (P < 0.05) than the respiratory strains. The two vaccine strains were also indistinguishable from one another, and were neutralized far more rapidly than either the respiratory or the genital strains.

The rates of neutralization of IBR-IPV virus strains by late anti-K22 19S AB are given in Fig. 7. The K and NK values of the virus strains...
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FIG. 6. Neutralization of IBR-IPV virus strains by late anti-650 (anti-IBR) 19S globulins (immunoglobulin M [IgM]). Note that the genital strains (K22 and Steiner) were neutralized more rapidly than the respiratory strains (650 and 1309). The vaccine strains (Diamond and Jensal), however, were neutralized more rapidly than the genital strains.

TABLE 2. Neutralization rate constants (K) and normalized neutralization rate constants (NK) of IBR-IPV virus strains using late anti-650 19S AB

<table>
<thead>
<tr>
<th>Virus</th>
<th>K</th>
<th>NK</th>
</tr>
</thead>
<tbody>
<tr>
<td>K22</td>
<td>0.078</td>
<td>169.6</td>
</tr>
<tr>
<td>Steiner</td>
<td>0.069</td>
<td>150.0</td>
</tr>
<tr>
<td>650</td>
<td>0.046</td>
<td>100.0</td>
</tr>
<tr>
<td>1309</td>
<td>0.047</td>
<td>102.2</td>
</tr>
<tr>
<td>Jensal</td>
<td>0.149</td>
<td>323.9</td>
</tr>
<tr>
<td>Diamond</td>
<td>0.156</td>
<td>339.1</td>
</tr>
</tbody>
</table>

TABLE 3. Neutralization rate constants (K) and normalized neutralization rate constants (NK) of IBR-IPV virus strains using late anti-K22 19S AB

<table>
<thead>
<tr>
<th>Virus</th>
<th>K</th>
<th>NK</th>
</tr>
</thead>
<tbody>
<tr>
<td>K22</td>
<td>0.080</td>
<td>100.0</td>
</tr>
<tr>
<td>Steiner</td>
<td>0.041</td>
<td>51.3</td>
</tr>
<tr>
<td>650</td>
<td>0.049</td>
<td>61.3</td>
</tr>
<tr>
<td>1309</td>
<td>0.045</td>
<td>56.3</td>
</tr>
<tr>
<td>Jensal</td>
<td>0.222</td>
<td>277.5</td>
</tr>
<tr>
<td>Diamond</td>
<td>0.265</td>
<td>331.5</td>
</tr>
</tbody>
</table>

with this AB preparation are listed in Table 3. A 1:2 dilution of late anti-K22 19S AB was found, in preliminary tests, to be the most suitable dilution for use in these kinetic neutralization trials. The two respiratory strains (650 and 1309) and the one genital strain (Steiner) were neutralized at approximately the same rate, whereas the homologous genital strain (K22) was neutralized significantly more rapidly ($P < 0.05$) than the former three strains. The two vaccine strains were neutralized at a similar rate, and both were neutralized much more rapidly than either the genital or the respiratory strains.

DISCUSSION

Preliminary results suggested that IBR-IPV virus strains could not be differentiated by their
neutralization rate using either early or late 7S AB. In contrast, both early and late 19S AB neutralized the homologous strain more rapidly than the heterologous strain. Late 19S AB was thus used for comparing the various virus strains by neutralization kinetics, since larger amounts of late antiserum were available and the difference in neutralization rate between the homologous and heterologous strain seemed greater with the late 19S AB. In addition, Hampar et al. (10) reported that late 19S AB was four to eight times more efficient than early 7S, late 7S, or early 19S AB in distinguishing herpes simplex virus strains by neutralization kinetics. Similar results were also observed when late 19S AB were compared with the other immunoglobulins in distinguishing strains of simian cytomegaloviruses (9) and in differentiating between SA8 herpesvirus and herpes simplex virus (24). The results reported here with IBR-IPV virus were similar except that early 19S AB seemed to approach the specificity of late 19S AB.

Neutralization kinetics with IBR-IPV virus strains using late 19S AB revealed significant differences between some of the strains. An unexpected and striking observation was the extremely rapid neutralization of the vaccine strains by both the antigenal (anti-K22) and the antirespiratory (anti-650) globulin preparations. The two vaccine strains could not, however, be distinguished from one another by their rates of neutralization with either anti-650 or anti-K22 19S AB. The two respiratory strains, 650 and 1309, had similar neutralization rates when anti-650 19S AB was used. The somewhat surprising result with the genital strains (K22 and Steiner) was that they were neutralized significantly more rapidly than the homologous respiratory strain. These genital strains were, however, indistinguishable using the later globulin preparation. Thus, with anti-650 19S globulin, the respiratory, genital, and vaccine strains each had their distinctive rates of neutralization. It seems that with this globulin preparation the potential does exist for distinguishing strains of IBR-IPV virus by neutralization kinetics according to their origin. However, before a definitive position can be adopted, a study including a larger number of strains should be undertaken. Such a study is presently being considered.

The distinction between strains was not as clear when anti-K22 19S AB was used. One genital strain (Steiner) behaved like the two respiratory strains. The homologous virus (K22) was, however, neutralized more rapidly than the two respiratory strains and the other genital strain.

In this study, several heterologous viruses were neutralized more rapidly than homologous strains, but the reason for this is not clear. Apart from the origin of these virus strains, the only other known difference between them is their passage level in cell cultures. The vaccine strains had been passaged 82 to 111 times in bovine cell cultures and in cells derived from other animal species. Steiner virus had been passaged approximately 11 times and K22 virus 29 times in bovine cell cultures. The two respiratory strains had been passaged the smallest number of times (approximately eight to nine times in bovine cell cultures). It is tempting to speculate that the rate by which these viruses were neutralized by late 19S AB was, in some way, a function of the number of passages they had undergone in cell cultures. This conclusion seems to correlate with the results of neutralization kinetics with anti-K22 19S AB, since Steiner virus was neutralized at a rate similar to the two respiratory strains. The passage level of Steiner virus is approximately equal to that of the respiratory strains, but less than half that of K22, the other genital strain. In addition, the two vaccine strains were neutralized considerably more rapidly than the homologous virus when either anti-650 or anti-K22 19S AB was used. In an earlier study with whole antiserum, Buening and Gratzek (4) also observed the phenomenon where a heterologous strain of IBR-IPV virus was neutralized more rapidly than the homologous strain. However, the cell culture passage levels of the virus strains were not considered in their study. The above theory would require that cell adaptation of a virus enhances the rate at which it is neutralized by AB to an unadapted strain. How cell adaptation could enhance neutralization of a virus is not known, but it is recognized that herpesviruses may acquire new antigens after numerous passages through cell cultures (8). It does not seem possible, however, that new antigens can influence the neutralization of a virus to antibodies which do not have a specificity directed against these antigens. The answer may lie in the reported alteration of the virion surface charge of viruses after passage in cell cultures (22). Since the binding of AB to antigen is known to be influenced by the relative charge on each (2), it is possible that the alteration in the viral charge due to passage in cell cultures could enhance the binding of specific neutralizing AB to the virus.

Many workers have reported that IBR-IPV
virus strains form an antigenically homogeneous group (1, 5, 6, 15, 18-20, 23, 27), but some did find strain differences by neutralization kinetics (4, 12). In these latter studies, whole serum was employed and strains could not be classified according to their origin. Matheka and Straub (22) and Straub et al. (26) reported that genital and respiratory strains were distinguishable by carrier-free zone electrophoresis based on differences in the surface charges of the virions. However, they found that these charge differences disappeared after several cell culture passages. The unique antigenic nature of the vaccine strains of IBR-IPV virus which we observed has apparently not been reported in the literature.

The action of complement on the neutralization of IBR-IPV virus by globulins purified from specific rabbit antisera was similar to what has been reported for herpes simplex virus (11). Early 7S, early 19S, and late 19S globulins derived from specific rabbit antisera were almost completely dependent on complement for the neutralization of IBR-IPV virus. In contrast, late 7S AB efficiently neutralized this virus in the absence of complement. Complement did appear, however, to slightly increase the rate by which IBR-IPV virus was neutralized by late 7S AB.

Neutralization of IBR-IPV virus by early whole antisera was also complement dependent. This was expected, since both 7S and 19S AB derived from early antisera were complement dependent. It is not known, however, to what extent either of the latter two globulin types in whole serum contributes to the neutralization of a virus in the presence of complement. The dependence of early antisera on complement for virus neutralization has also been observed with herpes simplex virus (14). In the present study, late immune sera were not dependent on complement for neutralization of IBR-IPV virus, indicating that the 7S globulin component of these sera dominated over the 19S component. The rate at which IBR-IPV virus was neutralized by late antisera was slightly enhanced by complement, which is similar to what was observed with 7S globulin derived from such serum.

The use of specific antisera in liquid medium to achieve plaquing with IBR-IPV virus was preferred to an agar overlay, since the former method was more convenient, clearer plaques were produced, and the results were more reproducible. Stevens and Groman (25) observed that secondary IBR-IPV virus plaques developed when this method was used, which they attributed to detachment, translocation, and reattachment of infected cells. They were, however, able to distinguish between the primary and the considerably smaller secondary plaques. In this study very few of the secondary plaques were observed, since the concentration of antisera used in the plaquing medium (0.5%) allowed the counting of the plaques after a relatively short postinfection period (30 to 48 h). Antiserum concentrations above 2% resulted in a degeneration of the cells, which was probably due to antibodies against cellular antigens, since absorption of antisera with cells prior to use in the liquid overlay markedly reduced this toxicity. It was not necessary to absorb the antisera when it was used at the working dilution of 0.5%, and such plaquing medium resulted in rapid plaque formation even after 11 months of storage at 4 C.

In conclusion, it does seem that the distinctive neutralization of the vaccine strains of IBR-IPV virus by specific rabbit 19S AB may be used to distinguish attenuated strains from wild strains of IBR-IPV virus. Notwithstanding the possibility that neutralization kinetics with 19S AB may be detecting antigens somehow related to cell culture adaptation, some preparations apparently have the potential of distinguishing between genital and respiratory strains of IBR-IPV virus.

ACKNOWLEDGMENT

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9. Hampar, B., L. M. Martos, D. V. Ablashi, R. F. Sigu-


