Serological Reactions of Bovine Diarrhoea Viruses with Anticellular and Antivirus Sera Produced in Rabbits

By A. L. FERNELIUS AND R. A. PACKER

National Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Box 70, Ames, Iowa 50010; and the Department of Veterinary Microbiology, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50010, U.S.A.

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SUMMARY

Rabbit antisera to bovine kidney cells neutralized several naturally occurring strains as well as laboratory modified strains of bovine diarrhoea virus. Antisera to porcine kidney cells failed to neutralize bovine diarrhoea virus even when cultured in porcine kidney cells. These anticellular sera failed to neutralize the viruses of infectious bovine rhinotracheitis, vesicular stomatitis, bovine enterovirus, vaccinia, and parainfluenza-3. Anticellular sera reacted specifically with homologous cells in an indirect immunofluorescent test, but did not form lines of precipitation with cell contents prepared by ultrasonic disruption. These preparations, however, formed multiple lines with antivirus sera, indicating that the antibody produced in rabbits against virus-infected cells differed in some way from the antibody produced in rabbits against whole cells or cell contents.

INTRODUCTION

Neutralization of viruses by anticellular sera was demonstrated with Rous sarcoma virus and antiserum against chicken tissues (Gye & Purdy, 1931; Amies & Carr, 1939; Rubin, 1956), the virus of avian myeloblastosis (Eckert et al. 1955) and avian erythroblastosis virus (Beard et al. 1957). Anticellular sera have been reported to inhibit the cytopathic effect (CPE) or to suppress virus replication in cell cultures of vaccinia, influenza, and parainfluenza viruses (Kosyakov, Rovnova & Schavelyova, 1966) and poliovirus, certain Coxsackie and ECHO viruses (Habel et al. 1958; Quersin-Thiry, 1958). Agglutination of enveloped herpes virus particles with anti-HeLa cell serum and of naked herpes virus particles with anti-herpes virus serum has been reported (Wildy & Watson, 1962). Complement-fixation reactions between vesicular stomatitis virus and anti-BHK or anti-PK sera were reported to be specific for the homologous host cell (Cartwright & Pearce, 1968).

We report here serological reactions between bovine diarrhoea viruses and antisera produced against uninfected and virus-infected host cells.
METHODS

Preparation of antigens. Primary embryonic bovine kidney (EBK) and porcine kidney established cell line (PK-15) cells were cultured in 11 Blake bottles by a method previously described (Malmquist, Fernelius & Gutekunst, 1965). Complete monolayers were washed twice with serum-free Earle's balanced salt solution to remove serum components of the growth medium, then harvested from the glass surfaces with a trypsin-EDTA mixture (Fernelius, 1967). After three washes with Earle's saline by centrifugation, cells were resuspended in serum-free medium at about half the volume of the original cell cultures. Half of the cell preparations were used as whole cell antigens, and the other half frozen and thawed thrice, then treated for 10 min. at 20,000 cyc./sec. in a 'Branson Sonifier' (Fernelius, 1968). As a precaution against the presence of latent adventitious bovine diarrhoea viruses in EBK cells (L. G. Classick & A. L. Fernelius, publication in preparation) these cells were tested by both a virus interference test (Malmquist et al. 1965) and an immunofluorescence test (Fernelius, 1964) before use.

Virus antigens used in neutralization tests consisted of the following bovine diarrhoea virus strains: NADL, C24V, CG-1220, and SAN propagated in EBK cells (Fernelius, 1964), strains adapted to a porcine cell line, NADL-PK22 and NADL-PK24, grown in PK-15 cells (Malmquist et al. 1965; Fernelius, 1967) and isolates C24V-PK22, C24V-PK24-EBK4, and NADL-RA adapted to porcine cell lines and rabbits (Fernelius, 1967). Other viruses employed in neutralization tests were the COLORADO strain of infectious bovine rhinotracheitis virus and the INDIANA strain of vesicular stomatitis virus grown in both EBK and PK-15 cell cultures; three strains of ECBO virus, rE, 4S, and LCR-4 (Van Der Maaten & Packer, 1967) and vaccinia virus; the latter viruses were grown in EBK cell cultures. Soluble antigens of strains of bovine diarrhoea virus were prepared by a method described by Gutekunst & Malmquist (1964).

Production of antiserum. Anticellular sera were produced in 12 New Zealand White male rabbits by the following schedule: groups of three rabbits were given intravenous injections of 0.5 ml. of one of four cellular antigen preparations on day 1 followed by 1.0 ml. on days 4, 6 and 13, and after a 14-day rest period, with 1.0 ml. of the same antigens. All rabbits were exsanguinated on the 43rd day. Cellular antigens given to groups of three rabbits were: (1) intact EBK cells, (2) EBK cells frozen and thawed and treated by ultrasonic vibration, (3) intact PK-15 cells and (4) PK-15 cells frozen and thawed and treated by ultrasonic vibration. Rabbit antisera were pooled according to treatments and used in neutralization tests with viruses cultured in EBK or in PK-15 cells.

Antivirus sera were produced in rabbits by the immunization schedule described above, and virus antigens were those used in neutralization tests. Titres of these antigens ranged from $10^{6.5}$ to $10^{8.0}$ TCD50/ml. as measured in susceptible cells directly or by an interference assay method (Gutekunst & Malmquist, 1964). These rabbit sera were used in cross-neutralization, cross-precipitation and in fluorescent antibody tests as controls on the anticellular systems.

Neutralization tests. Viruses were neutralized with antisera as described by Fernelius (1967).

Precipitation-in-gel tests. Immunodiffusion tests were made with soluble antigen of bovine diarrhoea viruses and anticellular and antivirus sera by a modification of the
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method of Ouchterlony (1949). Agar gel plates were prepared by the method described by Fernelius (1966) and contrast of precipitation lines was improved by addition of an orange dye to the agar (Glazier & Fernelius, 1967).

Fluorescent antibody tests. The fluorescent antibody reactions between anticellular sera and virus-infected and noninfected cells were performed by the indirect method of Cherry et al. (1960). The direct fluorescent antibody method was employed for reactions between cells infected with bovine diarrhoea virus and anti-bovine diarrhoea virus serum (Mengeling et al. 1964; Fernelius, 1964; Fernelius, 1969).

RESULTS

Neutralization reactions

Pooled antisera produced in rabbits against whole and sonically disintegrated EBK and PK-15 cells neutralized bovine diarrhoea viruses grown in EBK or PK-15 cells (Table 1). Both anti-EBK pooled sera diluted as much as 1/512 completely neutralized the NADL strain grown in either EBK or PK-15 cells. The C24V strain grown in EBK cells was neutralized to a greater degree by the anti-EBK pooled sera than was the NADL strain, but the noncytopathogenic strains CG-1220 and SAN were neutralized rather less than the NADL or C24V strains. Modified strains of virus were also neutralized less by anti-EBK sera than were NADL or C24C strains. The NADL-RA virus was neutralized by both antisera to whole cells and disrupted anti-cells, but the C24V-PK4 and C24V-PK20EBK4 viruses were neutralized only by the antisera to disrupted EBK cells. Neither of the pooled antisera to PK-15 cells neutralized the NADL virus grown in EBK or PK-15 cells, nor did they neutralize any other bovine diarrhoea virus.

Table 1. Neutralization of bovine diarrhoea viruses with pooled anticellular sera produced in rabbits

<table>
<thead>
<tr>
<th>Host cell</th>
<th>Virus strain</th>
<th>Anti-EBK cells</th>
<th>Anti-EBK disrupted cells</th>
<th>Anti-PK-15 cells</th>
<th>Anti-PK-15 disrupted cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBK</td>
<td>C24V</td>
<td>1024</td>
<td>2048</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NADL</td>
<td>512</td>
<td>512</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CG-1220</td>
<td>256</td>
<td>256</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>SAN</td>
<td>128</td>
<td>128</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NADL-RA</td>
<td>128</td>
<td>256</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C24V-PK20EBK4</td>
<td>0</td>
<td>128</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PK-15</td>
<td>C24V-PK4</td>
<td>0</td>
<td>128</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NADL-PK22</td>
<td>512</td>
<td>512</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NADL-PK51</td>
<td>256</td>
<td>1024</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Antiserum to disrupted EBK cells was absorbed for 1 hr at 37°C with a monolayer of EBK cells in a 1 l. Blake culture bottle, then titrated for neutralizing antibodies. Both NADL and C24V viruses were neutralized to exactly the same titre as those obtained from the serum before absorption. The cell monolayer on which the serum was absorbed was challenged with NADL virus, with the result that complete CPE occurred in 4 days. No neutralizing antibody was, therefore, removed by absorption with the cell monolayer, and no measurable degree of protection was given to the monolayer by
the anticellular sera. All anticellular sera failed to neutralize the COLORADO strain of infectious bovine rhinotracheitis virus and the INDIANA strain of vesicular stomatitis virus grown in either EBK or PK-15 cells. Three strains of ECBO virus or vaccinia virus grown in EBK cells were also not neutralized.

Precipitation-in-gel reactions

Anticellular sera would be expected to form lines of precipitation with soluble antigens extracted from homologous cell preparations because of the numerous protein constituents. This was not so with either anti-EBK or anti-PK-15 sera (Pl. 1). Both the EBK and PK-15 disrupted-cell antigens, however, did react with all four anti-bovine diarrhoea virus sera (Pl. 1). These findings were quite unexpected since disrupted cells of both EBK and PK-15 cultures failed to react with their homologous antisera. All four antivirus sera also cross-reacted with heterologous and homologous soluble antigens (Pl. 2, 3).

Table 2. Fluorescent antibody tests of rabbit anti-EBK and anti-PK-15 sera with infected and uninfected EBK and PK-15-cells

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>EBK cells</th>
<th>EBK cells NADL virus</th>
<th>PK-15 cells</th>
<th>PK-15, DNAL-PK29 virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-NADL (bovine) conjugate = (direct method)</td>
<td>-</td>
<td>+++++*</td>
<td>-</td>
<td>+++++</td>
</tr>
<tr>
<td>Anti-NADL (rabbit) + anti-rabbit conjugate</td>
<td>+ +</td>
<td>+++++</td>
<td>-</td>
<td>+++++</td>
</tr>
<tr>
<td>Anti-NADL-PK29 (rabbit) + anti-rabbit conjugate</td>
<td>-</td>
<td>+ + +</td>
<td>+</td>
<td>+++++</td>
</tr>
<tr>
<td>Anti-EBK (rabbit) + anti-rabbit conjugate</td>
<td>+ + + +</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anti-PK-15 (rabbit) + anti-rabbit conj.</td>
<td>-</td>
<td>-</td>
<td>+++++</td>
<td>+++++</td>
</tr>
<tr>
<td>Anti-rabbit conjugate only</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Indicates intensity of fluorescence.

Fluorescent antibody tests

Results of fluorescent antibody tests of anticellular sera with virus-infected and uninfected cells are given in Table 2 and Pl. 4. In the indirect fluorescent antibody test, anti-EBK sera reacted with both uninfected EBK cells and cells infected with bovine diarrhoea virus; likewise, anti-PK-15 sera reacted with both infected and uninfected PK-15 cells. There were, however, no cross-reactions between anti-EBK sera and PK-15 cells, or between anti-PK-15 sera and EBK cells unless antivirus sera and cells infected with a bovine diarrhoea virus were involved (Table 2). There were, however, slight immunofluorescent reactions between anti-NADL serum and EBK cells (+ + in line 2, column 1 of Table 2) and between anti-NADL-PK29 serum and PK-15 cells (+ in line 3, column 3 of Table 2). These antivirus sera from rabbits were produced from virus originally cultured in EBK and PK-15 cells respectively, so this slight fluorescence is probably cell-specific. This cell specific fluorescence did not occur in the direct method (Table 2, line 1, column 1) because the anti-NADL serum was absorbed with powdered bovine tissue to remove cell-specific antibodies from the conjugate.
Precipitation-in-gel reactions between soluble antigens extracted from uninfected cells and anti-cellular and antivirus sera. Antigens were placed in unmarked wells. (a, b, c) soluble antigens from EBK cells. (d, e, f) soluble antigens from PK-15 cells. Anticellular sera: (1) anti-PK-15 disrupted cells; (2) anti-PK-15 whole cells; (3) anti-EBK disrupted cells; (4) anti-EBK whole cells. Antivirus sera: (5) anti-cG-1220; (6) anti-SAN; (7) anti-NADL; (8) anti-c24v; (9) normal rabbit control.

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Precipitation-in-gel reactions between soluble antigens extracted from virus-infected cells and anticlassular and antivirus sera. Antigens were placed in unmarked wells. (a, b, c) soluble antigens from NAOL virus-infected cells. (d, e, f) soluble antigens from c24V virus-infected cells. Anticellular sera: (1) anti-PK-15 disrupted cells; (2) anti-PK-15 whole cells; (3) Anti-EBK disrupted cells; (4) anti-EBK whole cells. Antivirus sera: (5) anti-cG-I220; (6) anti-SAN; (7) anti-NADL; (8) anti-c24V; (9) normal rabbit control.

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Precipitation-in-gel reactions between soluble antigens extracted from virus-infected cells and antiscellular and antivirus sera. Antigens were placed in unmarked wells. (a, b, c) soluble antigens from CG-1220 virus-infected cells. (d, e, f) soluble antigens from SAN virus-infected cells. Anticellular sera: (1) anti-PK-15 disrupted cells; (2) anti-PK-15 whole cells; (3) anti-EBK disrupted cells; (4) anti-EBK whole cells. Antivirus sera: (5) anti-CG-1220; (6) anti-SAN; (7) anti-NADL; (8) anti-C24V; (9) normal rabbit control.

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Immunofluorescence of virus-infected and uninfected EBK and PK-15 cells.

(a) Direct fluorescent antibody method control—NADL virus in EBK cells plus NADL conjugate (+ + + +).

(b) NADL virus in EBK cells plus anti-NADL rabbit serum plus anti-rabbit conjugate (+ + + +).

(c) Uninfected EBK cells plus anti-EBK rabbit serum plus anti-rabbit conjugate (+ + + +).

(d) Uninfected EBK cells plus anti-rabbit conjugate (−).

(e) Uninfected PK-15 cells plus anti-PK-15 rabbit serum plus anti-rabbit conjugate (+ + + +).

(f) Uninfected PK-15 cells plus anti-rabbit conjugate (−).

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DISCUSSION

Anticellular sera have been reported either to inhibit the cytopathic effect of several viruses or to suppress virus multiplication or both in cell cultures and chick embryos. Theories concerning the mechanism of the inhibiting effect of anticellular sera vary. Some investigators say the sera act on the susceptible cell and possibly block attachment or entrance sites (Quersin-Thiry, 1958; Habel et al. 1958; Axler & Crowell, 1968). Others explain this phenomenon as a direct effect of anticellular sera on the virus particle as a result of antigenic similarity of viruses and host cells, perhaps because of the host-cell origin of the virus envelope (Kosyakov et al. 1966; Wildy & Watson, 1962; Laver & Webster, 1966).

The reason for neutralization of all strains of bovine diarrhoea virus by anti-EBK sera and failure of anti-PK-15 sera to neutralize any strains, even the NADL strain cultured after 22 and 24 passages in PK-15 cells (NADL-PK22 and NADL-PK24), is unknown (Table 1). Reasons for the wide variability in titres of the anti-EBK cell sera when reacted with the different strains of virus are also not apparent. The theory proposed by Quersin-Thiry (1958) and by Habel et al. (1958) that anticellular sera block attachment sites on susceptible cells does not explain the results of this study. When anti-EBK cell serum was absorbed by an EBK cell monolayer there was neither a reduction of neutralizing power of the serum for bovine diarrhoea viruses nor any inhibition of virus infection of the treated monolayer upon challenge. These results favour the theory of neutralization of the virus by the anticellular sera (Gye & Purdy, 1931; Amies & Carr, 1939; Kosyakov et al. 1966; Wildy & Watson, 1962; Laver & Webster, 1966).

Reasons for failure of the anticellular sera tested to neutralize infectious bovine rhinotracheitis, vesicular stomatitis, ECBO, and vaccinia viruses grown in EBK or PK-15 cells are unknown. Other workers have obtained neutralization of some related viruses with certain anticellular sera.

While there can be no doubt that anticellular sera contain antibodies, as shown by indirect fluorescent antibody tests and neutralization tests, there seems to be some sort of different anticellular antibody produced in animals as a result of injection of antigen from virus-infected cells. It is difficult to explain why soluble cell components from disrupted cells did not react in the agar double diffusion precipitation-in-gel tests, especially since many foreign proteins were involved. One possible explanation for the lack of lines of precipitation between disrupted cells and anticellular sera is that the disrupted cells may have been poor antigens which did not stimulate production of precipitating antibodies. The basis for this postulated lack of visible reaction could have been the formation of monovalent or blocking antibodies only. On the other hand, antivirus sera reacting with disrupted cells may have contained complete or bivalent antibodies which gave visible reactions with the disrupted-cell antigens. This theory would also account for the lack of a visible reaction between soluble antigen from infected cells and anticellular sera which may have contained a highly reactive antigen, but also contained the monovalent antibody which would have prevented precipitation in the test system.

Another possible explanation for the lack of precipitation is that cellular protein incorporated into the coat of the bovine diarrhoea virus may have an adjuvant effect on the production of serum antibodies. Lines of precipitation were formed by antisera
from such combinations of viral and cellular materials, even with disrupted cells, which by themselves did not stimulate the formation of precipitating antibodies.

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REFERENCES


**BD Virus reactions with anticellular sera**


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