Avirulent Rotavirus Infections Protect Calves from Disease with and without Inducing High Levels of Neutralizing Antibody

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(Accepted 14 May 1987)

SUMMARY

Two bovine rotaviruses, C3-160 and 17/4, which multiplied in calves without inducing disease, were studied for their ability to protect against disease caused by a virulent rotavirus, CP-1. Rotavirus 17/4 and the virulent virus CP-1 cross-neutralized poorly and, on the basis of 20-fold differences in neutralization titres, belonged to different serotypes. Rotaviruses C3-160 and CP-1 were more closely related: neutralization of the CP-1 virus by C3-160 antisera was within 20-fold of the homologous titre although neutralization of the C3-160 virus by CP-1 antisera was not. Nine gnotobiotic calves infected with either C3-160 or 17/4, had rotavirus antibody in their faeces and/or serum 21 days after oral inoculation as detected by indirect immunofluorescence and IgG, IgM and/or IgA antibodies by ELISA. As expected from the antigenic relationships between the viruses, the sera and faeces from the four calves infected with C3-160 contained moderate levels of neutralizing antibody to the virulent virus CP-1 and the sera and most of the faeces from the five calves infected with 17/4 contained undetectable or low levels. When challenged with CP-1 on day 21, four age-matched controls developed disease whereas all of four calves previously infected with C3-160 and four of five calves previously infected with 17/4 were protected from disease. It was concluded that avirulent rotavirus infection provided protection against disease caused by a virulent rotavirus even when one of the avirulent viruses was poorly related to the virulent virus by neutralization. Mechanisms other than neutralizing antibody appeared to be important in protection.

INTRODUCTION

Rotaviruses are enteric pathogens in many animal species but sub-clinical infections are also common in cattle (de Leeuw et al., 1980; Schusser et al., 1982; McNulty & Logan, 1983). Recent experiments have shown that bovine rotaviruses vary in virulence and one strain isolated from a sub-clinical infection multiplied without causing disease in calves aged 6 to 15 days (Bridger & Pocock, 1986). The possibility that sub-clinical infections protect against disease was suggested in a study in Australian children where asymptomatic infections during the neonatal period reduced the incidence and severity of later episodes of diarrhoea (Bishop et al., 1983). Whether these asymptomatic infections were due to avirulent human rotaviruses or strains whose virulence was suppressed by immunity is unknown.

Different isolates of bovine rotaviruses differ in their neutralization antigens (Murakami et al., 1983; Woode et al., 1983; Bridger & Brown, 1984) and these differences may be the reason for the lack of cross-protection that has been seen between some bovine rotaviruses (Woode et al., 1978, 1983; Murakami et al., 1986). Thus, protection after sub-clinical infection with one rotavirus may be restricted to viruses with similar neutralization antigens. The present study examines experimentally whether an infection with an avirulent bovine rotavirus protects calves against subsequent rotavirus disease either when the avirulent virus is related or only poorly related to the challenge virus by neutralization.
**METHODS**

*Viruses.* Two avirulent rotaviruses, C3-160 and 17/4, were used. Rotavirus C3-160 was obtained from a healthy calf and was passaged and cloned in cell culture (Bridger & Pocock, 1986). It was used either as cell culture fluid or as filtrates (pore size 0.45 μm) of faeces from gnotobiotic calves infected with virus grown in cell culture. Rotavirus 17/4 was obtained from a diarrhoea sample submitted to the Institute for Animal Disease Research from a Welsh dairy farm in 1982. In three calves, it was used as filtrates of faecal suspensions made from the original faeces or from faeces of infected gnotobiotic calves and in two calves it was used after two serial passages in MA104 cells. The virulent virus used for challenge was the cloned CP-1 virus grown in cell culture (Bridger & Brown, 1984; Bridger & Pocock, 1986).

*Animals and experimental design.* Gnotobiotic calves were produced and reared on a milk-based diet as described previously (Dennis et al., 1976; Hoare et al., 1976). On day 0, four calves aged 5 to 15 days were infected with $10^{5.0}$ to $10^{7.0} \log_{10} \text{TCID}_{50}$ of the cloned rotavirus C3-160 and four calves aged 5 to 7 days and one calf aged 21 days were infected with $<10^{5.5}$ to $10^{5.5} \log_{10} \text{TCID}_{50}$ of rotavirus 17/4. On day 21, the nine calves were challenged with $10^{7.9} \log_{10} \text{TCID}_{50}$ of the virulent rotavirus CP-1. Four previously uninfected calves served as challenge controls. They were infected with the same dose of CP-1 when three of them were aged 25 to 30 days and the fourth was aged 56 days. Three of the controls were infected during the period when the calves that had been infected with C3-160 or 17/4 were challenged with CP-1, and one was infected at the end of the experiment.

Faecal samples were collected daily. With male calves, the daily output was collected with the aid of a harness and collection bag, but with female calves, samples were collected when calves defaecated naturally. Diarrhoea was assessed by change in faecal colour and increase in the volume of faeces. Sera were obtained on days 0, 21 and 42.

*Virus detection.* Virus was detected in cell culture fluids and in filtrates of faecal suspensions by infectivity assays conducted in duplicate (Bridger & Pocock, 1986). Titres were expressed as $\log_{10} \text{TCID}_{50}$ per g of faeces or ml of culture fluid.

*Antibody detection in serum and faeces.*

**By indirect immunofluorescence (IIF).** One in 10 dilutions of sera or filtrates of faecal suspensions were diluted further in fourfold steps and immunofluorescence activity was determined indirectly using MA104 cells infected with the UK strain of bovine rotavirus (Bridger & Brown, 1984) and rabbit anti-bovine immunoglobulin conjugated with fluorescein isothiocyanate (Nordic Immunological Laboratories, Maidenhead, U.K.). Each sample was assayed on at least two occasions from separate starting dilutions and mean values were calculated.

**By neutralization.** Sera and filtrates of faecal suspensions were inactivated at 56 °C for 30 min and diluted in twofold steps from 1:5, 1:10 or 1:20 starting dilutions in Eagle's maintenance medium; neutralization titres were assessed by change in faecal colour and increase in the volume of faeces. Sera were obtained on days 0, 21 and 42.

**By ELISA.** Class-specific antibodies to rotavirus group antigens were measured by ELISA. Supernatant fluids from MA104 cells infected with the UK strain of rotavirus and with an infectivity titre of $10^{6.5} \text{TCID}_{50}$/ml were treated with an equal volume of 2 M-NaSCN for 15 min at room temperature as described by Inouye et al. (1984). The dissociated virus was then diluted with 3 vol. coating buffer (0.05 M-carbonate buffer pH 9.6) and 0.1 ml was added to the wells of ELISA microtitration plates (Flow Laboratories). After coating overnight at 4 °C, the plates were washed three times with ELISA buffer (phosphate-buffered saline, 0.05% Tween 20), drained and 0.1 ml of test samples or standards added to duplicate wells. After incubation for 3 h at room temperature, the plates were washed three times with ELISA buffer and 0.1 ml of anti-isotype antisera at optimal dilutions was added. For the detection of IgG antibodies, rabbit anti-bovine IgG conjugated with horseradish peroxidase (HRP) (Miles Laboratories) was used. For the detection of IgM and IgA antibodies, rabbit anti-bovine IgM (Miles Laboratories) and rabbit anti-bovine IgA (Miles Laboratories) were used. After a further 3 h incubation the plates were again washed and 0.1 ml of HRP-conjugated goat anti-rabbit IgG (Miles Laboratories) was added to each well. After overnight incubation at 4 °C, the plates were washed three times with ELISA buffer and 0.1 ml of substrate ($o$-phenylenediamine 340 μg/ml in citrate/phosphate buffer pH 5.0 with 3.9 mM-hydrogen peroxide) was added to each well. The reaction was stopped after 5 to 10 min by the addition of 2 M-H2SO4 (0.1 ml/well) and the A492 was read on an automatic plate reader (Titertek Multiskan).

Faecal extracts were prepared by suspending 1 g of faeces in 10 ml ELISA buffer, centrifuging at 100000 g for 1 h and the supernatant fluids were stored at −20 °C until assayed. They were assayed at a single dilution of 1:10. Sera were routinely assayed at a single dilution of 1:100. The validity of using a single dilution to obtain an endpoint titre had been previously assessed by comparing titres obtained in this way with those obtained using endpoint titrations.

For the IgG assay, twofold dilutions of a serum standard were used at dilutions from 1:100 to 1:25600, whereas for the IgM and IgA assays twofold dilutions of faecal standards were used at dilutions from 1:10 to 1:25600. Using a computer program, the data were subjected to logarithmic transformation, the regression equation determined and from this equation the antibody titres of the test samples were calculated.
RESULTS

Response of calves to infection on day 0 with avirulent rotaviruses C3-160 and 17/4

Virus was detected in the faeces of all nine calves infected with rotavirus C3-160 or 17/4 but there were no clinical signs of disease (Table 1). Faecal colour remained dark brown or green, faecal output did not increase (see also Bridger & Pocock, 1986), and anorexia did not occur.

On day 21, antibody to rotavirus group antigens was present in faeces and/or serum of all the calves infected with C3-160 or 17/4 by IIF and ELISA for IgG, IgA or IgM (Tables 2 and 3). Similar levels were produced by the two viruses and these levels were similar to those found in the challenge control calves on day 42, after infection with CP-1 (data not shown). Antibody was detected in the sera of all nine calves and in the faeces of eight (one calf not tested) by ELISA and in the sera of nine and the faeces of eight calves by IIF.

With both viruses, the mean levels of antibody in faeces were 7- to 38-fold lower than in sera by IIF and ELISA, except for IgA. The mean levels of IgA in sera and faeces of eight calves, infected with C3-160 or 17/4, were comparable. In the ninth calf, inoculated with 17/4, the level of IgA was six-fold lower in faeces than in serum; this calf was not protected from subsequent challenge with CP-1 (see below).

Table 1. Clinical effects and virus excretion in gnotobiotic calves given avirulent rotaviruses C3-160 and 17/4

<table>
<thead>
<tr>
<th>Virus</th>
<th>Calf age (days)</th>
<th>Dose ( \log_{10} ) TCID(_{50} )</th>
<th>No. calves tested</th>
<th>Faecal changes</th>
<th>Mean (range) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Faecal output</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>daily</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>output*</td>
</tr>
<tr>
<td>C3-160</td>
<td>5-15</td>
<td>5.0-7.0</td>
<td>4</td>
<td>Anorexia 0</td>
<td>ND</td>
</tr>
<tr>
<td>17/4</td>
<td>5-21</td>
<td>&lt;1.5-5.5</td>
<td>5§</td>
<td>Colour 0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Above 500 g; in uninoculated controls daily faecal output averaged 320 g (standard deviation ± 80 g).
† ND, Not measured in these calves but no increases were seen in calves reported previously (Bridger & Pocock, 1986).
§ Includes three calves infected with serial passes of the original faeces and two calves infected with cultured virus.
‖ Three calves tested.
‡ One calf was inoculated with the faeces of a farm calf, in which no virus was detectable by infectivity assay whereas four calves were inoculated with \( 10^{4.4} \) to \( 10^{5.5} \) TCID\(_{50}\).
Table 3. IgG, IgA and IgM antibodies to rotavirus group antigens on day 21 as determined by ELISA

<table>
<thead>
<tr>
<th>Primary inoculum</th>
<th>Clinical outcome after challenge</th>
<th>Serum Arithmetic mean titre (range)</th>
<th>Faeces Arithmetic mean titre (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3-160</td>
<td>Protected*</td>
<td>IgG 2226 (500-3795) IgA 863 (520-1050) IgM 1257 (350-1990)</td>
<td>IgG 158 (10-236) IgA 868 (276-1740) IgM 175 (33-399)</td>
</tr>
<tr>
<td>17/4</td>
<td>Protected†</td>
<td>IgG 1760 (736-3000) IgA 810 (470-1430) IgM 830 (380-1310)</td>
<td>IgG 133 (17-340) IgA 1642 (659-3442) IgM 77 (28-111)</td>
</tr>
<tr>
<td>None</td>
<td>Not protected‡</td>
<td>IgG 360  IgA 1140  IgM 4690</td>
<td>IgG 30  IgA 194  IgM 326</td>
</tr>
</tbody>
</table>

* Data from three calves.
† Data from the sera of four calves and faeces of three calves.
‡ Data from one calf.
§ Data from two calves.

On day 21, neutralizing antibody to the challenge virus CP-1 was present in sera and faeces of all four calves infected with C3-160 (Table 2). In contrast, all of five sera and four of five faecal samples from the calves infected with 17/4 contained low or undetectable levels of neutralizing antibody to CP-1 (Tables 2 and 6). The reason for the high level of neutralizing activity in the faeces of one of the 17/4-infected calves, which also had the highest level of neutralizing activity in its serum (Table 6), may be that this calf was inoculated with faeces from the second calf passage of uncloned faecal material.

The antigenic relationship between the avirulent rotaviruses, C3-160 and 17/4, and the challenge virus CP-1

The neutralizing activity to CP-1 found after infection with the avirulent viruses C3-160 and 17/4 was consistent with the antigenic relationships between the viruses (Table 4). On the basis of 20-fold differences in neutralization titres to differentiate rotavirus serotypes (Wyatt et al., 1982), rotaviruses 17/4 and CP-1 fall into two distinct serotypes and C3-160 and CP-1 have a one-way cross-relationship.

Response of calves to challenge on day 21 with rotavirus CP-1

All of four calves previously infected with C3-160 and four of five previously infected with 17/4 (a total of eight of nine) resisted challenge with the virulent virus CP-1 whereas all of four challenge controls developed disease (Table 5). The levels of neutralizing activity to CP-1 on day 21 in the sera and faeces of three of the four calves protected by 17/4 were low or undetectable (Table 6). The calves that were protected by previous infection with C3-160 or 17/4 showed little or no increase in faecal output and no changes in faecal colour after challenge, whereas in the challenge controls, the daily outputs of faeces were greater than 500 g for 3 to 7 days with a mean peak output of 1770 g and faeces of abnormal colour for 4 to 5 days (Table 5). The disease in the one unprotected calf, previously infected at 6 days of age with 10^5.4 TCID_{50} of rotavirus 17/4, was not significantly different to that in the challenge controls; the faeces were abnormal in colour and output was above 500 g per day for 6 days.

Virus was shed in the faeces of all four challenge control calves for an average of 5 days with a mean peak titre of 10^{6.1} TCID_{50} per g (Table 5). Virus was not detected in the faeces of the four calves previously infected with rotavirus C3-160 nor in two of the four protected calves previously infected with rotavirus 17/4. The other two protected calves excreted virus which was just detectable for 1 and 3 days. In the unprotected calf previously infected with 17/4, peak virus excretion was lower (10^{3.8} TCID_{50}/g) than that found in any of the challenge control calves (10^{5.4} to 10^{6.7} TCID_{50}/g).

On day 42, seroconversion had occurred in all four challenge controls. Seroconversion occurred also in all of the nine previously infected calves as demonstrated by greater than four-fold increases in neutralizing activity to CP-1 and/or the presence of secondary antibody
Table 4. Mean neutralization titres of convalescent antisera to rotaviruses C3-160, 17/4 and CP-1 with homologous and heterologous viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>C3-160</th>
<th>17/4</th>
<th>CP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3-160</td>
<td>1075</td>
<td>20</td>
<td>281</td>
</tr>
<tr>
<td>17/4</td>
<td>33</td>
<td>313</td>
<td>&lt;13</td>
</tr>
<tr>
<td>CP-1</td>
<td>43</td>
<td>21</td>
<td>4594</td>
</tr>
</tbody>
</table>

* Results based on sera taken from two to six calves 21 days after oral infection.

Table 5. Effect of challenge on day 21 with CP-1 on disease and virus excretion

<table>
<thead>
<tr>
<th>Primary inoculum</th>
<th>No. calves</th>
<th>Clinical outcome after challenge</th>
<th>No. days of abnormal colour</th>
<th>No. days output (&gt; 500 g)</th>
<th>Peak output/day (g)</th>
<th>No. days positive</th>
<th>Peak log_{10} TCID_{50} per g</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3-160</td>
<td>4</td>
<td>Protected</td>
<td>0</td>
<td>1* (0, 2)</td>
<td>449* (324, 574)</td>
<td>0</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>17/4</td>
<td>4</td>
<td>Protected</td>
<td>2</td>
<td>1.5* (1, 2)</td>
<td>547* (531, 562)</td>
<td>1 (0–3)</td>
<td>1.6 (&lt;1.5–1.7)</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>Not protected</td>
<td>1</td>
<td>6</td>
<td>1700</td>
<td>3</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Two calves tested for daily faecal output.
† Three calves tested for daily faecal output.

Table 6. Antibody levels on day 21 in the sera and faeces of the four calves protected by previous infection with 17/4

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>Source of inoculum</th>
<th>Neutralizing antibody to CP-1</th>
<th>ELISA IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>Neutralizing antibody to CP-1</th>
<th>ELISA IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>V81</td>
<td>Cell culture fluid</td>
<td>IIF &lt;7</td>
<td>736</td>
<td>860</td>
<td>1310</td>
<td>IIF &lt;10</td>
<td>&lt;23</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>V228</td>
<td>Original faeces</td>
<td>100</td>
<td>38</td>
<td>940</td>
<td>480</td>
<td>690</td>
<td>16</td>
<td>&lt;16</td>
<td>17</td>
</tr>
<tr>
<td>X24</td>
<td>V228</td>
<td>320</td>
<td>&lt;10</td>
<td>3000</td>
<td>1430</td>
<td>940</td>
<td>20</td>
<td>50</td>
<td>340</td>
</tr>
<tr>
<td>X54</td>
<td>X24</td>
<td>160</td>
<td>53</td>
<td>2365</td>
<td>470</td>
<td>380</td>
<td>20</td>
<td>353</td>
<td>43</td>
</tr>
</tbody>
</table>

* ND, Not done.

Responses to common group antigens as measured by ELISA to class-specific immunoglobulins (data not shown). Seroconversion was demonstrated only in the faeces of the calves infected with C3-160 whereas in the calves infected with 17/4 and the challenge controls it was found in both serum and faeces.

DISCUSSION

The present studies have shown experimentally that a sub-clinical infection with an avirulent rotavirus prevents subsequent rotavirus disease. This occurred even when the avirulent and virulent rotaviruses belonged to different serotypes (on the basis of 20-fold differences in neutralization titres; Wyatt et al., 1982) and when neutralizing activity was absent or low in faeces and serum. Thus, mechanisms other than neutralizing activity appear to be important in protection. Under natural conditions, sub-clinical infection of calves might limit the occurrence
and severity of rotavirus disease as has been suggested in children (Bishop et al., 1983). The present study indicates that protection against more than one rotavirus serotype should be achieved.

Rotavirus 17/4 is a further example of a rotavirus which multiplies without causing disease in calves aged about 1 to 2 weeks, the time when rotavirus diarrhoea is commonly found in natural conditions. In contrast to rotavirus C3-160, 17/4 was obtained from an outbreak of diarrhoea but the experimental data raise doubts as to whether it was the cause of the disease.

The avirulent rotavirus C3-160 induced good levels of neutralizing activity to the challenge virus and all calves were protected against challenge with no virus detected in the faeces after challenge. This protection was better than with 17/4 where one of five calves succumbed to challenge and two of the protected calves excreted low levels of virus. However, the protection achieved with 17/4 occurred with little detectable neutralizing activity to CP-1 in serum and faeces although an immune response to rotavirus group antigens was detectable at the time of challenge. One of the 17/4-infected calves which was inoculated with 10^{4.4} TCID_{50} of 17/4 at 21 days of age was protected from disease and failed to excrete virus after challenge; little or no neutralizing activity to the challenge virus was detected in its serum or faeces on the day of challenge (V81 in Table 6). This calf had excreted only 10^{1.7} TCID_{50} per g of faeces for 1 day after infection with 17/4, possibly due to its age at inoculation, but had developed an immune response to rotavirus group antigens by the time of challenge. Cross-protection in the absence of neutralizing antibody has been observed between strains of influenza virus (for review, see Stuart-Harris et al., 1985). It has also been shown that cytotoxic T cells generated by infection of mice with influenza virus show a broad pattern of cross-reactivity for influenza A viruses and antibody produced by the same infection is specific for virus subtype (Effros et al., 1977). Similar mechanisms may operate in rotavirus infections.

It is difficult to explain why one of the calves previously infected with rotavirus 17/4 developed disease and the other four did not. The calf had levels of immunofluorescent and neutralizing activity in faeces and serum at the time of challenge that were comparable to the four protected calves previously infected with 17/4. However, there were some differences in the levels of class-specific antibodies between the unprotected calf and the protected calves; IgM was present in serum and faeces at levels above the range for the protected calves and serum IgG and faecal IgA were below the ranges for the protected calves.

Cross-protection between rotaviruses has been studied with isolates from different animal species (Woode et al., 1978; Wyatt et al., 1979; Tzipori et al., 1980; Gaul et al., 1982; Zissis et al., 1983) and between rotaviruses from the same animal species, in calves (Woode et al., 1978, 1983; Murakami et al., 1986) and in pigs (Bohl et al., 1984). In some of these studies, cross-protection occurred but in others it did not. The reasons for these differences are unclear as the immune responses or the relationships between the rotaviruses were not always measured. Gaul et al. (1982), Woode et al. (1983) and Bohl et al. (1984) concluded that cross-protection occurred only when rotaviruses were closely related in their neutralization antigens. However, in addition to the results of the present study, protection has occurred between different serotypes (Woode et al., 1983; Wyatt et al., 1983) and when neutralizing antibody was undetected (Woode et al. 1978). Conversely, cross-protection does not always occur when neutralizing antibody is present (Woode et al., 1983).

The role of neutralizing antibody in protection was also examined by Kapikian et al. (1983) who failed to show a clear relationship between the presence of local neutralizing antibody and susceptibility to rotavirus disease in human volunteers. Furthermore, a bovine rotavirus vaccine has been used successfully in children to reduce the incidence of diarrhoea caused by human rotaviruses, which belong to serotypes different from that of the bovine virus (for review, see Vesikari, 1985).

Thus, the results of the present study and those of some others raise doubts about the importance in protection of the differences in neutralization antigens between rotaviruses. Neutralizing antibody may be just one of the immune responses involved in protective immunity. The mechanisms of protection and the role of avirulent rotavirus infections need to be more clearly understood to plan an effective strategy for the control of calf enteritis.
Protection from rotavirus disease

We thank J. F. Brown and A. Warrick for technical assistance and the staff of the gnotobiotic unit for rearing the calves.

REFERENCES


(Received 10 March 1987)