Immunological response to recombinant VP8* subunit protein of bovine rotavirus in pregnant cattle

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Bovine rotavirus VP8*, N-terminal trypsin cleavage product of VP4, was produced in Escherichia coli. To examine if this antigen could induce neutralizing antibody responses, different species of animals were immunized with the recombinant VP8* protein. The VP8* antigen was found to stimulate a neutralizing immune response in rabbits. When VP8*-immunized mice were exposed to bovine rotavirus strain C486, significantly higher antibody responses were observed than if they were only exposed to C486. To simulate a current vaccination protocol in the field with livestock, mice were exposed to live C486 virus first and then to VP8*. These mice had the elevated immune responses indicating that VP8* could boost immunity in primed mice. The immune response to VP8* was also tested in pregnant cows. The efficacy of VP8* in stimulating milk antibody was compared with a commercial inactivated vaccine. Differences in colostral antibody titres between VP8*-vaccinated and unvaccinated cows were statistically significant (P < 0.05) and equivalent to the commercial vaccine (P = 0.0569). The milk antibody titres on day 10 were comparable between VP8*- and commercial vaccine-vaccinated animals and were significantly higher (P < 0.05) than in unvaccinated controls. Furthermore, rabbit and bovine antibodies induced by VP8* were able to neutralize different P types of bovine rotaviruses to varying degrees, suggesting that serotype-specific and cross-reactive epitope(s) are present on the VP8* protein of rotavirus. Taken together, E. coli-expressed VP8* may be useful as a subunit vaccine candidate for bovine rotavirus.

Introduction

Neonatal calf diarrhea is one of the most common and economically devastating diseases of the cattle industry (Acres, 1977; Snodgrass et al., 1986). It has been estimated that approximately 5% of the calves born in North America die from diarrhea before they reach 1 month of age. These losses cost the cattle industry $1-7 billion annually (Ratafia, 1987). Since the discovery of bovine rotavirus in faeces of calves suffering from diarrhea (Mebus et al., 1969), rotavirus has been shown to be ubiquitous in most mammalian and avian species (Babiuk et al., 1985; Flewett & Babiuk, 1984). It is therefore important to develop control measures for the prevention of rotaviral disease.

Although cattle of all ages are susceptible to rotavirus infection (Bridger, 1994), disease occurs primarily in animals less than 1 month of age (Acres, 1977). Since calves are infected at birth, it is almost impossible to actively immunize them prior to exposure to virulent field virus. Therefore the best way to reduce economic losses in calves is to vaccinate dams to increase their colostral antibody levels, which is then passively transferred to the calves (Saif et al., 1983). It is postulated that colostral antibodies in the intestine can neutralize field virus and prevent disease. Depending on the balance between the dose of virus and antibody, animals can be infected and develop immunity without showing clinical disease. Thus, by the time milk antibody levels decline below a protective level the calves will have developed active immunity.

Rotaviruses are comprised of 11 segments of dsRNA molecules surrounded by a triple-layered capsid. The outermost capsid is comprised of protein VP4 and VP7. VP7, a 37 kDa glycoprotein, induces neutralizing antibodies which specify the glycoprotein G serotype (Greenberg et al., 1983). VP4, an 86 kDa non-glycosylated protein, also elicits neutralizing antibodies which specify P serotypes (Gorziglia et al., 1990). This protein is involved in various virus functions such as haemagglutination (HA; Kalica et al., 1983), trypsin-enhanced
The procedure for producing VP8* in incubating for 24 h at 37 °C, supernatant containing the virus was plaque formation (Clark et al., 1981) and virus attachment (Crawford et al., 1994). VP4 cleavage into VP5* and VP8* enhances viral infectivity (Estes et al., 1981) and assists in viral penetration. In studies using neutralizing monoclonal antibodies and a corresponding set of escape mutants, eight neutralization epitopes were identified on VP4 with five of them located on the VP8* subunit (Mackow et al., 1988). Monoclonal antibodies to VP8* were reported to neutralize rotavirus passively protect mice against rotavirus challenge in vivo (Matsui et al., 1989; Offit et al., 1986). Recently an Escherichia coli-expressed N-terminal portion of rotavirus SA114fM VP4 has been reported to induce neutralizing antibodies as well as to prime the immune system of mice (Lizano et al., 1991). These studies all suggest that VP8* plays an important role in viral infectivity, and immunity to this protein may be crucial in providing protection from infection.

In our attempt to determine if bovine rotavirus VP8* could elicit neutralizing immune response, we immunized animals with E. coli-produced recombinant VP8* (rVP8*) and assayed sera for their abilities to neutralize virus infectivity. Although rVP8* may not contain all the conformational epitopes involved in virus neutralization, it not only induced neutralizing antibodies but also primed the immune response of vaccinated animals.

Methods

Cells and viruses. All rotavirus strains were propagated in MA-104 cell monolayers as reported previously (Babiuk et al., 1977). After incubating for 24 h at 37 °C, supernatant containing the virus was collected and cell debris was removed by centrifugation at 5200 g for 20 min. For immunization studies, virus present in the supernatant was concentrated by centrifugation at 120000 g for 3 h at 15 °C through a 40% sucrose cushion containing 10 mM-CACl2. The virus pellet was resuspended in 100 μl of double-distilled water and then diluted 1:800 in distilled water. The amount of virus protein was measured spectrophotometrically using the formula: 183(A230)-75.8(A260) x 800 = μg/ml.

Construction of bacterial expression plasmid pGH432 VP8. The bovine rotavirus C486 VP8* gene from pTZ19RVP4 previously reported by Potter et al. (1987) was amplified by PCR using two primers: 5' ggatgcGATGCTTCATCTAATTAA 3' (BamHI site shown in lower case) for the forward primer and 5' ccggctgGTGTTATAGAC-AATA 3' for the reverse primer (lower case represents NcoI site). The cycles of PCR were repeated 30 times at 94 °C for 30 s, 5 °C for 30 s and 72 °C for 1 min. The PCR product was filled/phosphorylated with the Klenow fragment of DNA polymerase and then ligated into the PUC19 site of pGH432. The reamplified VP8* gene in E. coli strain DH5α cells was ligated into the BamHI-NcoI site of the pGH432 expression vector, resulting in pGH432VP8.

Preparation and solubilization of rVP8* protein produced in E. coli. The procedure for producing VP8* in E. coli and solubilization was a modification of that described previously (van Drunen Littel-van den Hurk et al., 1993). Briefly, DH5α cells containing pGH432VP8 were grown to late-log phase in L broth, followed by addition of IPTG (final concentration 2 mM) to induce VP8* gene expression under the control of the lac promoter. Six hours after induction, cells were harvested by low-speed centrifugation (5000 g) for 10 min. Cell pellets from 100 ml cultures were resuspended in 4 ml of 25% sucrose in 50 mM-Tris–HCl pH 8.0 and placed on ice. Lysozyme (10 mg/ml) was added and the suspension was incubated on ice for 10 min, followed by the addition of 30 ml of 2 x RIPA:TET buffer (5:4; 2 x RIPA containing 20 mM-Tris–HCl pH 8.0, 500 mM-NaCl and 2% sodium deoxycholate; TET containing 100 mM-Tris–HCl pH 8.0, 50 mM-EDTA and 2% Triton X-100). The preparation was sonicated three times for 30 s using a large probe (Sonics Materials) at the 50% duty cycle setting at power 2. The preparation was centrifuged at 15000 g for 15 min to pellet the VP8* inclusion bodies. To solubilize VP8* protein produced in E. coli, the inclusion bodies were resuspended in distilled water and the suspension was adjusted to a final concentration of 8 M-urea. The protein concentration was determined by SDS-PAGE against a BSA standard.

The solubilized protein was used directly for immunization studies.

Gel electrophoresis and Western immunoblot analysis. Samples containing area-treated VP8* were separated by electrophoresis through 10% polyacrylamide gels and transferred to nitrocellulose membranes. Antibody-bearing membranes were blocked with 3% horse serum in 10 mM-PBS for 1 h. After washing with PBS containing 0.05% Tween-20 (PBST), membranes were incubated for 1 h with a 1:100 dilution of rabbit antisera to bovine rotavirus C486. After washing with PBST, membranes were incubated for 1 h with biotin-conjugated goat anti-rabbit IgG diluted 1:500 in PBST. Membranes were washed and incubated for another 1 h with avidin-horseradish peroxidase (HRP) conjugate diluted 1:500 in PBST. After washing three times with PBST, bound antibody was visualized by a peroxidase substrate kit DAB (Vector Laboratories).

Immunization

(i) Mice. Seven- to 9-week-old female BALBc mice were screened on day 0 for neutralizing antibody to bovine rotavirus and group A antigen of rotavirus. Preimmune sera at a dilution of 1:25 failed to neutralize C486 and sera at a dilution of 1:50 did not react to single-shelled particles of C486 coated on ELISA plates, suggesting that the mice had not been previously exposed to rotavirus. A group of seven seronegative mice was inoculated with 100 μg of E. coli-expressed rVP8* protein in Freund’s complete adjuvant. This group was given two boosts separated by a 3 week interval with an identical formulation in Freund’s incomplete adjuvant. Mice were bled on day 50 (10 days after last boost).

For determination of the priming effect of rVP8* to C486 or vice versa, two strategies were used: (i) mice were first immunized with rVP8* and then boosted with C486. To do this, two groups of seven seronegative mice received two doses of 100 μg of either E. coli-expressed rVP8* or control E. coli proteins. Six weeks later, all mice were boosted subcutaneously with 50 μg of purified live C486. Sera were taken at day 50 after primary immunization. (ii) Alternatively, mice were first immunized with C486 and then boosted with rVP8*. This experiment was conducted by orally inoculating mice with 105.5 p.f.u. of live bovine rotavirus C486. Three weeks later, one group of mice was boosted with 10 μg of E. coli-expressed VP8* per mouse and the other group of mice was boosted with E. coli proteins. Ten days post-injection with rVP8*, mice were bled and sera were subjected to plaque reduction assay.

(ii) Rabbits. Prior to immunization, rabbits were screened for evidence of previous exposure to rotavirus by ELISA and virus neutralization assays. Preimmune sera did not neutralize bovine rotavirus C486 at a titre of 1:50 nor react to single-shelled particles of C486 coated on ELISA plates. Two seronegative rabbits were immunized with 50 μg of rVP8* protein emulsified with an equal volume of Freund’s complete adjuvant on day 0. Three weeks later, rabbits were boosted with rVP8* emulsified in an equal volume of
Expression and enrichment of rVP8* protein produced in E. coli

E. coli DH5α cells transformed with pGH432 carrying the VP8* gene (pGH432VP8) of bovine rotavirus were grown to late-log phase in L broth containing IPTG. The VP8* inclusion bodies were solubilized in 8 M-urea and the presence of the protein was confirmed by SDS-PAGE. As shown in Fig. 1 (a) and (b), lanes 2 and 4, respectively, clearly indicate the presence of VP8*. To determine whether or not urea destroyed the conformational integrity of E. coli-expressed VP8*, HA and HI assays were performed using the urea-treated rVP8*. As shown in Table 1, urea-treated rVP8* haemagglutinated guinea-pig erythrocytes and its HA activity was completely inhibited by the rabbit antisera against bovine rotavirus C486.

Table 1. Conformational integrity and immunoreactivity of E. coli-expressed rVP8* following urea treatment

<table>
<thead>
<tr>
<th>Assays (titres*)</th>
<th>HI</th>
<th>ELISA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunogens (50 μg)</td>
<td>HA</td>
<td>2E8</td>
</tr>
<tr>
<td>----------------------</td>
<td>----</td>
<td>------</td>
</tr>
<tr>
<td>rVP8* ⁸</td>
<td>65536</td>
<td>20480</td>
</tr>
<tr>
<td>C486</td>
<td>16384</td>
<td>5120</td>
</tr>
<tr>
<td>E. coli proteins</td>
<td>0</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Titres are expressed as the reciprocal of the highest dilution that completely inhibited HA of erythrocytes by rVP8* or C486.

† Neutralizing monoclonal antibody 2E8 was coated and reacted with twofold dilutions of immunogens.

² Antiserum was raised in rabbit against bovine rotavirus C486.

§ Aggregated E. coli-expressed rVP8* was solubilized with urea followed by dialysis to PBS. NT, Not tested.
was inhibited by a neutralizing monoclonal antibody (2E8) and polyclonal antibodies, indicating conformational integrity. The urea-treated rVP8* was also detected using a monoclonal antibody-based ELISA for reactivity with 2E8, specific to VP8* of bovine rotavirus C486.

Immune response to E. coli-expressed VP8* protein

(i) Immune response in mice
To test the capability of E. coli-expressed VP8* protein to elicit an immune response, rVP8* was injected into one group of mice and control bacterial antigen injected into a control group. Mice immunized with rVP8* developed neutralizing and HA-inhibiting antibodies with mean titres of 192 and 60, respectively (Fig. 2), indicating that rVP8* was not only immunogenic but induced antibody that could inhibit the biological function of rotavirus. Mice immunized twice with rVP8* first and then boosted with whole virus (C486) developed significantly higher neutralizing and HA-inhibiting antibody titres than mice immunized with rVP8* alone (Fig. 2), suggesting that rVP8* primed mice to respond to whole virus. Under the normal field situation, cattle are often exposed to field strains of rotavirus and then vaccinated. To determine whether vaccination with rVP8* could increase the immune response to rotavirus under the simulated field condition (i.e. previous exposure to virus), mice were first orally exposed to bovine rotavirus C486 and then boosted with rVP8*. On day 30 (10 days after boosting), the boosted group had higher neutralizing antibody, with a titre of 160, than the non-boosted animals (titres 1:60).

(ii) Immune responses in rabbits and cattle
To confirm neutralizing immune responses of other animal species to E. coli-expressed VP8* protein, rabbits were immunized twice with rVP8*, with a 3 week interval, and bled 10 days after the second immunization. Virus-neutralizing antibody titres of 1250 and ELISA titres of 6250 were detected in the rVP8*-immunized rabbits, while control animals retained no increase in neutralizing (< 1:50) and ELISA (< 1:100) titres. This result encouraged us to carry out a clinical trial in cattle.

One hundred and twenty pregnant cows were randomized into three groups of 40 each. All groups were vaccinated with Ecolan (a commercial vaccine to prevent diarrhoea caused by enterotoxigenic E. coli). One group served as a control and was vaccinated with adjuvant alone, another group was immunized with E. coli-expressed VP8*, and a third group was vaccinated with a commercial inactivated rotavirus/coronavirus vaccine (ScourGuard). In order to assess the degree of natural immunity, all cows were bled at the time of immunization. All groups had a mean neutralizing antibody titre of 1000–2700. These results indicated that all animals had previously been exposed to rotavirus, as expected. Three weeks later, all cows were revaccinated with the respective vaccines. At 12 h and 10 days after calving, colostra and milk were collected to assay for the presence of neutralizing antibodies. Geometric mean neutralizing antibody titres (Fig. 3 a) to bovine rotavirus C486 in colostra from vaccinated cows were 17000 and 17154 in ScourGuard- and rVP8*-immunized animals, respectively. Colostra from control cows had a titre of 9461. Although the differences in colostral antibody titres between the group of animals immunized with the commercial vaccine and rVP8*-vaccinated cows was not quite significant (P = 0.0569), titres from those animals immunized with the E. coli-expressed VP8* protein were significantly different from the controls (P = 0.0421). The colostral neutralizing antibody titre induced by the rVP8* and commercial vaccines rapidly decreased so that by 10 days post-calving, neutralizing milk antibody titres were 510 and 466, respectively (Fig. 3b). The control group neutralizing titre also decreased to 221. The difference in antibody titres between vaccinated
animals had been generated against rVP8*. Since cattle sera carrying the highest neutralizing antibody titres were collected from those carrying the highest neutralizing antibody titres. Since milk antibodies decline rapidly in cattle, the calf may become naturally infected with rotavirus as early as 2 months post-calving. These antibodies present in the blood and colostrum of most cows are detectable in the blood and colostrum of most cows (Saif & Smith, 1983). Colostral antibodies protect the offspring against rotaviral diarrhoea for the first several days of life by bathing the intestinal lumen. These antibodies present in the intestinal tract of the calf are much more effective than those found in the circulation (Saif & Smith, 1983). Since milk antibodies decline rapidly in cattle, the calf often becomes naturally infected with rotavirus as maternal milk antibody titres decline (Acres & Babiuk, 1978). It has been postulated that calves can develop rotavirus B641 shares the G6 serotype but not the P type with C486, the antibody titre of 14580 that neutralized strain B641 was probably due to the heterotypic neutralization activity of the rVP8*. This is consistent with the data of heterotypic antibody titres using rabbit antisera, since rVP8*-immunized rabbits are all rotavirus-naive (Table 2). Table 2 indicates that P1 was recognized most strongly, as would be expected since the rVP8* used was cloned from a P1 serotype. P5 was neutralized at an intermediate level and P11 was weakly neutralized.

**Cross-reactivity of antiserum to E. coli-expressed rVP8**

<table>
<thead>
<tr>
<th>Rotavirus strains</th>
<th>Similarity† with C486 (%)</th>
<th>Neutralizing antibody titres*</th>
<th>Colostrum†</th>
<th>Rabbit antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>C486 (P1)</td>
<td>100</td>
<td>81000</td>
<td>1250</td>
<td></td>
</tr>
<tr>
<td>B641 (P5)</td>
<td>53</td>
<td>14580</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>B223 (P11)</td>
<td>44</td>
<td>3240</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Cross-reactivity of colostrum and rabbit antiserum to E. coli-expressed rVP8

* Neutralizing antibodies were tested for their ability to neutralize the indicated viruses. The test was performed by a modification of the procedure of Brüssow et al. (1991). Titres are expressed as the reciprocal of the highest dilution of the serum or colostrum that neutralized at least 50% of foci in the immunofluorescent focus reduction test.
† Amino acid similarities in a variable region of the VP8* (amino acids 71-204; Hardy et al., 1992).
‡ Colostrum from five animals was randomly selected from those carrying the highest neutralizing antibody titres.

(rVP8*- and commercial vaccine-immunized) and control cows was statistically significant (P < 0.05) at day 10 post-calving.

**Discussion**

It has been reported that 10 M-urea does not destroy the cell binding and HA activities of reovirus σ1 protein (Yeung et al., 1987). These authors also reported that urea-treated σ1 protein could induce neutralizing antibodies in animals. This report suggested that 8 M-urea may provide an excellent way to solubilize aggregated proteins since it may not affect the native conformation of functional proteins. As shown in Table 1, binding of monoclonal antibody 2E8 to urea-treated rVP8* suggested that an epitope involved in binding to neutralizing antibodies was maintained. Furthermore, the rVP8* could bind to erythrocytes and agglutinate them. These results indicate that at least some functional epitope on rVP8* is intact following urea treatment.

It has been reported that some proteins or synthetic peptides of viral origin can prime the immune system of animals, as indicated by enhanced antibody or cell-mediated immune responses following exposure to the virus from which the peptides were derived (Arias et al., 1989; Earl et al., 1986; Ijaz et al., 1991) as well as from a heterologous virus (Emini et al., 1985). In the latter case, it is especially interesting to see that poliovirus-specific peptides could prime the immune system of rabbits to hepatitis A virus. Since peptides from supposedly unrelated viruses could prime the immune response, it is not surprising that a protein (VP8*) could dramatically increase the neutralizing and HI (functional) antibody response following exposure to wildtype virus (Fig. 2).

Rotaviral disease is pandemic in cattle and antibodies are detectable in the blood and colostrum of most cows (Babiuk et al., 1985; Snodgrass et al., 1990; Woode, 1978). Colostral antibodies protect the offspring against rotaviral diarrhoea for the first several days of life by bathing the intestinal lumen. These antibodies present in the intestinal tract of the calf are much more effective than those found in the circulation (Saif & Smith, 1983). Since milk antibodies decline rapidly in cattle, the calf often becomes naturally infected with rotavirus as maternal milk antibody titres decline (Acres & Babiuk, 1978). It has been postulated that calves can develop...
active intestinal (mucosal) immunity in the presence of passive antibody (Besser et al., 1988; Mebus et al., 1973). Thus, by the time all the milk antibody has decayed, there has been sufficient time for active mucosal immunity to develop. Therefore, considerable efforts are under way in order to develop vaccines which will prolong the duration of protective antibody levels in milk, thereby preventing early infection and allowing time for development of active immunity (Babiuk et al., 1985). In one study, diarrhoea did not develop in calves experimentally infected with rotavirus if the colostrum had a neutralizing antibody titre of 320 (Bridger & Woode, 1975). Another study indicated that 7-day-old colostrum with a neutralizing titre of 640 only delayed the onset of disease as well as the extent of virus shedding (Snodgrass et al., 1980). The explanation for these discrepancies was that the quantity of challenge virus can overcome the protective capacity of the antibody (Snodgrass et al., 1980). These studies all indicate that antibody in the milk is protective but as with any viral infection, immunity can be overcome with a massive challenge. In the present study, maternal antibody titres to E. coli-expressed VP8* protein were found to be maintained above 510 for 10 days after birth. Taken together, this indicates that calves born to cows immunized with E. coli-expressed VP8* protein may be protected under normal management conditions against natural infection of bovine rotavirus for at least the first 10 days after birth. This would be sufficient time for the animal to begin mounting an active immune response to infection.

The initial bovine rotavirus isolated from cattle (Mebus et al., 1969) was serotype G6 and subsequently a G10 serotype has been reported in cattle (Snodgrass et al., 1990). Recently, serotype G6 has been reported to contain at least two P serotypes (P1 and P5) and G10 at least one P serotype (P11) (Parwani et al., 1993). Although little is known regarding the global distribution and frequency of bovine rotavirus P types, one study reported that G6P5 serotypes were detected most frequently among the bovine rotavirus strains in the USA (Parwani et al., 1993). These studies suggest that a G6P5 strain may be a better vaccine candidate than a G6P1 strain, presently used as a vaccine in North America. It has been reported that neutralizing antibodies to VP8* cross-react among different serotypes (Larralde et al., 1991) to various degrees. Wood et al. (1983) reported that although antisera of cattle vaccinated with strain NCDV (G6P1) could cross-react with B641 (G6P5) they failed to cross-protect. One of the reasons for the lack of cross-protection between G6P5 and G6P1 may be due to the difference in P types. This view is supported by the analysis of the variable region of VP8* which demonstrated an amino acid similarity of 53% between B641 and NCDV (Hardy et al., 1991).

Since the immunogen used in this study was produced from the VP8* gene encoded by serotype P1, it was of interest to determine whether E. coli-expressed VP8* protein could elicit neutralizing antibodies that cross-reacted with other serotypes, especially P5 which is widespread in the cattle population (Parwani et al., 1993). It is encouraging that neutralizing antibodies elicited by rVP8* did cross-react to a considerable level with bovine rotavirus serotype P5 (Table 2). However, this reactivity was much lower with a P11 serotype. Since the amino acid similarity between P1 and P11 is only 44% (Table 2), the lower cross-reactivity is not surprising.

In summary, the results of the present study demonstrate that E. coli-expressed VP8* protein elicited neutralizing antibodies in seronegative mice and rabbits and significantly increased antibody responses in colostrum and milk of cows (P < 0.05) under normal condition (i.e. previously exposed to rotavirus). Ten days after calving, the rotavirus-neutralizing antibody titres to the vaccine P type in the milk of vaccinated cows remained above the threshold which is considered to be protective for calves. The present data strongly indicate that E. coli-expressed VP8* protein may be useful as a subunit vaccine for prevention of serotype P1 rotavirus infection and that other P types will need to be incorporated into the vaccine to provide broad spectrum protection.

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