Expression of Bovine Rotavirus Neutralization Antigen in \textit{Escherichia coli}

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\textbf{SUMMARY}

A 646 bp fragment derived from a full length cDNA clone of genomic segment 9 of bovine rotavirus (NCDV strain) was inserted into \textit{Escherichia coli} expression plasmid pEX1. The fragment encodes amino acids 50 to 265 of the major viral neutralization antigen VP7, a 326 amino acid long outer shell glycoprotein. Several transformed bacterial clones were isolated in which the recombinant plasmid directed the synthesis of a \textit{cro-\beta-galactosidase-VP7} fusion protein that was recognized by rabbit polyclonal antibodies against NCDV rotavirus. Sera from rabbits immunized with the fusion protein specifically reacted with VP7 among NCDV virion polypeptides. The chimeric polypeptide was also specifically recognized by two monoclonal antibodies against UK strain rotavirus VP7 that exhibited virus-neutralizing activity. However, immune sera to the chimeric polypeptide showed no neutralizing activity against bovine rotavirus. These results are discussed in view of a recent report that a fusion VP7-\textit{\beta-galactosidase} polypeptide comprising 35 more amino acids at the carboxy terminus was able to induce neutralizing antibodies in mice to simian rotavirus SA11.

Rotaviruses are a major cause of acute neonatal gastroenteritis in many mammalian and avian species. In humans, they represent the single most important cause of diarrhoeal disease in infants and, in developing countries, an important cause of infant mortality. The need for immunoprophylaxis against rotavirus-induced gastroenteritis has been widely recognized (Kapikian \textit{et al.}, 1980; Chanock, 1981; Cukor & Blacklow, 1984). Recently, the bovine NCDV strain has been proposed as a vaccine to protect young children against diarrhoeal disease caused by human rotavirus (Wyatt \textit{et al.}, 1979; Vesikari \textit{et al.}, 1984), in spite of the different serotypes of human and bovine strains. However, more recent results suggest this vaccine to be ineffective (De Mol \textit{et al.}, 1986). Simian rotavirus RRV has also been evaluated as a potential live vaccine for humans (Kapikian \textit{et al.}, 1985). Several reports have recently described atypical rotaviruses (pararotaviruses) lacking the group antigen (VP6) common to most human and animal rotaviruses (Pedley \textit{et al.}, 1983, 1986).

Rotaviruses are double-shelled virions containing 11 segments of double-stranded RNA (Estes \textit{et al.}, 1983). The outer shell viral proteins VP7 and VP3 are the antigens responsible for induction of neutralizing antibodies against the virus. The genomic segments coding for VP7 in human, bovine and simian rotaviruses have been identified and their sequence determined (Richardson \textit{et al.}, 1984; Dyall-Smith & Holmes, 1984; Elleman \textit{et al.}, 1983; Glass \textit{et al.}, 1985; Both \textit{et al.}, 1983; Arias \textit{et al.}, 1984). In rotaviruses that infect humans and other mammals, VP7 is a polypeptide of 326 amino acids with one or more potential glycosylation sites. Production of large quantities of VP7 polypeptide in prokaryotic or eukaryotic systems by recombinant DNA techniques could be of value in evaluating the effectiveness of a subunit vaccine against...
rotavirus infections, and in the characterization of the relevant epitopes involved in neutralization. We report here the expression of a large portion of the VP7 gene of bovine rotavirus Nebraska calf diarrhoeal virus (NCDV) as a fusion protein in *Escherichia coli*, and the characterization of immunological properties of the chimeric polypeptide. Expression of a portion of the VP7 gene of simian rotavirus SA11 as a fusion protein in *E. coli* has been recently described by Arias *et al.* (1986).

The NCDV strain of bovine rotavirus, the kind gift of C. A. Mebus (Plum Island Animal Disease Center, Greenport, N.Y., U.S.A.), was propagated in MA104 cells and purified as described (Gerna *et al.*, 1984). Viral dsRNA was purified by phenol–chlooroform extraction as described (Kalica *et al.*, 1978). Genomic segments were separated by electrophoresis on a preparative polyacrylamide slab gel. Segments 7, 8 and 9, which comigrated in overlapping bands under these conditions, were recovered together by electroelution and DEAE–Sephacel adsorption. cDNA copies of genomic segments were synthesized essentially as described (McCrae & McCorquodale, 1982). A poly(A) tail of approximately 20 nucleotides was added to the 3′ termini of the dsRNA segments using *E. coli* poly(A) polymerase (New England Nuclear). cDNA to genomic segments 7, 8 and 9 was synthesized with avian myeloblastosis virus reverse transcriptase (Life Sciences, St Petersburg, Fla., U.S.A.) and self-annealed. Single-stranded 3′ protruding extremities were filled with the Klenow fragment of *E. coli* DNA polymerase (Bethesda Research Laboratories) and the ds cDNA was cloned following oligo dG–dC tailing into the *PstI* site of pBR322. Clones containing cDNA to genomic segment 9 were identified by cross-hybridization to human rotavirus (Wa) RNA fractionated in an acrylamide gel (segment 9 is clearly distinguishable from segments 7 and 8 in this strain), using an incomplete cDNA clone of human rotavirus segment 4 as an internal size marker (data not shown). Restriction analysis and comparison to published sequences (Elleman *et al.*, 1983; Glass *et al.*, 1985) allowed identification of full length clones. Clone 13b was chosen for further manipulations.

The sequence of NCDV rotavirus gene 9 (Glass *et al.*, 1985) shows two in-phase initiation codons, 84 nucleotides apart, each followed by a signal sequence that codes for a domain rich in hydrophobic amino acids. Both initiation codons are apparently used in the infected cell, although only the second one has the optimal sequence for initiation of translation. The primary product synthesized from the first AUG is trimmed by post-translational cleavage to a size corresponding approximately to the smaller gene product (Chan *et al.*, 1986). Since highly hydrophobic polypeptide regions are often the cause of inefficient expression of cloned foreign genes in bacteria, we decided to eliminate both hydrophobic signal sequences for expression in *E. coli*. Restriction endonuclease *HhaI* cuts the VP7 cDNA at two sites, corresponding to codons 50 and 266 from the first AUG. An oligo(dC) tail was added to the 3′ protruding ends of the fragment, thus regenerating the codon for amino acid 50. The oligo(dC)-tailed *HhaI* fragment was then inserted into the *PstI* cloning site of expression plasmid pEX1 (Stanley & Luzio, 1984, the kind gift of K. K. Stanley), previously tailed with oligo(dG). These manipulations resulted in the cloning of the coding sequence for 216 amino acids from the VP7 gene, with the exclusion of 21 amino acids from the second AUG (49 from the first AUG) and 61 amino acids from the carboxy terminus. The variable length of both oligo(dC) and oligo(dG) tails (average lengths 17 and 30, respectively) randomized the reading frame of the viral insert, so that approximately one-sixth of the recombinant plasmids were expected to contain the insert in the correct reading frame, flanked by a variable number of glycine codons (GGG) at the 5′ end and of proline codons (CCC) at the 3′ end.

pEX1, after induction at 42 °C, directs the synthesis of large quantities of a highly insoluble *cro–β*-galactosidase hybrid protein of approximately 115000 *M*<sub>r</sub> (Stanley & Luzio, 1984). The hybrid protein from recombinant pEX1 containing the VP7 insert should then be approximately 138000 *M*<sub>r</sub>. Transformation of *E. coli* strain pop2136 yielded approximately 600 insert-containing clones (identified by colony hybridization), of which 121 contained the insert in the correct reading frame (identified by colony immunoblotting using a rabbit anti-NCDV serum). Positive clones were screened for production of the hybrid protein containing the VP7 insert by electrophoresis of lysates from small induced cultures, as shown in Fig. 1. Six clones were selected for further analysis of the fusion protein by immunoblotting, using a rabbit anti-NCDV...
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Fig. 1. SDS–PAGE of proteins produced in transformed clones. Exponential cultures (10 ml) of transformants pre-selected by colony hybridization and colony immunoblot for the presence of the VP7 insert and production of fused VP7 peptide were induced at 42 °C for 2 h. Cells were collected by centrifugation, resuspended in 500 μl of PBS and 15 μl samples were added to 30 μl of sample buffer. After boiling for 5 min, the bacterial proteins were electrophoresed on a 7.5% acrylamide gel (Laemmli, 1970). Lane 1, E. coli strain pop2136 containing plasmid pEX1 with no insert; lane 2, same strain as in lane 1, after induction; lanes 3 to 9, clones 62, 71, 75, 78, 79, 81 and 85, after induction; lane 10, molecular weight standards (Bio-Rad) myosin (200K), β-galactosidase (β-gal) (116.2K), phosphorylase B (92.5K), bovine serum albumin (66.2K), ovalbumin (45K), carbonic anhydrase (31K).

serum. The results of such an experiment, shown in Fig. 2, indicate that the insert-containing hybrid protein is specifically recognized by anti-rotavirus (NCDV) rabbit antibodies. The slight reactivity to the same antibodies shown by the cro-β-galactosidase hybrid protein (with no insert) probably reflects the presence in the immune serum of a low level of antibodies against E. coli β-galactosidase.

In order to test the immunogenic properties of the chimeric product, 200 ml cultures of producer clones were grown to mid-exponential phase at 30 °C and induced at 42 °C for 2 h. The bacteria were collected by centrifugation, resuspended in 1/100 of the original volume of 50 mM-Tris–HCl pH 8.0, 1 mM-EDTA, 50 mM-NaCl, and sonicated. Samples of the extracts were added to 2 vol. sample buffer (62 mM-Tris–HCl pH 8.8, 3% SDS, 0.004% bromophenol blue, 20% glycerol). After boiling for 5 min, the bacterial proteins were fractionated by electrophoresis in a polyacrylamide gel (Laemmli, 1970) and the band containing the fusion protein was excised, homogenized in phosphate-buffered saline (PBS) and used for immunization. The immunization schedule consisted of five weekly multiple subcutaneous and intramuscular inoculations of the fusion protein into 2-month-old, rotavirus antibody-free rabbits. Final bleeding was done, following a preliminary testing of immune response, 45 days after the first inoculum. The specificity of immune sera was tested by immunoblot against the chimeric protein, and against rotavirus polypeptides dissociated from NCDV virions, as shown in Fig. 3. Since the whole fusion protein was used for immunization, antibodies were raised also against the cro-β-galactosidase portion of the product. Antibodies with this specificity were saturated by pre-incubating the hyperimmune sera with a large excess of a crude preparation of the cro–β-galactosidase polypeptide from clones with no VP7 insert. Fig. 3 shows that after such quenching, immune sera specifically recognized the VP7 peptide in the fusion protein from clone 62 (lane 2). The same sera specifically reacted with VP7 among all NCDV or UK strain rotavirus structural proteins when virion polypeptides were separated by electrophoresis (lane 4). On the other hand, rabbit immune sera obtained by multiple immunizations with a fusion product that had been treated with 0.7 M-2-mercaptoethanol showed almost undetectable
Fig. 2. Immunoblot of fusion proteins in transformed clones. Bacterial cell samples were electrophoresed as in Fig. 1. The separated proteins were transferred to a nitrocellulose sheet which was then incubated with rabbit anti-NCDV serum overnight at 4 °C, then with goat anti-rabbit peroxidase-conjugated serum for 1 h at room temperature. Reactivity was detected by 4-chloro-l-naphthol. Lane 1, *E. coli* containing pEX1 with no insert; lane 2, same as lane 1, after induction; lanes 3 to 7, clones 62, 63, 65, 69 and 85, after induction.

Fig. 3. Immunoblot reactivity of rabbit immune serum to the hybrid protein. Lane 1, *cro-*β-galactosidase produced by pEX1 with no insert; lane 2, insert-containing hybrid protein produced in clone 62; lane 4, total proteins from bovine rotavirus particles (UK strain). Lane 3 shows no reactivity of rabbit preimmune serum with the hybrid protein produced in clone 62. Serum dilution was 1/50 for all samples. Other experimental methods as described in the text and in the legend to Fig. 1 and 2.

Fig. 4. Immunoblot reactivity of two neutralizing monoclonal antibodies to VP7 of bovine rotavirus (UK strain) with the insert-containing hybrid protein produced by clone 62. Lanes 3 and 5, reactivity of monoclonal antibodies 1C1 and 3B4, respectively, with the insert-containing fusion protein produced by clone 62; lanes 4 and 6, reactivity of the same monoclonal antibodies with *cro-*β-galactosidase coded by pEX1 with no insert. Lane 1, polypeptides of bovine rotavirus (UK strain) stained by NCDV rabbit immune serum; lane 2, viral polypeptides stained with monoclonal antibody 3B4.
reactivity with NCDV VP7 (data not shown), indicating that reduction by this reagent substantially alters the immunogenic properties of the chimeric polypeptide.

Two neutralizing monoclonal antibodies prepared in our laboratories were found to neutralize at a titre of 1:10000 both the UK and NCDV strains of bovine rotavirus (but not the four human rotavirus serotypes or SA11) and were shown to react with VP7 of the UK strain (but not with VP7 or other viral proteins of the four human serotypes or SA11) by immunoblotting (data not shown). Both monoclonal antibodies were found to react with the insert-containing hybrid protein produced by clone 62, but not with the cro-\(\beta\)-galactosidase polypeptide produced by pEX1 with no insert (Fig. 4). Thus, the insert-containing fusion protein was recognized by both polyclonal and neutralizing monoclonal antibodies to bovine rotavirus, whereas the immune serum to the protein was able to react specifically with VP7 of both NCDV and UK strains of bovine rotavirus. These two strains have recently been shown to possess similar VP7, but distinct VP3 proteins (Offit et al., 1986). However, when the immune serum to the chimeric protein was tested for neutralization against bovine rotavirus, no neutralizing activity was detected either by conventional or anti-globulin-enhanced neutralization assays (data not shown). This finding was surprising, since recognition of the fusion protein by neutralizing monoclonal antibodies suggests that at least one neutralization epitope is present in the portion of VP7 expressed from pEX1. Recently, it has been shown that at least two distinct regions of VP7 of simian rotavirus SA11 form a single immunodominant antigenic site containing different epitopes (Dyall-Smith et al., 1986). Both these regions appear to be included in our hybrid protein. Arias et al. (1986) have recently shown that a VP7-\(\beta\)-galactosidase hybrid polypeptide expressed in E. coli and including amino acids 63 to 300 of the viral protein did induce neutralizing antibodies when injected into mice. Compared to our product, their VP7 portion started 12 amino acids downstream at the N terminus and included 35 more amino acids at the C terminus. More recently, Andrew et al. (1987) introduced a complete cDNA copy of the simian rotavirus SA11 gene for VP7, or a deleted cDNA lacking the codons for amino acids 47 to 61, into the vaccinia virus genome. When either recombinant virus was injected into rabbits, neutralizing antibodies were induced. These observations suggest that the carboxy-terminal region of VP7 may be important for the proper folding of the polypeptide to generate the relevant neutralization epitope(s). Alternatively, induction of neutralizing antibodies by our fusion protein might have been prevented by the overall structure of the cro-\(\beta\)-galactosidase-VP7 polypeptide. For example, the fact that the fusion polypeptide described by Arias et al. (1986) had the VP7 portion in an amino-terminal position with respect to the \(\beta\)-galactosidase moiety, whereas in our case VP7 was at the carboxy terminus, may have played a crucial role in determining the antigenic properties of the two products. Finally, the major VP7 neutralization epitopes in our chimeric product might have been irreversibly denatured during purification in SDS-polyacrylamide gel. However, rabbits immunized with a partially purified fusion product that had not been SDS-solubilized were also unable to produce a neutralizing immune response (our unpublished results).

With respect to a possible protective activity of our fusion protein in vivo, it must be pointed out that monoclonal antibodies lacking neutralizing activity may exert passive protection in animals (Schmaljohn et al., 1982). In addition, it has been shown that immunization with a hybrid protein may elicit an efficient priming immune response in the absence of detectable neutralizing antibodies. These can appear at a substantial level following a booster injection of a sub-immunizing amount of the intact antigen (Jacob et al., 1985). Experiments to verify these possibilities are under way.

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REFERENCES


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