Expression of the bovine viral diarrhoea virus Osloss p80 protein: its use as ELISA antigen for cattle serum antibody detection

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The putative gene encoding the cytopathic bovine viral diarrhoea virus (BVDV) Osloss strain p80 protein was amplified by PCR and inserted into a T7 promoter-based vector for expression in Escherichia coli. Bacterial expression led to cytoplasmic insoluble inclusion bodies which were denatured by urea treatment and renatured by dialysis. Rabbit antisera were raised against this p80 recombinant antigen and assayed for the immunoprecipitation of either p120 or p80 protein from cytopathic or non-cytopathic BVDV biotype-infected bovine cells. The p80 gene sequence was also integrated into a baculovirus genome for its expression in Spodoptera frugiperda insect cells. The recombinant proteins isolated from bacteria or insect cells showed distinct antigenic properties when analysed by ELISA. Their ability to detect anti-BVDV specific antibodies was examined in a monoclonal antibody-based competitive ELISA performed on a series of field cattle sera. This comparative assay revealed the superiority of the insect cell-mediated expression to mimic the natural BVDV antigen produced by cell culture. The baculovirus/insect cell recombinant antigen gave the highest correlation between the ELISA-detected antibodies and the corresponding virus neutralization data.

Bovine viral diarrhoea virus (BVDV) is a member of the Pestivirus genus in the Flaviviridae family (Francki et al., 1991; Mennen & Plagemann, 1992). Hog cholera virus (HCV) and Border disease virus (BDV) are the two other members of this group of positive-stranded RNA viruses. The genomes of two BVDV isolates, Osloss and NADL, and two HCV isolates, Alfort and Brescia, have been cloned and sequenced (Collett et al., 1988a; Renard et al., 1987; De Moorloose et al., 1993; Meyers et al., 1989; Moorman et al., 1990). They contain a single open reading frame encoding a large polyprotein which is co- and post-translationally processed by either host or viral proteases (Collett et al., 1988b, 1991; Wiskerchen & Collett, 1991; Wiskerchen et al., 1991). Field isolates of BVDV and BDV viruses can be divided into two biotypes, cytopathic (cp) and non-cytopathic (ncp), according to their ability to induce cytopathic effects in cell culture; HCV virus, however, usually multiplies without producing a cytopathic effect. The role of the two biotypes in the pathogenesis of BVDV infections has been reviewed by Brownlie (1990). At the molecular level, the major difference between the cp and ncp biotypes is between the p125 and p80 non-structural proteins. The 125K protein detected in ncp biotypes, is not observed in cp isolates. In the latter case large amounts of 80K protein are present as well as a p125 amino-terminal product which varies in size from 39K to 54K (Donis & Dubovi, 1987; Pocock et al., 1987; Collett et al., 1988b; Akkina, 1991).

The p80 protein is a trypsin-like serine protease related to the NS3 protein of flaviviruses (Bazan & Fletterick, 1989; Gorbalenya et al., 1989; Wiskerchen & Collett, 1991). It is a major viral protein in virus-infected cells. Its location has been defined in the NADL polyprotein by Collett et al. (1988b, 1991) within the region most conserved between the four known pestiviral polyproteins (more than 80% amino acid identity). This abundant and well-conserved antigen appeared therefore to be of use in the development of pestivirus antibody detection assays (Kwang et al., 1991; Lecomte et al., 1991; Paton et al., 1991; Petric et al., 1992). Here we report the expression of the Osloss p80 protein in Escherichia coli and in baculovirus-infected insect cells. The antigenicity of the recombinant proteins was examined in a competitive ELISA carried out on a series of field cattle sera. The ELISA data were compared with virus neutralization titres.

The p80 sequence location was deduced from immunoprecipitation experiments (Collett et al., 1988b, 1991), but the exact position of its cleavage sites has not been identified by amino acid sequence analysis. Our own attempts to determine the p80 N-terminal sequence...
were unsuccessful and we have therefore positioned p80 in the BVDV cp Osloss polyprotein by comparison with the conserved flavivirus cleavage sites (Chambers et al., 1990). An RR/G conserved site was found at amino acid position 1715 in the Osloss polyprotein and is also present in the three other known pestiviral sequences, those of NADL, Alfior and Brescia. The carboxy terminus of the p80 protein was positioned on the basis of the calculated Mₜ as well as experimental data on the immunoprecipitation of in vitro translation products and epitope scanning (Lecomte et al., 1991). From these data, two oligonucleotide primers were chosen at positions 5532 (primer A) and 7715 (primer B) in the Osloss genomic sequence. Primer A contained a translation initiation codon within a Ncol restriction site, and primer B included a translation stop codon in a Nhel site. The corresponding 2183-nucleotide fragment was amplified by reverse transcription and PCR as follows. Total RNA was extracted from fetal sheep kidney cells (OCK, Rhône-Mérieux) infected with the BVDV Osloss strain (Liess, 1967) at an m.o.i of 0.1 and lysed at 48 h post-infection. Total RNA (20 μg) was used for cDNA synthesis under the recommended conditions with 10 μg of pDN6 random primer (Pharmacia), 1 mM-dNTPs and 800 units of Moloney murine leukaemia virus RNase H-negative reverse transcriptase (Superscript, Life Technologies). After RNA alkaline hydrolysis, one-hundredth of the cDNA was used for PCR amplification in a 100 μl mixture containing unit concentration PCR buffer (Promega), 1.5 mM-MgCl₂, 2 units of Taq DNA polymerase (Promega) and 1 μg of each specific primer. After thirty cycles of denaturation, annealing (at 55 °C) and elongation, the PCR product was restricted by the appropriate enzymes for cloning in the expression vectors.

For expression in E. coli, the amplified p80 sequence was inserted into the pARHS3 plasmid (De Moerlooze et al., 1992) under the control of the T7 RNA polymerase promoter. The recombinant plasmid, pAR80, was introduced into the BL21-DE3 strain (F-ompTrB-m B). This lambda lysogenic E. coli strain contains the T7 RNA polymerase gene downstream from the IPTG-inducible lacUV5 promoter (Studier & Moffatt, 1986). Expression of the p80 protein was induced with 1 mM-IPTG (Fig. 1a). The integrity of p80 was checked by Western blot analysis (NovaBlot, Pharmacia) using anti-p80 monoclonal antibodies (MAbs) (Fig. 1b). The expressed protein formed inclusion bodies that were harvested after enzyme-induced bacterial lysis (Klempnauer et al., 1983) or French Press disruption. For the mechanical method, centrifuged cells were resuspended in TE8 buffer (10 mM-Tris–HCl pH 8.0, 1 mM-EDTA) were twice disrupted in a French Press, at 4 °C and a pressure of 110 MPa. For small-scale inclusion
body production (less than 11 of initial culture), the enzymatic method usually gave better results in terms of p80 integrity and purity. The mechanical method was required for culture on a larger scale. Inclusion bodies obtained using the two methods are compared in Fig. 1(c). Fig. 1(d) shows inclusion bodies after the denaturation/renaturation process, which clearly allows removal of most of the contaminating proteins, and thus constitutes an essential purification step for the recombinant protein. Inclusion body denaturation was performed in 8 M-urea, 10 mM-DTT, 25 mM-Tris-HCl pH 8·0, then the p80 protein was renatured by dialysis against 20 mM-NH$_4$HCO$_3$, pH 7·9, 0·5 mM-PMSF, at 4 °C. We also noticed a similarity in the patterns observed with or without 2-mercaptoethanol which might indicate an absence of intramolecular disulphide bonds.

Lyophilized p80 protein inclusion bodies were used to immunize three rabbits selected on the basis of their low non-specific reactivity against the immunization antigen. The antisera were tested by radioimmunoprecipitation assays (RIPA) performed on $[^{35}S]$-methionine-BVDV cp and ncp Osloss strain-infected cell lysates (Fig. 2b). Osloss virus was allowed to adsorb to MDBK cells (m.o.i. of 1) for 1 h in Eagle’s MEM containing 10 % horse serum. After 23 h of infection, cells were incubated in MEM lacking methionine for 1 h and radiolabelled for 17 h with $[^{35}S]$-methionine (Amersham, 100 μCi/ml) in the same methionine-free medium. Mock-infected and virus-infected cells were harvested in RIPA lysis buffer (0·05 M-Tris–HCl pH 7·2, 0·15 M-NaCl, 1 % Triton X-100, 0·1 % SDS, 1 % sodium deoxycholate and 1 mM-PMSF). RIPS were carried out as previously described (Boulanger et al., 1991) except that the serum antibodies were directly coupled to the Protein A–Sepharose in contrast to the MAbs. Rabbit antisera gave similar patterns when compared with those obtained using specific anti-p80 MAbs (Lecomte et al., 1990; Fig. 2). However, the p125 protein precipitated from BVDV ncp-infected cell extracts appeared as a more intense band, indicating that this antiserum might be more appropriate to bind the p125 protein natural precursor than MAbs that recognize discrete epitopes. This property was exploited for developing an ELISA that detects BVDV p125/p80 antigens in persistently infected animal crude blood samples efficiently (Crevat et al., unpublished).

The p80 sequence was integrated into the Autographa californica nuclear polyhedrosis virus (AcNPV) baculovirus genome under the control of the polyhedrin gene promoter for expression in insect cells. The p80 gene isolated from the NeoI–BamHI-restricted pAR80 was inserted into the pACYM1 transfer vector (Matsuura et al., 1987). The resulting pACY80 was used to co-transfect Spodoptera frugiperda cells (SF9) together with wild-type AcNPV DNA. Transfections were performed according to the lipofection technique (Life Technologies). The recombinant viruses were isolated after three to five rounds of plaque assays (Summers & Smith, 1987), followed either by hybridization with the $^{32}$P-labelled p80 cDNA or by direct PCR detection of the p80 sequence in the culture medium (Tessier et al., 1991). The isolated recombinant baculoviruses (AcNPV-p80) were used to infect SF9 cells at an m.o.i. of 0·02, and the p80 protein expression level was analysed by SDS-PAGE and Western blotting. The highest level of expression was observed after 66 h of cell infection, but this level remained lower than that achieved in bacteria since Western blot analysis was required to detect the expressed protein (data not shown).

A competitive binding assay was designed to compare the antigenicity of the p80 proteins produced in E. coli and insect cells with that present in BVDV-infected cells.
Table 1. Reactivity of recombinant and natural p80 antigens in a comparative MAb-based competitive ELISA

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* SN titre, Neutralization titre expressed as serum dilution.
† 0, no effect; vir., viraemic animals.

The assay was a MAb-based ELISA allowing the detection of pestivirus antibodies directed against conserved epitopes of the p80 protein. Plates were coated overnight at 4 °C with the capture anti-p80 MAb diluted in carbonate buffer pH 9·6, and washed three times with PBS. After the blocking step had been performed for 1 h at 37 °C with PBS containing 10% horse serum, the antigen or the control was incubated for 16 h at 4 °C. Plates were washed three times with PBS and 0·1% Tween 20, and bovine serum dilutions were incubated for 2 h at 37 °C, followed by a further three PBS and Tween 20 washes. The competitive peroxidase-conjugated anti-p80 MAb was added and incubated for 1 h at 37 °C. After three washes with PBS and Tween 20 and one with H2O, o-phenylenediamine substrate was used to reveal the amount of peroxidase-conjugated competitive antibodies. The reactivity of the p80 antigens in the competitive assay and their ability to detect anti-p80-specific antibodies in cattle sera were compared using a series of field-collected bovine samples (Table 1).

In a previous report, we described a comparison between the bacterial recombinant p80 protein and the natural one, using 350 bovine serum samples. These data showed a good correlation between the ELISA values and the seroneutralization titres (Lecomte et al., 1991). In this study, we have, however, identified a few samples that were positive in seroneutralization but negative in ELISA, one example being sample 6 shown in Table 1. At a cut-off value of 45% inhibition, the ELISA based on insect cell antigen gave results that correlated closely with the ELISA and neutralization data obtained with the natural protein. This recombinant p80 seems to be closely related to the natural antigen and more discriminatory than the bacterial one, giving a test of greater sensitivity. This increased specificity and sensitivity is especially shown by the data obtained from vaccinated, non-vaccinated or persistently viraemic tested animals. An additional advantage of the baculovirus-expressed antigen was the use of crude insect cell lysates, as compared to the time-consuming denaturation/renaturation and dialysis steps required for the bacterial antigen. Compared with E. coli, this expression system provides an easier approach for the production of an optimal recombinant ELISA antigen.

In conclusion, the data we have obtained show that the bacterial p80 protein can be used to develop essential immune reagents such as specific antiserum of high binding efficiency and that the recombinant p80 produced in a baculovirus-based expression system provides a good alternative to the classical cell culture for the production of an optimal, well-characterized ELISA antigen useful for detecting pestiviral antibodies in cattle sera. Compared to the use of the natural viral antigen, the recombinant antigen will allow discrimination between
vaccinated animals and naturally infected ones in a
strategy of vaccination that would employ structural
protein-based recombinant vaccine.

This work was supported in part by Thöne-Mérieux (Lyon, France),
within a research contract with the Eurogentec company (Seraing,
Belgium).

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(Received 15 October 1992; Accepted 3 March 1993)