Inhibition of pestivirus infection in cell culture by envelope proteins Erns and E2 of classical swine fever virus: Erns and E2 interact with different receptors

M. M. Hulst and R. J. M. Moormann

Institute for Animal Science and Health (ID-DLO), Virology Department, PO Box 365, NL-8200 AJ Lelystad, The Netherlands

Introduction

Classical swine fever virus (CSFV), bovine viral diarrhoea virus (BVDV) and border disease virus (BDV), members of the genus Pestivirus of the family Flaviviridae (Francki et al., 1991), are small, enveloped, positive-stranded RNA viruses (Moennig, 1988). The viruses are structurally, antigenically and genetically closely related. BVDV and BDV can infect ruminants and pigs, while CSFV infections are restricted to genetically closely related. BVDV and BDV can infect ruminants and pigs, while CSFV infections are restricted to porcine and bovine cells with the pestiviruses CSFV and bovine viral diarrhoea virus (BVDV). Almost 100% inhibition of infection of porcine kidney cells with CSFV was produced by 100 μg/ml Erns. After removal of the virus no Erns was needed in the overlay medium (growth medium) to maintain this level of inhibition. In contrast, 100% inhibition of infection of porcine kidney cells with CSFV by 10 μg/ml E2 was only achieved when E2 was added to the overlay medium. When E2 was omitted, a maximum of 50% inhibition was achieved. This indicated that after the virus and E2 were removed from the cells, infection still occurred, by virus particles which were still bound to the cell surface. Treatment with 100 μg/ml Erns released these particles from the cell surface. Furthermore, Erns bound irreversibly to the surface of cells susceptible or unsusceptible to pestivirus infection and cell-to-cell spread of CSFV was completely inhibited by E2 but not by Erns. These results demonstrated that Erns and E2 interacted with different cell surface receptors. Inhibition of BVDV infection of porcine and bovine cells by CSFV E2 suggested that CSFV E2 and BVDV E2 share an identical receptor. BVDV strain 5250 isolated from pigs was efficiently inhibited by CSFV Erns, whereas several BVDV strains isolated from cattle were not, suggesting that the conformation of Erns plays a role in host tropism.

Pure preparations of envelope glycoproteins Erns and E2 of classical swine fever virus (CSFV) synthesized in insect cells were used to study infection of porcine and bovine cells with the pestiviruses CSFV and bovine viral diarrhoea virus (BVDV). Almost 100% inhibition of infection of porcine kidney cells with CSFV was produced by 100 μg/ml Erns. After removal of the virus no Erns was needed in the overlay medium (growth medium) to maintain this level of inhibition. In contrast, 100% inhibition of infection of porcine kidney cells with CSFV by 10 μg/ml E2 was only achieved when E2 was added to the overlay medium. When E2 was omitted, a maximum of 50% inhibition was achieved. This indicated that after the virus and E2 were removed from the cells, infection still occurred, by virus particles which were still bound to the cell surface. Treatment with 100 μg/ml Erns released these particles from the cell surface. Furthermore, Erns bound irreversibly to the surface of cells susceptible or unsusceptible to pestivirus infection and cell-to-cell spread of CSFV was completely inhibited by E2 but not by Erns. These results demonstrated that Erns and E2 interacted with different cell surface receptors. Inhibition of BVDV infection of porcine and bovine cells by CSFV E2 suggested that CSFV E2 and BVDV E2 share an identical receptor. BVDV strain 5250 isolated from pigs was efficiently inhibited by CSFV Erns, whereas several BVDV strains isolated from cattle were not, suggesting that the conformation of Erns plays a role in host tropism.

Introduction

Classical swine fever virus (CSFV), bovine viral diarrhoea virus (BVDV) and border disease virus (BDV), members of the genus Pestivirus of the family Flaviviridae (Francki et al., 1991), are small, enveloped, positive-stranded RNA viruses (Moennig, 1988). The viruses are structurally, antigenically and genetically closely related. BVDV and BDV can infect ruminants and pigs, while CSFV infections are restricted to pigs (Carbury et al., 1976).

The genome of pestiviruses varies in length from 12.5 to 16.5 kb (Becher et al., 1994; Collett et al., 1988a; Meyers et al., 1989; Moormann & Hulst, 1988; Qi et al., 1992; Renard et al., 1985) and contains a single large open reading frame which after translation is converted into mature proteins by virus and host cell proteases (Rümenapf et al., 1993). The core protein, C, and three glycoproteins, Erns, E1 and E2, which are the structural proteins, are encoded in the 5’ region of the genome (Becher et al., 1994; Collett et al., 1988b; Rümenapf et al., 1993; Stark et al., 1990). Although all three glycoproteins are associated with the virus envelope (Thiel et al., 1991), up to now only antibodies against the two largest glycoproteins have been detected in animals infected with pestiviruses (Kwang et al., 1992; Terpstra & Wensvoort, 1988). The most immunodominant protein present in the virus envelope of pestiviruses is E2 (Donis & Dubovi, 1987; Wensvoort et al., 1990). When E2 is used as a subunit vaccine, it elicits high levels of neutralizing antibodies and protects pigs from classical swine fever (Hulst et al., 1993; van Zijl et al., 1991). The role of this glycoprotein in virus–cell interactions has been studied for BVDV. Using anti-idiotypic antibodies, Xue & Minocha (1993) identified a 50 kDa cell surface protein as an E2-specific receptor. No data are available about whether envelope protein Erns is involved in attachment to or entry of pestiviruses into susceptible cells. Although neutralizing monoclonal antibodies (MAbs) against Erns have been prepared (Weiland et al., 1992; Wensvoort, 1989), no data have been published to show that Erns can induce neutralizing antibodies in pigs (Rümenapf et al., 1991; König et al., 1995).
Recently, envelope protein E\textsuperscript{NS} of CSFV was identified as a ribonuclease (Hulst et al., 1994; Schneider et al., 1993). This unexpected finding stimulated us to investigate the function of this envelope protein in the life cycle of the virus. In this report, we have studied the role of glycoproteins E2 and E\textsuperscript{NS} in the infection process of pestiviruses in cell culture. Pure and concentrated preparations of biologically active E2 (Hulst et al., 1993) and E\textsuperscript{NS} (Hulst et al., 1994) of CSFV synthesized in insect cells were used to inhibit both CSFV and BVDV infection of susceptible porcine and bovine cell lines.

Methods

- **Cells.** Swine kidney cells Sk6 (Kasza et al., 1972) and PK15 (ATCC CCL33) and foetal bovine epithelium (FBE) cells (P. J. Straver, personal communication) were maintained as described previously (Hooft van Iddekinge et al., 1992). Foetal bovine serum (FBS) and cells were free of BVDV and the FBS was free of anti-BVDV antibodies. Baby hamster kidney cells (BHK-21) and monkey kidney cells (CL2621) were maintained as described previously (van Nieuwstadt et al., 1996).

- **Viruses.** CSFV strain Brescia was cloned by limiting dilution (three times) on PK15 and passaged twice on PK15 and Sk6 cells to prepare virus stocks. Cytopathogenic BVDV strains 1138, NADL and Odsoss, isolated from cattle, were plaque-purified three times on FBE cells, and passaged once on FBE cells. Non-cytopathogenic BVDV strain 3520, isolated from pigs, was cloned by limiting dilution (three times) on FBE cells and passaged twice on FBE or PK15 cells. The origin of the pestivirus strains used has been described elsewhere (Hooft van Iddekinge et al., 1992; Wensvoort et al., 1989a). Transmissible gastroenteritis virus (TGEV) strain Purdue was used as a control virus (Bohl et al., 1972).

- **Purification of CSFV E2 and E\textsuperscript{NS} produced in insect cells.** E2 of CSFV strain Brescia was purified from the serum-free medium of Spodoptera frugiperda cells (Sf21) infected with recombinant Autographa californica nuclear polyhedrosis virus (AcNPV) expressing E2 of CSFV strain Brescia by immunofinity chromatography as described previously (Hulst et al., 1993). Serum-free medium of Sf21 cells infected with this recombinant virus and with wild-type AcNPV was also used directly in E2 inhibition experiments. The concentration of E2 in the medium was determined by ELISA using a pure E2 preparation as a standard (see below). E\textsuperscript{NS} of the C strain of CSFV was purified from the lysate of Sf21 cells infected with recombinant AcNPV expressing E\textsuperscript{NS} by immunoaffinity chromatography as described by Hulst et al. (1994). The ribonuclease specific activity of this preparation was 525 A\textsubscript{260} units/ml (Hulst et al., 1994). To prepare a control fraction, a lysate of Sf21 cells infected with wild-type AcNPV was chromatographed on the same column material (Hulst et al., 1994). As judged by SDS-PAGE, E\textsuperscript{NS} and E2 preparations were more than 95% pure.

- **Inhibition experiments.** These were done using a plaque assay. Confluent monolayers of Sk6, PK15 or FBE cells, grown in 2 cm\textsuperscript{2} tissue culture wells (M24 plates; Costar), were washed twice with Earle’s MEM without FBS and antibiotics (EMEM). The cells were pre-incubated at 37 °C for 30 min with 100 µM EMEM with different concentrations of E2, E\textsuperscript{NS} or control preparations. Dilutions (100 µl) of a virus stock (prepared using the same cell line as inhibition experiments were performed with) in EMEM were added to the wells, mixed and incubated as described above. When the virus solution was added, the concentration of E2 and E\textsuperscript{NS} in the wells was diluted twofold. The concentration given in the rest of this report corresponds to this diluted concentration (the concentration at which inhibition is actually measured). After 30 min the virus was removed, and the wells were washed once with 200 µl EMEM and supplied with EMEM supplemented with 10% FBS, antibiotics and 1% Nycodenz (overlay medium). In E2 inhibition experiments, E2 was also added to the overlay medium at the same concentration as was used during the 30 min of virus adsorption. Cells were grown for 24, 48 or 72 h at 37 °C, and infection centres (hereafter denoted as plaques) were detected by immunostaining as described by Wensvoort et al. (1986). An E2-specific MAb (MAb 3) was used to detect CSFV strain Brescia (Wensvoort et al., 1986). BVDV strains 1138, NADL, Odsoss and 5250 were detected using MAbs produced against BVDV strain 1138 (G. Wensvoort, unpublished data). TGEV plaques were detected using a MAb directed against the spike protein (van Nieuwstadt et al., 1988). The number of plaques in a well was counted with a microscope. The percentage inhibition in M24 wells was calculated using the formula 100 × [1 – (c/e)], where c is the number of plaques in a well to which no E2 or E\textsuperscript{NS} was added (control well), and e is the number of plaques in wells containing E2 and E\textsuperscript{NS}.

- **Detection of E2 and E\textsuperscript{NS} in medium and cells.** Confluent monolayers of Sk6, PK15, FBE, BHK-21, CL2621 and Sf21 cells grown in 96-well tissue culture plates (± 10\textsuperscript{3} cells per well) were washed twice with EMEM. EMEM (34 µl) with different concentrations of E2 or E\textsuperscript{NS} was added to the wells and the cells were incubated for 2 h at 37 °C. The medium was collected and the wells were washed twice with 50 µl EMEM. Cells were lysed in 34 µl 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, containing 1% NP40. Cell lysate and medium were analysed for E2 and for E\textsuperscript{NS} in a direct E2-specific ELISA (anti-geno capture assay; Hulst et al., 1993), and for E\textsuperscript{NS} in a direct E\textsuperscript{NS}-specific ELISA as described by Hulst et al. (1994).

- **Detection of E\textsuperscript{NS} on the surface of swine kidney cells.** Confluent monolayers of Sk6 cells grown in 1 cm\textsuperscript{2} tissue culture slides (Nunc culture chamber slides) were washed twice with EMEM. EMEM (100 µl) containing 100 µg/ml E\textsuperscript{NS} was added to the wells and the cells were incubated for 2 h at 37 °C. The medium was removed and the wells were washed three times with 0.2 ml EMEM without E2 or E\textsuperscript{NS}. The cells were fixed with acetone and stained with an appropriate dilution of an E\textsuperscript{NS}-specific horseradish peroxidase-conjugated Mab (MAb C5; Wensvoort; 1989).

- **Transfection of Sk6 cells with CSFV RNA.** Cytoplasmic RNA was isolated from Sk6 cells infected with CSFV strain Brescia as described by Moormann & Hulst (1988). RNA (0.5 µg) was dissolved in 50 µl HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) and gently mixed with 50 µl 0.25 mg/ml Lipofectin reagent (Gibco-BRL). The mixture was incubated for 15 min at 20 °C. Confluent monolayers of Sk6 cells grown in 10 cm\textsuperscript{2} tissue culture wells (M6 plates; Greiner) were washed twice with Dulbecco’s Modified Eagles’ Medium (DMEM) without FBS and antibiotics (DMEM). DMEM (1 ml) was added to the wells and to each well, gently, 100 µl of the RNA–Lipofectin mixture was mixed with 1 ml overlay medium containing different concentrations of E2 or E\textsuperscript{NS} was added. After incubation for 2 h at 37 °C, the transfection mixture was removed and 1 ml overlay medium containing different concentrations of E2 or E\textsuperscript{NS} was added. The cells were grown for 24 h and plaques (± 1200 per well) were detected by immunostaining as described above.

- **Nomenclature.** The nomenclature of the pestivirus proteins used in this report will be proposed to the International Committee on Taxonomy of Viruses by the Flaviviridae Study Group. The nomenclature of the pestivirus envelope proteins used by the CSFV research group in Lelystad in previous publications (Hulst et al., 1993, 1994; Moormann et al., 1990; van Zijl et al., 1991; Wensvoort, 1989; Wensvoort et al., 1986, 1989a, b, 1990) was: E1, renamed E2; E2, renamed E\textsuperscript{NS}; E3, renamed E1.
**Results**

**Inhibition of CSFV infection by E2 and E^ns**

The effect of glycoproteins E2 and E^ns, synthesized in insect cells, on the infection of SK6 cells with CSFV strain Brescia was studied. For both glycoproteins, the percentage inhibition of infection in 2 cm² tissue culture wells at different concentrations of E2 and E^ns was determined in a plaque assay (Fig. 1). About 90% inhibition of infection was achieved at an E^ns concentration of 100 µg/ml. In contrast, only a maximum inhibition of 50% was achieved with 25 µg/ml (Fig. 1; −E2). One hundred per cent inhibition of infection by E2 was achieved only when freshly applied E2 was added to the overlay medium, after removal of the virus solution from the wells (Fig. 1; +E2). Identical results were obtained when the unfractionated medium of Sf21 cells infected with E2 expressing AcNPV was used as a source of E2 and the unfractionated medium of Sf21 cells infected with wild-type AcNPV was used in control wells (results not shown). Infection was also not inhibited by 100 µg/ml BSA or by a control fraction, prepared identically to the purified E^ns fraction, from the lyase of insect cells infected with wild-type AcNPV (see Methods).

To establish that E2 and particularly the ribonuclease E^ns do not inhibit virus infection by interfering with the replication of the virus RNA in the cytoplasm of the cell, the effect of E2 and E^ns on the infection of SK6 cells with the RNA virus TGEV was studied in the plaque assay (Table 1). No inhibition of TGEV infection was observed at concentrations of E2 and E^ns that inhibited CSFV infection by nearly 100%. Furthermore, it appeared that when SK6 cells were infected with concentrations of CSFV that were 10-fold higher (10^4 TCID₅₀ per well) than in the experiments described above, inhibition of infection by both E2 and E^ns was still nearly 100%.

**Inhibition of BVDV infection by CSFV E2 and E^ns**

Inhibition of infection of both porcine and bovine cells with BVDV by CSFV E2 and E^ns was studied for the BVDV strains 1138, NADL and Osloss isolated from cattle and for the BVDV strain 5250 isolated from pigs. Strain 5250 grows on porcine and bovine cells; therefore, this strain could be tested on both these cell types. Strains 1138, NADL and Osloss do not grow on swine kidney cells and were tested on bovine cells only. In these experiments, inhibition by E2 and E^ns was measured in a plaque assay in a dose-dependent manner. E2 was included in the overlay medium at the same concentration as was used during virus adsorption. The highest concentrations tested in these experiments were 150 µg/ml E^ns and 50 µg/ml E2. The results of these experiments are summarized in Table 1. Infection of PK15 cells with strain 5250 and infection of FBE cells with strains 1138 and Osloss were inhibited efficiently by E2. However, inhibition of infection of FBE cells with strains 5250 and NADL reached a plateau of 50% at E2 concentrations of 25–50 µg/ml.

Although infection of FBE cells with strain 5250 was inhibited by 85% at a concentration of 150 µg/ml E^ns, no significant inhibition of infection of FBE cells with BVDV strains isolated from cattle (1138, NADL and Osloss) was observed at a concentration of 150 µg/ml E^ns. Efficient infection of FBE cells with these strains and efficient infection of SK6 cells with TGEV virus demonstrated that virus attachment and penetration of these viruses were not affected by E^ns. This indicated that the cell surface of SK6 and FBE cells was not damaged by high concentrations of E^ns.
reached a plateau of 50% at a concentration of 10 µg/ml. Whether E\textsuperscript{rns} could interfere with infection by virus particles which were already bound to the cell surface but were prevented from entering the cell due to competition with E2 was again able to infect the cells in the absence of E2. It is likely that inhibition of CSFV infection by E2 is a reversible process. The observation that nearly 100% inhibition was achieved when E\textsuperscript{rns} was only present during the 30 min of infection indicated that E\textsuperscript{rns} irreversibly prevented the binding of virus particles to the cell surface and/or the entry of virus particles into the cell. To seek evidence for this, we analysed whether E\textsuperscript{rns} could interfere with infection by virus particles which were already bound to the cell surface of SK6 cells. Infection of SK6 cells with CSFV in nine wells was inhibited directly for about 30% by 10 µg/ml E2 (see Fig. 1a; —E2), creating a situation where virus particles were still attached to the cell surface. After the virus–E2 solution was removed, the wells were ‘chased’. Three wells were incubated with EMEM containing 10 µg/ml E2 (Fig. 2, column A), three wells were incubated with EMEM without E2 or E\textsuperscript{rns} (Fig. 2, column B) and three wells were incubated with EMEM containing 100 µg/ml E\textsuperscript{rns} (Fig. 2, column C). After 30 min incubation, these ‘chase media’ were collected and assayed for CSFV by titration in a plaque assay. To ensure that the E2 and E\textsuperscript{rns} in these media did not inhibit the infection of SK6 cells, in this assay the collected ‘chase media’ were diluted 10–20-fold.

Overlay medium with 10 µg/ml E2 was added to the A wells and overlay medium without E2 was added to the B and C wells. The mean number of plaques in these wells after 24 h growth (open bars) and the mean number of plaques recovered from the ‘chase media’ (shaded bars) are presented. Treatment of the cells with E\textsuperscript{rns} after the virus–E2 solution was removed resulted in a drop of virus infection from 70% (B wells) to 10% (C wells). This percentage corresponded with the level of infection (14%) observed in wells where E2 was also present in the overlay medium (A wells), indicating that after removal of the ‘chase medium’ with E\textsuperscript{rns} from the C wells, cells in these wells were no longer infected during the 24 h growth. Significantly more virus particles were recovered from the ‘chase medium’ of the wells treated with 100 µg/ml E\textsuperscript{rns} (column C) than from the wells treated with E2 (column A) or treated with medium without E2 or E\textsuperscript{rns} (columns B and V). This indicated that virus particles which were already bound to the cell surface were released from the cell surface by treatment of the cells with an E\textsuperscript{rns} concentration that completely inhibited CSFV infection.

### Interaction of E\textsuperscript{rns} with cells

Irreversible inhibition of CSFV infection by E\textsuperscript{rns} and the interference of E\textsuperscript{rns} with virus binding suggests that E\textsuperscript{rns} interacts strongly with cells. To confirm this interaction, porcine kidney cells (SK6 and PK15), FBE cells, monkey kidney cells (CL2621), baby hamster kidney cells (BHK-21) and insect cells (SF21) were incubated for 2 h with different concentrations of E2 and E\textsuperscript{rns}. The medium was collected, the cells were washed twice with EMEM without E2 or E\textsuperscript{rns} and the cells were lysed. The medium and lysate of the cells were analysed.

### Table 1. Inhibition of pestivirus infection by E\textsuperscript{rns} and E2 of CSFV

<table>
<thead>
<tr>
<th>Virus Strain*</th>
<th>Origin</th>
<th>log\textsubscript{10} p.f.u.†</th>
<th>Cell line tested</th>
<th>E2</th>
<th>E\textsuperscript{rns}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSFV Brescia</td>
<td>Pig 4</td>
<td>SK6</td>
<td>95(10)</td>
<td>95(100)</td>
<td></td>
</tr>
<tr>
<td>TGEV Purdue</td>
<td>Pig 2 and 4</td>
<td>SK6</td>
<td>0(10)</td>
<td>0(100)</td>
<td></td>
</tr>
<tr>
<td>BVDV 5250</td>
<td>Pig 3</td>
<td>PK15</td>
<td>90(10)</td>
<td>95(75)</td>
<td></td>
</tr>
<tr>
<td>1138 Cattle</td>
<td>3-7</td>
<td>FBE</td>
<td>± 50(50)</td>
<td>65(150)</td>
<td></td>
</tr>
<tr>
<td>NADL Cattle</td>
<td>2</td>
<td>FBE</td>
<td>90(25)</td>
<td>&lt; 5(150)</td>
<td></td>
</tr>
<tr>
<td>Osloss Cattle</td>
<td>2</td>
<td>FBE</td>
<td>± 50(50)</td>
<td>&lt; 10(150)</td>
<td></td>
</tr>
</tbody>
</table>

* Virus stocks were prepared using the same cell line that inhibition experiments were performed with.
† log\textsubscript{10} p.f.u. per 2 cm\textsuperscript{2} tissue culture well.
‡ Inhibition experiments were performed as described in the legend of Fig. 1. The percentage inhibition was calculated as described in Methods. The concentration (µg/ml) of E2 or E\textsuperscript{rns} at which the indicated inhibition was achieved is indicated in parentheses. In these experiments, E2 was added to the overlay medium at the same concentration as was used during virus adsorption.
Inhibition of pestivirus infection in cell culture

Fig. 2. Inhibition of infection with cell-bound virus particles by Erns. Nine 2 cm² tissue culture wells with SK6 cells were pre-incubated for 30 min with 100 µl medium containing 20 µg/ml immunoaffinity-purified E2. Subsequently, 100 µl of a virus dilution with 1400 p.f.u. CSFV strain Brescia was added to the wells and virus infection was inhibited for 30 min by 10 µg/ml E2. After the virus–E2 solution was removed, three wells were chased with 100 µl medium containing 10 µg/ml E2 (column A), three wells were chased with 100 µl medium without E2 and Erns (column B) and three wells were chased with 100 µl medium containing 100 µg/ml Erns (column C). After incubation for 30 min, the chase media were collected and overlay medium containing 10 µg/ml E2 was added to the A wells and overlay medium without E2 was added to the B and C wells. After incubation for 24 h, wells were immunostained and plaques were counted. The mean numbers of plaques of three wells are presented by open bars. Error bars represent the standard deviation (n = 1). After 10–20-fold dilution in EMEM supplemented with 10% FBS, the collected chase media were used to infect 2 cm² tissue culture wells. After 90 min infection, these media were removed and overlay medium without E2 or Erns was added. After 24 h growth, the wells were stained and plaques were counted (shaded bars, mean of three observations).

Fig. 3. Interaction of Erns with cells from different species; amount of Erns (µg/10⁵ cells) absorbed by SK6, CL2621, Sf21, BHK-21, FBE and PK15 cells after incubation of 10⁵ cells (2 h) with different amounts of Erns (1–7, 3 ± 4, 6 ± 8 or 13 ± 6 µg/10⁵ cells). Plot symbols represent the mean of three observations.

in an ELISA for the presence of E2 and Erns. No E2 could be detected in the lysates of the cell lines tested, and also no decrease in the level of E2 in the medium was observed after 2 h incubation (results not shown). In contrast, significant amounts of the Erns applied to the cells were detected in the lysates of cells susceptible (SK6, PK15 and FBE) and unsusceptible (CL2621, BHK-21 and Sf21) to pestivirus infection (Fig. 3). Except for PK15 and FBE cells, the amount of Erns absorbed by cells increased almost linearly with the amount of Erns added. It is likely that SK6, CL2621, BHK-21 and Sf21 cells can bind significantly more Erns than FBE and PK15 cells, which become saturated with 2 µg Erns/10⁵ cells.

To determine the localization of Erns, SK6 cells were incubated with 100 µg/ml for 2 h, washed three times and fixed with acetone. Staining with an Erns-specific horseradish peroxidase-conjugated MAb clearly showed that Erns irreversibly interacts with the plasma membrane of SK6 cells (Fig. 4a). In contrast, no Erns staining was observed in the cytoplasm or nucleus of these cells. A clear Erns staining was observed in the cytoplasm of CSFV-infected cells (Fig. 4b).

E2 prevents cell-to-cell spread of CSFV

In E2 inhibition assays, we observed that the number of CSFV- and BVDV-infected cells within a plaque was significantly reduced when E2 was included in the overlay medium. This suggested that E2 interfered with cell-to-cell spread of CSFV and BVDV. To determine whether E2 and Erns are able to prevent infection of neighbouring cells, SK6 cells were transfected with cytoplasmic RNA isolated from SK6 cells infected with CSFV strain Brescia. Two hours after transfection, the cells were provided with overlay medium containing...
different concentrations of E2 or E\textsuperscript{rns}. After growth for 24 h at 37 °C, the transfected monolayers were immunostained and the number of infected cells within a plaque of 40 independent plaques was counted. At a concentration of 12.5 µg/ml E2, the mean number of infected cells within a plaque was reduced to 1.6 ± 0.5 (Fig. 5a). Taking into account that cells infected with CSFV strain Brescia divide normally (once in 24 h), cell-to-cell spread of CSFV was completely blocked by E2. Although the mean number of infected cells within a plaque was reduced by 150 µg/ml E\textsuperscript{rns} from 11.3 to 5.3 (Fig. 5b) the variation (SD ± 3.0) was much higher than for high concentrations of E2, and comparable to that of wells without E2 or E\textsuperscript{rns}, indicating that E\textsuperscript{rns} did not prevent infection of neighbouring cells significantly.

**Discussion**

In this report, we used purified CSFV-specific E2 and E\textsuperscript{rns} synthesized in insect cells to study pestivirus infection in cell culture. We demonstrated that both envelope proteins E2 and E\textsuperscript{rns} can prevent infection of porcine and bovine cells with the pestiviruses CSFV and BVDV. The mechanism of inhibition of pestivirus infection by E\textsuperscript{rns} is different from that of E2. Inhibition of infection by E\textsuperscript{rns} is irreversible. To maintain 100% inhibition of infection, no E\textsuperscript{rns} is needed in the overlay medium after removal of the virus. In contrast, interaction of E2 with the cell surface seems to be reversible. Only 50% inhibition of infection could be achieved when E2 was omitted from the overlay medium. This observation that inhibition by E2 reaches
a plateau of 50% when no E2 was included in the overlay medium indicated that in this situation cells could still be infected by virus particles which were already attached to the cell surface. Treatment of these cells with an E\textsuperscript{NS} concentration that completely inhibited CSFV infection resulted in the abolishment of infection by these particles and in the release of these particles from the cell surface. It is likely that E\textsuperscript{NS} interfered with binding of virus particles to the cell surface. Together with the finding that E\textsuperscript{NS} irreversibly binds to the cell surface of SK6 cells, these results indicated that interaction of E\textsuperscript{NS} with the cell surface is indispensable for attachment of CSFV to the surface of swine kidney cells. The binding studies with several cell lines from different species not only confirmed that interaction of E\textsuperscript{NS} with the cell surface is much stronger than interaction of E2 with its receptor, but also showed that this interaction is not limited to cells susceptible to pestivirus infection. It is likely that E\textsuperscript{NS} interacts with a cell surface component which is present on the surface of eukaryotic cells originating from various species.

The concentration of E\textsuperscript{NS} (100 µg/ml) needed to establish complete inhibition of CSFV infection was much higher than that for E2 (10 µg/ml). Because the molecular mass of both proteins is almost the same, and both proteins are synthesized in insect cells as homodimers (M. M. Hulst & R. J. M. Moorman, unpublished data), this difference in concentration suggests that there are far more binding sites for E\textsuperscript{NS} on the cell surface of SK6 cells than for E2. The results of the ELISA and plasma-membrane staining showed that indeed large amounts of E\textsuperscript{NS} irreversibly bind to the surface of cells. Because we used insect cell-derived E2 and E\textsuperscript{NS}, we can not exclude that this difference in concentration is due to differences in protein processing in insect and mammalian cells. Such differences could influence the avidity of binding of both proteins to the cell surface of SK6 cells. Furthermore, we used E2 homodimers in our experiments and no E2–E1 heterodimers. E2–E1 heterodimers are present at a higher molarity in virions than E2 homodimers (Thiel et al., 1991). However, with respect to receptor binding, no data are available about which form represents the most active form of E2. Inoculation of pigs with the same preparation of E2 that was used in this study induced high levels of neutralizing antibodies (Hulst et al., 1993). These results indicated that the conformation of E2 homodimers synthesized in insect cells perfectly mimics the surface structure of E2 in virions. Furthermore, the ribonuclease specific activity of the E\textsuperscript{NS} preparation used in this study was comparable to that of native E\textsuperscript{NS} (Hulst et al., 1994), indicating that the conformation of E\textsuperscript{NS} synthesized in insect cells also reflects the conformation of native E\textsuperscript{NS}. Therefore, we conclude that, in addition to the observation that E\textsuperscript{NS} irreversibly binds to the cell surface while E2 does not, this difference in concentration needed to establish complete inhibition indicates that the two envelope proteins do not interact with the same surface component. Also, no synergism of inhibition was observed when CSFV infection was inhibited simultaneously with concentrations of E2 and E\textsuperscript{NS} that, when used apart, gave an inhibition of about 25% (not shown).

Another indication that E\textsuperscript{NS} and E2 do not interact with the same surface component was the observation that E2 completely inhibited cell-to-cell spread of pestivirus while E\textsuperscript{NS} could only induce a moderate reduction of plaque size. Thus, interaction of E\textsuperscript{NS} with the basolateral plasma membrane is probably not essential for cell-to-cell spread of pestiviruses, while interaction of E2 with its receptor on this membrane is essential. It is possible that no binding sites for E\textsuperscript{NS} are present on the basolateral plasma membrane and entry of virus particles by cell-to-cell spread proceeds by a different mechanism to entry at the apical plasma membrane.

Sera induced in pigs infected with CSFV and BVDV and in ruminants infected with BVDV cross-react in neutralization assays (Wensvoort et al., 1989a), indicating that the immunodominant E2 proteins of CSFV and BVDV are structurally and antigenically related. Therefore, we were not surprised that E2 of CSFV was able to inhibit BVDV infection of bovine epithelium cells and porcine kidney cells and that E2 could also prevent cell-to-cell spread of BVDV. It is likely that CSFV E2 and BVDV E2 interact with an identical receptor present on both pestivirus-susceptible bovine and porcine cells. This is in agreement with the data reported by Flores et al. (1996) that swine and ruminant pestiviruses use the same cell surface component to enter bovine cells. The E2 concentration needed to almost completely inhibit BVDV infection varied between bovine epithelium and swine kidney cells but was not dramatically higher than that observed for inhibition of infection of CSFV. Besides the variation in cell density and differences in molarity of the E2 receptor on the cell surface of different cell lines, differences in avidity for the E2 receptor between CSFV E2 and E2 of different BVDV strains could be responsible for the variation in the E2 concentration needed to establish complete inhibition. That differences in avidity for the E2 receptor are critical for the level of inhibition of infection was demonstrated by the moderate inhibition (50%) of infection of FBE cells with BVDV strain 5250 by CSFV E2. In contrast, this protein almost completely inhibited the infection of PK15 cells with this strain and the infection of FBE cells with BVDV strains 1138 and Osloss. It is likely that the structure of the E2 receptor is important as well as the structure of E2 and E2 of CSFV and 5250 interacted with structurally different receptors on the cell surface of FBE cells. This is in line with the suggestion of Xue & Minocha (1993) that multiple receptors for E2 with different avidity for different BVDV strains may exist on the cell surface of bovine cells.

Inhibition of infection of PK15 and FBE cells with strain 5250 by E\textsuperscript{NS} indicated that BVDV E\textsuperscript{NS} is also involved in attachment of BVDV to the cell surface. A remarkable finding, however, was that infection of FBE cells with BVDV strains 1138, NADL and Osloss, isolated from cattle, could not be inhibited by a high concentration (150 µg/ml) of E\textsuperscript{NS}. This lack of inhibition suggests that the conformation of E\textsuperscript{NS} in virions
accomplished by the interaction of at least two structural components. For E2, Xue & Minocha (1993) identified a 50 kDa cell surface protein as a specific receptor; for E1, the nature of the interaction of E1 with the cell surface has to be established before conclusions can be drawn. Recently, Schelp et al. (1995) precipitated two bovine-specific cell surface proteins of 60 and 93 kDa with MAbs which efficiently inhibited the infection of bovine cells with BVDV. Their results suggested that additional surface proteins might be involved in pestivirus entry. Further studies regarding the interaction of E1 with the cell surface and further characterization of the surface proteins identified by Xue & Minocha (1993) and Schelp et al. (1995) are needed to unravel the mechanism of pestivirus entry.

In conclusion, the mechanism of pestivirus infection is accomplished by the interaction of at least two structural proteins, E1 and E2, which interact with different cell surface components. For E2, Xue & Minocha (1993) identified a 50 kDa cell surface protein as a specific receptor; for E1, the nature of the interaction of E1 with the cell surface has to be established before conclusions can be drawn. Recently, Schelp et al. (1995) precipitated two bovine-specific cell surface proteins of 60 and 93 kDa with MAbs which efficiently inhibited the infection of bovine cells with BVDV. Their results suggested that additional surface proteins might be involved in pestivirus entry. Further studies regarding the interaction of E1 with the cell surface and further characterization of the surface proteins identified by Xue & Minocha (1993) and Schelp et al. (1995) are needed to unravel the mechanism of pestivirus entry.

We thank B.J.L. Hooft van Iddekinge for his assistance in the inhibition experiments with E2, and G. Kok and F. Wagenaar for their assistance with the surface detection of E1.

References


Schelp, C., Greiser-Wilk, I., Wolf, G., Beer, M., Moennig, V. & Liess, B.
Inhibition of pestivirus infection in cell culture


Received 5 March 1997; Accepted 23 May 1997