Vaccinia virus-expressed bovine ephemeral fever virus G but not GNS glycoprotein induces neutralizing antibodies and protects against experimental infection

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Two related glycoproteins (G and GNS) encoded in the bovine ephemeral fever virus (BEFV) genome were expressed from recombinant vaccinia viruses (rVV). Both proteins were detected in lysates of rVV-infected cells by labelling with D-[6-3H]glucosamine or by immuno-blotting. The recombinant G protein (mol. mass 79 kDa) appeared slightly smaller than the native G protein but reacted with monoclonal antibodies directed against all defined neutralizing antigenic sites (G1, G2, G3a, G3b and G4). The recombinant GNS protein (mol. mass 90 kDa) was identical in size to the native GNS protein and failed to react by immuno-fluorescence with anti-G protein monoclonal or poly-clonal antibodies. Antisera raised in rabbits against rVV-G or rVV-GNS both reacted strongly by immuno-fluorescence and immuno-electron microscopy with BEFV-infected cells. The G protein was localized intracellularly in the endoplasmic reticulum/Golgi complex and at the cell surface associated with budding and mature virus particles. The GNS protein also localized intracellularly in the endoplasmic reticulum/Golgi complex; however, at the cell surface it was associated with amorphous structures and not with budding or mature virions. Rabbits vaccinated with rVV-G developed high levels of antibodies which neutralized BEFV grown in either mammalian or insect cells. Cattle vaccinated with rVV-G also produced neutralizing antibodies and were protected against experimental BEFV infection. In contrast, rVV-GNS vaccinated rabbits and cattle failed to produce neutralizing antibodies and, after challenge, BEFV was isolated from two-thirds of the vaccinated cattle.

Introduction

Bovine ephemeral fever virus (BEFV) is an arthropod-borne rhabdovirus, genus Ephemerovirus (Wunner et al., 1995), which causes an acute febrile disease in cattle and water buffalo. In tropical and sub-tropical regions of Africa, Asia and Australia, seasonal epidemics can have significant economic consequences. The BEFV virion has the characteristics typical of rhabdoviruses with bullet-shaped morphology, a single-stranded (-) RNA genome, a lipid envelope and five structural proteins designated L, G, N, M1 and M2 (Della-Porta & Brown, 1979; Walker et al., 1991). The virion G protein is an 81 kDa transmembrane glycoprotein which contains type-specific, neutralizing antigenic sites (Cybinski et al., 1990; Walker et al., 1991). Vaccination of cattle with G protein purified from BEFV virions induces neutralizing antibodies and protects against subsequent experimental infection (Uren et al., 1994). However, unlike other rhabdoviruses, the genomes of BEFV and the closely related Adelaide River virus (ARV) encode a second glycoprotein gene (GNS) which is located immediately downstream of the G gene. The 90 kDa BEFV GNS protein is a highly glycosylated product which is related in structure and amino acid sequence to G protein and appears to have arisen by gene duplication (Walker et al., 1992; Wang & Walker, 1993). The GNS protein is present in infected cells in similar abundance to the G protein but has not been detected in virions and its function is presently unknown (Walker et al., 1991).

In this paper, we report the construction and analysis of recombinant vaccinia viruses (rVV) which express the BEFV G or GNS proteins. We show that native G protein is transported to the surface of BEFV-infected cells via the endoplasmic reticulum/Golgi complex, is associated with budding and mature virus particles and that
vaccination with rVV-G induces neutralizing antibodies and protects cattle against experimental infection. The GNS protein, although similarly located in the endoplasmic reticulum/Golgi complex and transported to the surface of BEFV-infected cells, is not associated with budding or mature virions, is not related antigenically to the G protein and does not induce virus neutralizing or protective immune responses.

Methods

Viruses and cells. BEFV strain BB7721 was isolated from a cow with clinical bovine ephemeral fever (Doherty et al., 1969). The passage history and growth of the virus in BHK-21 cells (BSR clone) have been described (Walker et al., 1991). The challenge virus used in vaccination experiments was isolated from the same source and had been passaged only 11 times in cattle (Uren et al., 1994). BEFV strain CSIRO 1927 was isolated from the blood of an infected cow at Kairi, Queensland, Australia in 1975 and had been passaged only three times in insect cells. Aedes albopictus cells (clone C6/36) were grown in Leibovitz L-15 medium containing 10% fetal calf serum (FCS) and 5% tryptose phosphate broth as described previously (Uren et al., 1992). Vaccinia virus strain NYBH (VV-NYBH) was used as parental virus for the construction of recombinant vaccinia viruses (rVV) and as control virus. The viruses were grown in human TK-143B cells in Eagle’s minimal essential medium containing 5% FCS.

Construction of recombinant vaccinia viruses. DNA manipulations were performed according to standard procedures. BEFV G and GNS genes were amplified by PCR from genomic cDNA clone g207 (Walker et al., 1992) by using synthetic oligonucleotide primer pairs G1.1B (5’ GGCCAGATCTACAAATGTGTCGAATTCAATA-ATT 3’)/G1.2B (5’ GGCCAGATCTTTATATGATCAAAGAACCTATCA-ATT 3’) and G2.1AB (5’ GGCCGGATCCATCATGTTCCTGTTTCTGGACTTATTATATCG 3’) and G2.2AB (5’ GGCCGGATCCGCTCAATAATTCGAAATCTAAAG 3’) respectively. The PCR products were cloned into the Smal site of plasmid pUC18 and then subcloned into the BamHI site of VV transfer vector pFN24 in which the VV G gene is interrupted by a fowlpox early-late promoter (Uren et al., unpublished). The G gene was subcloned as a BglII fragment derived from clone G1.A6 and the GNS gene as a BamHI fragment from clone G2.H4, placing both genes in frame with the ATG of PEL. Human TK-143B cells infected with VV strain NYBH were used to construct rVV-G and as a control. The viruses were grown in human TK-143B cells in Eagle’s minimal essential medium containing 5% FCS.

Glucosamine labelling. Confluent monolayers of BHK cells in 24-well plates were infected with BEFV or VV at a multiplicity of 20 p.f.u./cell. At 10 h post-infection (VV) or 25 h post-infection (BEFV), infected cells were washed once with PBS and then incubated for 2 h in glucose-free RPMI 1640 (Gibco BRL) supplemented with 10 mM-HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% bovine serum albumin Fraction V (BSA). After glucose starvation, 200 μCi/ml D-[6-3H]glucosamine hydrochloride (Amersham) was added. After 2 h, the cells were washed twice with PBS and harvested into 50 μl of SDS gel loading buffer (50 mM-Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 100 mM-DTT). Radio-labelled proteins were analysed by electrophoresis in 12% SDS-polyacrylamide gels and fluorography (Walker et al., 1991).

Protein immunoblotting. Confluent monolayers of BHK cells grown in 25 cm² flasks were infected with BEFV or VV at a multiplicity of 10 p.f.u./cell. At 12 h post-infection (VV) or 26 h post-infection (BEFV), infected cells were washed twice with PBS and harvested into 10 μl of SDS gel loading buffer. The proteins were resolved by electrophoresis in 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell) by electroblotting. The membranes were blocked with 5% non-fat dry milk in 0.02% Tween 20 in PBS, incubated for 1 h with BEFV G protein monoclonal antibody 17B1 or rabbit antisera diluted in blocking solution, washed three times, incubated with sheep anti-mouse or sheep anti-rabbit IgG conjugated with horseradish peroxidase (Silenus), washed three times and reacted with 4-chloro-1-naphthol and hydrogen peroxide.

Immunofluorescence assays. Confluent monolayers of BHK cells in Lab-Tek tissue culture chamber slides (Miles Scientific) were infected at a multiplicity of 1 p.f.u./cell with BEFV or VV. At 10 h post-infection (VV) or 24 h post-infection (BEFV), cells were treated with BEFV G protein monoclonal antibody (diluted 1:500 to 1:2000 in PBS) or rabbit antisera (diluted 1:100 in PBS). For total cell staining, cells were fixed in methanol prior to antibody treatment. For cell surface staining, primary antibodies were added directly to the culture medium for 30 min at 37 °C and the cells were subsequently fixed with methanol. Binding of specific antibodies was detected with fluorescein isothiocyanate-conjugated sheep anti-mouse or sheep anti-rabbit IgG (Silenus).

Immunization of rabbits. Seven 12 to 16-week-old Half Lop rabbits were vaccinated by scarification on days 1 and 21 respectively. Blood samples were collected on days 0, 21 and 49. Three rabbits each were vaccinated with rVV-G or rVV-GNS and one rabbit with VV-NYBH as control.

Immunization and challenge of cattle. Six-month-old Hereford cattle were vaccinated intradermally on days 1 and 15. Six were vaccinated with VV-G, six with rVV-GNS, three with a combination of rVV-G and rVV-GNS and three with VV-NYBH. On day 29 all cattle were challenged with 5 ml of blood from a BEFV-infected cow (10⁶ TCID₅₀/ml). Blood samples were collected prior to vaccination, revaccination and challenge (days 0, 15 and 29), twice daily for 10 days and on days 39 and 45 after challenge. Buffy coat fractions were prepared for virus isolation and sera were tested for anti-viral antibodies.

Blocking ELISA and virus neutralization assays. The blocking ELISA was performed according to the method of Zakrzewski et al. (1992) using BEFV G protein monoclonal antibody DBS. Virus neutralization tests in BHK cells were done according to the method of Cybinski et al. (1978) using BEFV strain BB7721. Virus neutralization tests in Aedes albopictus cells were done using BEFV strain CSIRO 1927 according to the method described by Cybinski et al. (1992).
Vaccinia virus-expressed BEFV glycoproteins

Fig. 1. SDS-PAGE and protein immunoblotting of BEFV-, rVV-G-, rVV-G<sub>NS</sub>- and control, VV-NYBH-infected BHK cell lysates: (A) d-[3H]glucosamine-labelled cell lysates; (B) unlabelled cell lysates reacted with BEFV site G<sub>1</sub> monoclonal antibody 17B.

Conventional and immuno-electron microscopy. Uninfected and BEFV-infected BHK cells were processed for conventional ultra-thin sections, pre-embedding immuno-electron microscopy and for the generation of cryo-sections.

Preparations for conventional electron microscopy were fixed (40 min) with 2.5% (v/v) glutaraldehyde buffered with 0.1 M-cacodylate buffer (pH 7.2, 300 mM-OsO<sub>4</sub>) washed in the same buffer (3 × 20 min), post-fixed in 1% (w/v) osmium tetroxide (1 h), washed in water (4 × 5 min), dehydrated through graded alcohol and embedded in Spurr's low viscosity resin. Pre-immuno embedding electron microscopy was performed as described by Hyatt (1991). Briefly, infected cells were fixed with 0.1% (v/v) glutaraldehyde (10 min) and incubated for 1 h at room temperature with a 1:625 dilution of rabbit anti-G or G<sub>NS</sub> (diluted with 1% cold water fish gelatine in PBS-wash buffer). After six rinses in wash buffer the cells were incubated with a 1:40 dilution of 9 nm gold-labelled goat anti-rabbit (Amersham) for 1 h (room temperature). Cells were then rinsed (six times) and processed as described by Hyatt (1991).

For cryo-sectioning, uninfected and infected cells were fixed as described for pre-embedding immuno-electron microscopy. After six rinses in wash buffer, cells were scraped and pelleted in 5% gelatine. The resulting pellets were fixed for a further 10 min in 0.1% (v/v) glutaraldehyde, rinsed in wash buffer and infiltrated with 2.3 M-sucrose-10% polyvinylpyrrolidone. Cryo-sections were incubated with the above antibodies and processed as described by Hyatt & Wise (1994).

Results

Identification of rVV-expressed BEFV G and G<sub>NS</sub> proteins

BHK cells were infected with BEFV, vaccinia virus (VV-NYBH) or recombinant vaccinia viruses in which the BEFV G gene (rVV-G) or G<sub>NS</sub> gene (rVV-G<sub>NS</sub>) were expressed under the control of a fowlpox early-late promoter. At 12 h post-infection (VV) or 27 h post-infection (BEFV), cells were labelled for 2 h with d-[6-<sup>3</sup>H]glucosamine hydrochloride and cell lysates were analysed by SDS-PAGE and fluorography (Fig. 1 A). As described previously (Walker et al., 1991), the 81 kDa BEFV G protein and 90 kDa BEFV G<sub>NS</sub> protein were detected in BEFV-infected cells. In rVV-G-infected cells, only one band of similar mobility to the BEFV G protein was visible. In rVV-G<sub>NS</sub>-infected cells, a 90 kDa band corresponding to BEFV G<sub>NS</sub> protein was detected. However, a band of similar mobility to the BEFV G protein was also detected and this also occurred in VV-NYBH-infected cells, suggesting co-migration of the BEFV G protein with a VV glycoprotein. To verify this, immunoblots were done on lysates prepared from cells infected with BEFV, VV-NYBH, rVV-G or rVV-G<sub>NS</sub>, using BEFV G protein monoclonal antibody 17B. The results indicated that the G protein was present only in lysates of BEFV and rVV-G-infected cells and the recombinant G protein (mol. mass 79 kDa) appeared slightly smaller than the native form.

Antigenic analysis and cellular localization of BEFV G and G<sub>NS</sub> proteins

BHK cells infected with rVV-G, rVV-G<sub>NS</sub> or VV-NYBH were examined by immunofluorescence microscopy for reactions with BEFV G protein using monoclonal antibodies directed against all defined neutralizing antigenic sites (G1, G2, G3<sub>a</sub>, G3<sub>b</sub> and G4) (Cybinski et al., 1990, 1992). As shown in Fig. 2 for representative epitopes, all monoclonal antibodies to G protein reacted with rVV-G-infected cells, indicating that the native antigenic structure was preserved in the recombinant protein. Immunofluorescence was detected at the cell surface where the G protein appeared to accumulate in intensely staining patches (Fig. 2A–E) and in the cytoplasm (Fig. 2 F–J), indicating that the recombinant G protein was anchored in the cell membrane and transported efficiently from the endoplasmic reticulum/Golgi complex to the cell surface. In contrast, all BEFV G protein-specific monoclonal antibodies did not stain rVV-G<sub>NS</sub>-infected cells (Fig. 2K–O). A polyclonal rabbit
Fig. 2. For legend see facing page.
Rabbits were vaccinated with rVV-G, rVV-G_{NS} or VV-NYBH and revaccinated after 21 days. Sera collected on day 0 (pre-bleed), and on days 21 and 49 after initial infection were tested for virus-neutralizing antibodies. Neutralization tests were done in BHK cells and in Aedes albopictus cells using, respectively, homologous BB7721 strain adapted to BHK cells and CSIRO 1927 strain passaged only in Aedes albopictus cells after isolation from an infected cow (Table 1). Three rabbits vaccinated with rVV-G developed neutralizing antibodies after the first vaccination and serum antibody titres increased after the second vaccination. Neutralization titres were similar for viruses grown in mammalian and insect cells. In contrast, rabbits vaccinated with rVV-G_{NS} or VV-NYBH did not develop antibodies that neutralized BEFV grown in either cell line (Table 1).

BEFV-infected BHK cells were examined by immunofluorescence for reactions with rVV-G, rVV-G_{NS} or VV-NYBH rabbit antisera. As observed for rVV-G-infected cells, the BEFV G protein was detected in the endoplasmic reticulum/Golgi (Fig. 3A) and on the cell surface (Fig. 3B) where it accumulated in intensely staining patches. In contrast, BEFV G_{NS} protein was detected in the endoplasmic reticulum/Golgi (Fig. 3C) but at the surface the staining was diffuse, apparently not associated with the intensely staining patches observed for G protein. VV-NYBH rabbit antiserum failed to react with BEFV-infected BHK cells (data not shown).

Electron microscopic examination of BEFV-infected BHK cells showed characteristic bullet-shaped particles budding from the plasma membrane. When these were incubated with rabbit anti-G antiserum the viruses were specifically gold-labelled; the adjacent areas of the plasma membrane were not gold-labelled (Fig 4A). In contrast, when infected cells were incubated with rabbit anti-G_{NS} antiserum, the gold-labelling was associated with amorphous matrix on the extracellular aspect of the plasma membrane and not with budding or mature virions (Fig. 4B). Underlying the plasma membrane at the sites of G_{NS} localization were smooth-surfaced vesicles (Fig. 4B). Labelling of cryo-sections with anti-G or anti-G_{NS} antisera showed intracellular gold-labelling to be associated with the juxta-nuclear smooth-surfaced vesicles (Fig. 5A). Examination of similar regions within conventionally fixed cells (Fig 5B) showed that these structures are associated with the endoplasmic reticulum.
Vaccination of cattle twice at an interval of 2 weeks with infected cow. Cattle vaccinated with rVV-G alone or in combination with rVV-GNs developed neutralizing and blocking ELISA antibodies which continued to rise after challenge. Four of six cattle vaccinated with rVV-GNs and from two of three cattle vaccinated with YW-NYBH. In each case, and, in three animals, virus was isolated on two to three consecutive days. BEFV was isolated from blood samples collected from the second vaccination and challenge (Fig. 6). Viraemia was not detected in these animals for 10 days nor on day 16 after challenge. Cattle vaccinated with rVV-GNs alone or VV-NYBH did not develop neutralizing antibodies.

The BEFV GNs protein contains corresponding signal neutralizing antigenic sites (Cybinski et al., 1990) and, in three animals, virus was isolated on two to three consecutive days. BEFV was isolated from blood samples collected from the second vaccination and challenge (Fig. 6). Viraemia was not detected in these animals for 10 days nor on day 16 after challenge. Cattle vaccinated with rVV-GNs alone or VV-NYBH did not develop neutralizing antibodies.

The role of BEFV G and G~s glycoproteins in the synthesis and transport of BEFV G and G~s is a structural component of the viral envelope (Walker et al., 1989). Like the native G protein, rVV-expressed BEFV G is a structural component of the viral envelope and retains the native antigenic structure, reacting with monoclonal antibodies representing all five defined neutralizing antigenic sites. Despite its reduced size, the recombinant G protein appears slightly smaller than the native protein, perhaps due to the absence of one of the native protein's potential N-glycosylation sites.

The large open arrow shows the presence of gold particles with an amorphous matrix associated with the plasma membrane. This region of the plasma membrane was associated with large numbers of vesicles. Plasma membrane not associated with the amorphous matrix was not gold-labelled. N, nucleus; C, cell. Bars represent 100 rim.

Panel (A) shows BEFV specifically labelled with anti-G. Panel (B) shows BEFV-infected cells pre-labelled with anti-G~s. Fig. 4. Transmission electron micrographs of sections of BEFV-infected cells which were pre-labelled with rabbit anti-G and anti-G~s.

The glycoproteins to the cell surface.

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reticulum/Golgi system. No gold labelling was observed on or within control cells reacted with antiserum to G or G\textsubscript{NS}. These results are consistent with an active involvement of the endoplasmic reticulum/Golgi system in the synthesis and transport of BEFV G and G\textsubscript{NS} glycoproteins to the cell surface.

**Vaccination of cattle with rVV-G and rVV-G\textsubscript{NS} viruses**

The role of BEFV G and G\textsubscript{NS} glycoproteins in the induction of protective immunity was determined by vaccination of cattle twice at an interval of 2 weeks with rVV-G, rVV-G\textsubscript{NS}, rVV-G\textsubscript{NS}, or VV-NYBH, and challenge 2 weeks later with blood from a BEFV-infected cow. Cattle vaccinated with rVV-G alone or in combination with rVV-G\textsubscript{NS} developed neutralizing and blocking ELISA antibodies which continued to rise after the second vaccination and challenge (Fig. 6). Viraeemia was not detected in these animals for 10 days nor on day 16 after challenge. Cattle vaccinated with rVV-G\textsubscript{NS} alone or VV-NYBH did not develop neutralizing antibodies following vaccination but seroconverted after challenge. BEFV was isolated from blood samples collected from four of six cattle vaccinated with rVV-G\textsubscript{NS} and from two of three cattle vaccinated with VV-NYBH. In each case, virus was isolated between days 3 to 6 after challenge and, in three animals, virus was isolated on two to three consecutive days.

**Discussion**

Rhabdoviruses of the genus *Ephemerovirus* have the unusual characteristic of consecutive genes encoding related class 1 transmembrane glycoproteins. The BEFV G protein, like those of vesicular stomatitis virus and rabies virus, is a structural component of the viral envelope (Walker et al., 1991) which contains virus-neutralizing antigenic sites (Cybinski et al., 1990) and induces protective immunity in cattle (Uren et al., 1994). The BEFV G\textsubscript{NS} protein contains corresponding signal and transmembrane domains, and shares regions of significant amino acid sequence similarity with the virion G proteins of BEFV and other rhabdoviruses (Walker et al., 1992). The G\textsubscript{NS} protein is expressed in mammalian cells at similar levels to the G protein but, although it has the structural characteristics of a rhabdovirus envelope glycoprotein, it has not been detected in virions (Walker et al., 1991). The results reported here indicate that, despite their structural similarity, the BEFV G and G\textsubscript{NS} proteins are antigenically unrelated and functionally distinct. Recombinant G\textsubscript{NS} protein failed to bind polyclonal BEFV G antisera or monoclonal antibodies directed at each of the five G protein neutralization sites and did not induce neutralizing antibody or protective immunity in cattle. Anti-G\textsubscript{NS} rabbit serum also failed to neutralize BEFV grown in BHK or *Aedes albopictus* cells indicating that, at least in these cell lines, G\textsubscript{NS} does not function as a cell attachment protein.

Other families of negative-strand RNA viruses (paramyxoviruses, filoviruses, bunyaviruses, arenaviruses and orthomyxoviruses) possess two transmembrane glycoproteins that are packaged into the viral envelope. In bunyaviruses, both G1 and G2 are class 1 membrane proteins and, when co-expressed, both are retained in the Golgi complex (Chen et al., 1991; Ruusala et al., 1992) where virus maturation occurs. For the bunyavirus La Crosse, it has been proposed that the G1 and G2 glycoproteins have differential roles in vertebrate and insect cell attachment during natural infections (Ludwig et al., 1989). We have found that in BEFV both G and G\textsubscript{NS} are transported via the endoplasmic reticulum/Golgi to the plasma membrane but only the G protein is packaged into mature virions. The signals responsible for the packaging of BEFV G protein whilst excluding G\textsubscript{NS} from mature virions have not been characterized. Although antiserum to G\textsubscript{NS} failed to neutralize BEFV in either vertebrate or invertebrate cell cultures, G\textsubscript{NS} may play a role in extending BEFV cell tropism if packaged into mature virions in other tissues during natural infections.

Like the native G protein, rVV-expressed BEFV G effectively protected cattle against experimental infection. The recombinant G protein appeared slightly smaller than the native protein, perhaps due to the absence of N-glycosylation at one of the five potential sites. Despite its reduced size, the recombinant G protein retained the native antigenic structure, reacting with monoclonal antibodies representing all five defined neutralizing antigenic sites and was efficiently trans-
Fig. 5. Transmission electron micrograph (A) of a cryo-section from a BEFV-infected cell incubated with rabbit anti-G and 10 nm goat anti-rabbit. Arrows indicate gold-labelled smooth-surfaced vesicles. Bars represent 100 nm. (B) Conventional ultrathin section of a BEFV-infected cell. The arrow indicates the presence of Golgi stacks and vesicles within the cytoplasm of an infected cell. The structures labelled (arrows) in (A) are consistent in size and intracellular location with those identified (arrows) in (B), namely the smooth-surfaced vesicles of the Golgi complex. Bar represents 200 nm. N, nucleus; m, mitochondrion.
ported to the cell surface. The recombinant $G_{NS}$ protein was indistinguishable in size from the highly glycosylated native protein. Although $G_{NS}$ failed to protect against challenge with BEFV-infected cattle blood, its role in infection requires further investigation and challenge experiments using infected insects will be required to exclude totally its possible involvement in protection. The efficacy of current live attenuated or live-killed BEFV vaccines has, in general, been less than satisfactory (Uren et al., 1992, 1994). This study suggests that recombinant glycoproteins expressed by VV, other viral vectors or naked DNA may be useful alternatives to current vaccines.

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References


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