Mapping of B-Cell Epitopes of Hemagglutinin Protein of Rinderpest Virus

G. J. Renukaradhya,* S. Mitra-Kaushik,†† G. Sinnathamby,†² M. Rajasekhar,* and M. S. Shaila†³

*Project Directorate on Animal Disease Monitoring and Surveillance, Hebbal, Bangalore-560 024, India; and
†Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore-560 012, India

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Monoclonal antibodies (mAbs) against secreted hemagglutinin (H) protein of rinderpest virus (RPV) expressed by a recombinant baculovirus were generated to characterize the antigenic sites on H protein and regions of functional significance. Three of the mAbs displayed hemagglutination inhibition activity and these mAbs were unable to neutralize virus infectivity. Western immunoblot analysis of overlapping deletion mutants indicated that three mAbs recognize antigenic regions at the extreme carboxy terminus (between amino acids 569 and 609) and the fourth mAb between amino acids 512 and 568. Using synthetic peptides, aa 569–577 and 575–583 were identified as the epitopes for E2G4 and D2F4, respectively. The epitopic domains of A12A9 and E2B6 mAbs were mapped to regions encompassing aa 527–554 and 588–609. Two epitopes spanning the extreme carboxy terminal region of aa 573 to 587 and 588 to 609 were shown to be immunodominant employing a competitive ELISA with polyclonal sera from vaccinated cattle. The D2F4 mAb which recognizes a unique epitope on RPV-H is not present on the closely related pestes des petits ruminant virus HN protein and this mAb could serve as a tool in the seromonitoring program after rinderpest vaccination.

Key Words: rinderpest virus; hemagglutinin protein; monoclonal antibodies; deletion proteins; synthetic peptides; epitope mapping.

INTRODUCTION

Rinderpest virus (RPV) is an important member of the morbillivirus genus in the Paramyxoviridae family. The virus affects domestic cattle and buffaloes as well as many cloven-hoofed animals in the wild and therefore the disease is economically significant; the disease is enzootic in south Asia and the Near and Middle East (Barrett and Rossiter, 1999). RPV consists of two cell surface glycoproteins—hemagglutinin H, which binds to the cellular receptor to begin the process of infection, and the fusion protein F, which helps in virus entry by way of the fusion of the virus envelope with the host cell membrane. These two proteins together are responsible for protective immunity (Yilmə et al., 1988; Romero et al., 1994) and thus are considered promising candidates for subunit vaccines.

The expression of a recombinant H protein of RPV in secreted (Sec H) as well as membrane-bound forms using baculovirus systems (Naik and Shaila, 1997) facilitated the examination of the immunogenic properties of the recombinant baculovirus expressing the membrane-bound H protein as extracellular baculovirus particles (rECV-H) in cattle (Naik et al., 1997). Subsequently, the H protein presented on rECV particles was shown to generate virus neutralizing antibodies, helper T cell responses in cattle (Sinnathamby et al., 2001a).

We and others (Bhavani et al., 1989; Sugiyama et al., 1989; Anderson and McKay, 1994) have earlier reported the production and characterization of a panel of mAbs to H protein. However, the linear and/or conformational epitopes for these mAbs were not mapped on the protein, which could provide an insight into the antigenic structure of the protein. Recently, neutralizing immunodominant epitopes on RPV-H have been mapped using selected mAb-resistant mutants of a lapinized strain (Sugiyama et al., 2002). However, there is no information concerning the major antigenic region(s) on the H protein, which carries epitopes unique to RPV-H and not present on the corresponding protein of pestes des petits ruminant virus (PPRV), recognized by non-cross-reactive monoclonal antibodies. In the present work, we report the generation of mouse mAbs against RPV-H using recombinant H produced in a secretory form in insect cells as the immunizing antigen in mice. Of several clones isolated, four have been extensively characterized. B-cell epitopes recognized by these mAbs were mapped to the extreme carboxy terminus of H using truncated proteins derived from overlapping gene dele-
tion constructs of the H gene, expressed and purified from *Escherichia coli* (Sinnathamby et al., 2001a) and a panel of synthetic peptides representing the sequences of H and closely related measles virus H protein.

**RESULTS**

**Specificity and biological properties of mAbs**

A secretory form of the hemagglutinin protein (membrane anchorless) of RPV was employed to generate hybridomas. The reactivity of mAbs was tested using RPV (RBOK vaccine strain)-infected Vero cell extracts. Thirty-nine parental clones secreting antibodies reactive with H protein were chosen for subcloning by limiting end point dilution. In order to obtain mAbs with different specificities, one clone from each parental clones was selected after subcloning. Finally, four subclones secreting the mAbs in a stable manner were chosen (D2F4, E2G4, E2B6, and A12A9) for the generation of ascitic fluid. As PPRV-HN and RPV-H are antigenically closely related, it was necessary to assess the cross-reactivity of the mAbs with PPRV (Nig 75/1)-infected cell extracts, while the fourth clone, D2F4, was non-cross-reactive (Table 1). The immunoglobulin class and subclass of the mAbs were defined using isotype-specific ELISA and the results are summarized in Table 1.

The reactivity patterns of mAbs were also tested using intracellular and cell surface immunofluorescence with either RPV-infected Vero cells or PPRV-infected Vero cells (Fig. 1). The cross-reactivities of three of the four mAbs (data not shown) and the non-cross-reactivity of D2F4 are shown in Fig. 1, which matched the ELISA results. When tested for their ability to neutralize the infectivity of RPV (RBOK) and PPRV (Nig 75/1) on Vero cells, it was observed that none of the antibodies neutralized virus infectivity (data not shown). Three of the cross-reactive mAbs (E2G4, A12A9, and E2B6) inhibited hemagglutination activity, while D2F4 did not, which indicated that the determinants recognized by these two groups of antibodies are different (Table 2).

**Mapping of B-cell epitopes on the hemagglutinin protein**

The immunoreactivity pattern of D2F4 in Western blot analysis using six different RPV-H deletion fragments indicated that only two deletion mutants, namely, N^Δ448 and N^Δ511, were recognized (Fig. 2A and Table 2). Mabs E2G4 and E2B6 in the Western blot analysis gave similar immunoreactivity patterns (data not shown). A12A9 recognized three deletion mutants, namely, N^Δ448, N^Δ511, and N^Δ359C^Δ41 (Fig. 2B and Table 2). From the immunoreactivity pattern in Western analysis employing deletion mutants of RPV-H, it was deduced that the domain recognized by three mAbs (D2F4, E2G4, and E2B6) lies in the region comprising aa 569–609 (extreme 41 aa in the carboxy terminus of H protein) and the domain recognized by mAb A12A9 is aa 512–568. By employing a recombinant fragment N^Δ241 of PPRV (AP94/1) hemagglutinin neuraminidase (HN), it was further confirmed that E2G4, A12A9, and E2B6 cross-reacted with PPRV-HN and D2F4 failed to recognize this protein (Fig. 3).

In order to further narrow down the mAb binding region, two 15-mer overlapping peptides (peptides 51 and 52, Partidos et al., 1991) specific for the highly homologous MV-H protein spanning the region aa 563–587 (with a 5-aa overlap) were used. These MV peptides have been shown to elicit helper T-cell response in mice (Partidos et al., 1991). Comparison of the amino acid sequences for MV-H and RPV-H in this region reveals about 67% identity of peptides 51 and 52 and conservative substitutions making up the rest of the changes, thus bringing a high degree of homology. The results of the peptide ELISA performed with all four mAbs (Table 3) indicated that E2G4 and D2F4 showed binding to aa 563–577 and 573–587, respectively. Subsequently, to narrow down the sites recognized by the mAbs to the precise epitope level, two 9-mer synthetic peptides for

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**TABLE 1**

**Properties of Rinderpest Virus Hemagglutinin Protein Monoclonal Antibodies**

<table>
<thead>
<tr>
<th>RPV-H mAbs</th>
<th>Antibody class</th>
<th>Light chain</th>
<th>Cross-reactivity with PPRV</th>
<th>Intracellular</th>
<th>Cell surface</th>
<th>Intracellular</th>
<th>Cell surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2F4</td>
<td>IgM</td>
<td>κ</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>E2G4</td>
<td>IgG 2b</td>
<td>κ</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>E2B6</td>
<td>IgG 2b</td>
<td>κ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>A12A9</td>
<td>IgM</td>
<td>κ</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*Note.* (+) Low reactivity in ELISA or IF; (++) moderate reactivity in ELISA or IF; (+++) high reactivity in ELISA or IF; (−) negative reaction in ELISA or IF.
RPV-H protein in the region spanning aa 569–583 (with a 3-aa overlap) were used. The peptide ELISA results (Table 3) successfully identified the epitopes for the mAbs E2G4 and D2F4 to be aa 569–577 and 575–583, respectively.

In order to localize the epitope for A12A9 mAb, two each of non-overlapping 6-mer and 7-mer synthetic peptides spanning the region aa 527–554 were tested for their reactivities in peptide ELISA (results not shown). The results showed that A12A9 mAb reacted with three peptides spanning the region aa 527–554, indicating the possibility of the coming together of the epitope associated with the structural form adopted by amino acids 527–554. Based on the reactivity of mAb E2B6 in Western blot analysis and nonreactivity with any of the synthetic peptides used, it is likely that E2B6 recognizes an...
epitopic determinant in a stretch of 21 aa, between aa 588 and 609.

Identification of immunodominant epitopes

To determine if the epitopes recognized by the mAbs are immunodominant, we carried out a competitive ELISA in which sera from cattle vaccinated with tissue culture rinderpest vaccine were used as competitors for mAbs for binding to the corresponding epitopes, mapped in the present work (Fig. 4). The ascites of all four mAbs had titers of over 1500 in indirect ELISA using Sec H as well as RPV-infected cell lysate. Binding of two mAbs to their epitopes was inhibited by immune sera (Fig. 5), suggesting that these epitope/epitopic regions are immunodominant (aa 573–587 and 588–609). The immunodominant epitopic region as identified in the present work also contains the neutralizing epitope of H (aa 587–592) identified recently (Sugiyama et al., 2002) on the H protein of lapinized rinderpest virus.

DISCUSSION

We have earlier demonstrated that baculovirus-expressed membrane-bound RPV-H (Naik et al., 1997; Sinnathamby et al., 2001b) and PPRV-HN (Sinnathamby et al., 2001c) generate virus neutralizing antibodies and cell-mediated immune responses in the natural hosts cattle and goats, respectively. Thus, baculovirus-expressed protective antigens of complex viruses can be

### TABLE 2
Reactivity of RPV-H mAbs with Hemagglutinin Deletion Mutants of Hemagglutinin Protein in Western Blot Analysis

<table>
<thead>
<tr>
<th>RPV-H protein region (aa)</th>
<th>Name of the deletion fragment</th>
<th>mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D2F4</td>
</tr>
<tr>
<td>449–609</td>
<td>NΔ448</td>
<td>+</td>
</tr>
<tr>
<td>512–609</td>
<td>NΔ511</td>
<td>-</td>
</tr>
<tr>
<td>360–568</td>
<td>NΔ359Δ41</td>
<td>-</td>
</tr>
<tr>
<td>113–182; 425–450</td>
<td>NΔ112Δ183–424; CΔ160</td>
<td>-</td>
</tr>
<tr>
<td>357–424</td>
<td>NΔ356CΔ185</td>
<td>-</td>
</tr>
<tr>
<td>113–182</td>
<td>NΔ112CΔ427</td>
<td>-</td>
</tr>
</tbody>
</table>

*Note.* (+) Signals with a specific deletion protein fragment; (−) negative with that specific deletion protein fragment. All the deletion fragments reacted with polyclonal hyperimmune serum against rinderpest virus.

![FIG. 2](image-url) Reactivity of RPV-H mAbs with RPV-H deletion proteins by Western immunoblot. Purified proteins (1 μg) were electrophoresed on a 13.5% SDS–polyacrylamide gel (A) and 12% (B); the blots were probed with mAbs at dilutions 1 in 5000 for D2F4 and 1 in 3000 for A12A9 ascitic fluid. (A) Lanes: 1, NΔ359; CΔ41; 2, NΔ112; Δ183–424; CΔ160; 3, NΔ448; 4, NΔ511; 5, NΔ112; CΔ427; and 6, NΔ356; CΔ185. (B) Lanes: 1, NΔ359; CΔ41; 2, NΔ448; 3, NΔ511; 4, NΔ112; Δ183–424; CΔ160; 5, NΔ112; CΔ427; and 6, NΔ356; CΔ185.
employed in a wide variety of applications to strengthen our knowledge of the immunological properties of such antigens in isolation. Further, the recombinant H protein was engineered for secretion into the medium by removing the membrane anchor region (Naik and Shaila, 1997). This soluble form of H is similar to the membrane-bound form, as shown by its reactivity with convalescent sera from animals infected with RPV or sera from vaccinated animals; therefore, this soluble recombinant H was used to generate monoclonal antibodies with the objective of identifying and characterizing a monoclonal antibody unique to rinderpest virus for use in seromonitoring work.

The immunoreactivity pattern of the three mAbs to RPV-H deletions identified the antigenic domain to be aa 569–609 on RPV-H protein, while for the fourth one, it is the aa 512–568 region. As the epitope recognized by the mAb A12A9 spans a region of 57 aa, it is likely that the epitope may have been formed by the conformation attained by the amino acids in this region. Earlier work on glycoprotein B of pseudorabies virus has shown that continuous epitopic domains could be successfully identified in recombinant fragments (Zaripov et al., 1999).

Lack of authentic higher order structure of recombinant proteins expressed in E. coli (Cason, 1994) can be exploited to identify linear epitopes on protein antigens. Synthetic peptides have been successfully used to identify B-cell epitopes (Ziegler et al., 1996). Hence we used a panel of synthetic peptides to narrow down the antigenic domains recognized by the mAbs. The reactiv-

<table>
<thead>
<tr>
<th>mAb designation</th>
<th>ELISA reactivity with MV-H peptides</th>
<th>ELISA reactivity with RPV-H peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peptide 51</td>
<td>Peptide 52</td>
</tr>
<tr>
<td>D2F4</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>E2G4</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E2B6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A12A9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCRV vaccinated serum (positive)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Preimmune serum (negative)</td>
<td>–</td>
<td>–</td>
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</tbody>
</table>

Note. Peptide 51, 563PIELOVECFTWDQKL577, and peptide 52, 577WDQKLWCRHFCVLAD583. All the peptides showed positive signals with rinderpest hyperimmune serum.
ity of the two mAbs E2G4 and D2F4 with two 15-mer MV-H synthetic peptides (peptides 51 and 52) indicated that closely related viral peptides can be made use of while searching for epitopes. Finally, the precise B-cell linear epitopes of E2G4 and D2F4 (aa 569–577 and 575–583) were successfully mapped based on their reactivity with two 9-mer RPV-H peptides and similarly, based on the reactivity of mAb A12A9 with two 6-mer and one 7-mer RPV-H peptides, the epitopic domain of A12A9 mAb was identified to be aa 527–554 (28 aa). Based on its reactivity in Western blot and nonreactivity with any of the synthetic peptides used in this study, the binding region of the fourth mAb, E2B6, was localized to a 21-aa stretch (between aa 588 and 609); hence, the epitopes of the two mAbs A12A9 and E2B6 may constitute a linear or even a conformation-dependent epitope.

Two major T-helper (Th) epitopes on RPV-H in cattle have been identified (Sinnathamby et al., 2001a). One of the epitopes was identified at the extreme carboxy terminus (aa 575–583). This region overlaps the B-cell epitopic region (aa 569–583) identified in this work. This is the first demonstration of the occurrence of overlapping B-cell and Th epitopes on the H protein.

In a recent study, a number of mAbs against epitopes 587 to 592 on RPV-H were identified compared to those against other epitopes (Sugiyama et al., 2002); the immunodominant epitopes recognized by two of the mAbs in the present work (D2F4 and E2B6) fall in the same region as epitopes D and E reported by these authors. Our finding also demonstrates that all the four mAbs are from the extreme carboxy terminal domain and the region encompassing the epitopes for two mAbs is beyond aa 575 (Fig. 4). The two mAbs which recognize the immunodominant epitope/epitopic domain are nonneutralizing, which suggests that all the immunodominant B-cell epitope/epitopic domain need not elicit neutralizing antibody. The characterization of the linear non-cross-reactive epitope recognized by the mAb D2F4 has provided a valuable tool for the development of a highly specific C-ELISA kit for seromonitoring as well as for serosurveillance for rinderpest virus infection (Renukaradhy et al., to be published).

PPRV has been shown to possess hemagglutination activity, whereas RPV is devoid of this activity, although antibodies to RPV-H inhibit the hemagglutination by PPRV (Ramachandran et al., 1995). Therefore, the hemagglutination inhibition (HI) activity of RPV-H mAbs was assessed. Three of the four RPV-H mAbs, E2G4, A12A9, and E2B6, showed HI activity, with titers of 200–400, while the fourth mAb, D2F4, did not show any HI activity.
as it is non-cross-reactive with PPRV-HN (Table 1). Monoclonal antibodies can also be grouped based on their reactivity in various functional assays, such as virus neutralization, HI, and hemolysis inhibition (HLI) activity (Rydbeck et al., 1986). Four RPV-H mAbs can be classified into two groups based on their functional inhibitory activities: group I, which showed HI activity, but did not have both virus neutralization and HLI activities (three non-cross-reactive mAbs, E2G4, A12A9, and E2B6), and group II, which did not show HI activity, virus neutralization, and HLI activities (RPV-H-specific and PPRV-HN non-cross-reactive mAb, D2F4). Since the epitopes for the HI− mAb D2F4 (aa 575–583) overlaps one of the HI+ mAb E2G4 (aa 569–577), one or more aa in the 569–577 region seems to be a major determinant for the hemagglutination or cell attachment function of the H protein. This is in close proximity with residue 546 on MV-H protein involved in HA activity (Hummel and Bellini, 1995).

RPV and PPRV are two distinct, but antigenically closely related viruses, which are the causative agents of RP and PPR. PPRV also causes subclinical infection in large ruminants, which in turn serves as a carrier of infection to sheep and goats, apart from protecting the bovine population against RPV infection (Dardiri et al., 1977). This posed a problem earlier in differentiating them serologically; it is very essential to differentiate these two diseases serologically to achieve a successful RP serosurveillance program. Similarly, to assess vaccine efficacy, it is essential to carry out seromonitoring using rinderpest-specific C-ELISA. In order to differentiate the antibodies made against RPV from those against PPRV during serosurveillance and seromonitoring studies, it is important to use a highly specific monoclonal antibody which recognizes a unique epitope on the RPV antigen. The generation of such a mAb recognizing a unique epitope on RPV-H protein as shown in this work could serve such a role.

**MATERIALS AND METHODS**

**Cells and viruses**

Vero cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Himedia, India) supplemented with 5% fetal calf serum (FCS, Gibco-BRL, U.S.A.). *Spodoptera frugiperda* (Sf)-21 cells were grown in TC-100 insect cell culture medium (Gibco BRL, U.S.A.) supplemented with 10% fetal bovine serum (FBS, Gibco BRL). Hybridoma cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM, Gibco BRL) supplemented with 10% FBS. Baculo recombinant viruses expressing soluble, Sec H and the membrane-bound form have been described earlier (Naik and Shaila, 1997) and were propagated in Sf-21 cells. PPRV Nig 75/1 was obtained from Dr. A. Diallo, CIRAD-EMVT, France, and RPV (RBOK vaccine strain) was obtained from the Institute of Animal Health and Veterinary Biologicals, Bangalore. They were propagated on Vero cells. Parental clones of hybridoma were selected using HAT medium (hypoxanthine, amionopterin, and thymidine, Gibco BRL) and HT medium (hypoxanthine and thymidine, Gibco BRL).

**Titration of recombinant baculovirus by TCID_{50} assay**

Sf-21 cells were plated on flat-bottomed 96-well tissue culture plates (Costar, U.S.A.) at a density of 1 × 10^5 cells/ml; 10-fold dilutions of the virus was performed in complete TC-100 medium. Virus dilutions (100 μl) were then added in replicate wells and incubated at 27°C for 4 days and the development of cytopathic effect (CPE) was recorded. TCID_{50} was calculated employing the Reed and Muench formula (Burleson et al., 1992).

**Expression and purification of deletion mutants of RPV-H protein**

Construction of deletion mutants of RPV-H and PPRV-HN protein, expression as His-tagged fusions in *E. coli* strain BL21(DE3), purification, and also stably expressed deletion mutants were confirmed by Western blot, probed with rabbit hyperimmune serum to RPV (RBOK) as described earlier (Sinnathamby et al., 2001a). The deletion mutants of PPRV-HN, N_{424} and RPV-H N_{448}, N_{511}, NΔ359;C_{41}, N_{112};Δ183-424;C_{160}, NΔ356;Δ185, and NΔ112;Δ427 were employed in this study, where NΔ112;Δ183-424;C_{160} is a deletion mutant that does not express the N-terminal 112 aa, the C-terminal 160 aa, an internal stretch between aa 183 and 424, and so on.

**Western blot analysis of the recombinant proteins**

Equal amounts of deletion proteins were electrophoresed on SDS–polyacrylamide gels and proteins were transferred onto nitrocellulose membrane (Amer sham, UK) using a semidry transfer apparatus (LKB, U.S.A.). The blot was then blocked with blocking buffer (3% gelatin in PBS) for 1 h at room temperature and then incubated with appropriately diluted mAbs in blocking buffer. After incubation for 1 h at room temperature, horseradish peroxidase (HRP)-conjugated antimouse antibody (Boehringer Manheim, Germany) in blocking buffer was added and the blot was incubated for 1 h at room temperature. Then the blot was developed using hydrogen peroxide and diaminobenzidine (DAB) (Sigma Chemicals Co, St. Louis, MO) in PBS. The membrane washed after each incubation step three times using PBS containing 0.1% Tween 20 (Merck, Germany) (PBST).

**Synthetic peptides**

MV-H peptides were a kind gift from Dr. M. Steward, London School of Hygiene and Tropical Medicine, United Kingdom. Sequences of MV-H peptides that were
employed in this study correspond to peptide 51, PIELOVECFTWQDKL (positions 563–677), and peptide 52, WDQKLCWRHFCVVLAD (positions 573–687). Two 9-mer synthetic overlapping peptides were synthesized corresponding to the region aa 569–577 (peptide 1, ECF-PWDRKL) and aa 576–583 (peptide 2, RKLWHCHF) on RPV-H protein. Four non-overlapping peptides corresponding to different specified locations on the H protein of RPV, peptide 1, ATYDISR (aa 527–533); peptide 2, SSYYYYP (aa 549–554); peptide 3, FHMTNYL (aa 270–278); and peptide 4, EHAIVY (aa 535–540), were also used for reactivities in peptide ELISA with H mAbs.

Competitive ELISA

C-ELISA was performed essentially as described previously (Anderson and McKay, 1994). Briefly, the baculo recombinant Sec H of RPV prepared in serum-free medium was used as an antigen for C-ELISA. Each well of a 96-well microtiter plate was coated with the antigen (5 μg/ml) at 4°C overnight and treated with blocking buffer (PBS supplemented with 0.1% (v/v) Tween 20 and 0.3% (v/v) normal bovine serum seronegative for RPV) for 1 h at 37°C. After washing five times in phosphate-buffered saline (0.2 X PBS), appropriate dilutions of test sera (10 negative and 50 positive sera) and anti-H mAbs (D2F4, E2B6, A12A9, and E2G4) were added. Controls were included, such as strong positive, weak positive, and negative bovine sera and a mAb (0% competition) control. Monoclonal antibody control wells (0% competition) contained antigen, mAb, and enzyme conjugate (no test serum). Following incubation at 37°C for 1 h in an orbital shaker, plates were washed and antimouse HRPO conjugate (predetermined dilution) was added. After a final incubation, substrate/chromogen (OPD/H2O2) was added and the color allowed to develop for 10–15 min. Plates were read on a ELISA reader at 490 nm and analyzed using enzyme immunoassay (EIA) software (Biologicals Diagnostic Supplies Ltd., United Kingdom) and the OD values were converted to percentage of inhibition (P) values using the following formula. P = 100 – (OD in test well/OD in 0% control well) × 100.

Purification of recombinant Sec H protein

SF-21 cells were adapted gradually to serum-free medium (SF-900 II SFM, Gibco-BRL). Then the cells were infected with the recombinant baculovirus expressing H in a secretory form (Naik and Shaila, 1997) at an m.o.i. of 10. The culture supernatant was collected after 72 h when more than 90% CPE was observed, and the supernatant was clarified by centrifugation at 4000 g for 30 min. The supernatant was precipitated with ammonium sulfate at 60% saturation. The precipitate was dialyzed against column binding buffer (Tris–HCl, pH 7.8, 10 ml, 50 mM NaCl, 1% Triton X-100, and 1 mM PMSF) and purified on a rabbit anti-RPV-H antibody affinity column as described by Naik et al. (1997). Briefly, immunoglobulins from 2 ml of monospecific polyclonal rabbit antiserum were precipitated at 50% ammonium sulfate saturation and the dialyzed immunoglobulins were coupled to CNBr-activated Sepharose 4B, blocked with ethanolamine, and used to make the affinity column. This column was equilibrated with 20 vol of wash buffer (the same as binding buffer). After recycling the supernatant (a dialysate of Sec H) thrice at a flow rate of about 10 ml/hour, the column was washed with 20–25 ml of wash buffer at a flow rate of 12 ml/hour at 4°C. The column-bound protein was eluted with elution buffer (0.2 M glycine, pH 2.8, and 0.2% Triton X-100) and the eluate was immediately neutralized with 1 M Tris solution. Ten fractions of 1 ml each were eluted, pooled, and lyophilized until further use. The purified protein was used for immunization in BALB/c mice.

Generation of RPV-H hybridoma clones

BALB/c mice were immunized subcutaneously (sc) with 100 μg of purified Sec H protein in Freund's complete adjuvant and they were boosted twice with 50 μg of Sec H in Freund's incomplete adjuvant at 3-week intervals. A week following the second booster injection, mice were bled and H-specific ELISA titers were determined. A titer of 1:100,000 was considered optimum for carrying the fusion. Three weeks after the second booster, mice were further boosted with 100 μg of purified Sec H intraperitoneally. Four days later, one of the immunized animal was sacrificed to make a spleen cell suspension which was fused with Sp2/0 myeloma cells by standard PEG-mediated fusion as described by Bhavan et al. (1989).

Parental clones were subjected to sub-cloning to achieve monoclonality of the clones. One clone from each parental clone was selected based on its reactivity with Sec H and RPV-RBOK infected cell extracts in ELISA. These hybridomas (1 X 10⁵ cells per ml) were injected in PBS intra-peritonealy into pristine primed Balb/c mice for ascites production and after 10 to 14 days ascitic fluid was collected. The antibody titer in the ascitic fluid was determined using Sec H or infected cell extracts of RPV (RBOK) as coating antigen in indirect ELISA.

RPV (RBOK)- and PPRV (Nig 75/1)-infected cell extracts

RPV (RBOK) and PPRV (Nig 75/1) viruses were propagated in Vero cells. At 60–70% confluency, Vero cells were infected at an moi 10. After 48–72 hrs when 60–70% cpe was visible, infected cells were scraped and were gently washed once with PBS and then resuspended in PBS. The cell suspension (1 X 10⁶ cells ml⁻¹) was sonicated in a VibraCell (U.S.A.) ultrasonic processor and clarified at 10,000X g for 10 min. Protein concentration
was estimated by Lowry method (1972) and used in indirect ELISA.

Indirect ELISA

ELISA plates were coated with lysates of Vero cells infected with PPRV Nig 75/1 and RPV-RBOK strain at a concentration of 1 μg/well or culture supernatants containing Sec H (500 ng/well) for 1 h at 37°C or overnight at 4°C. The plate was washed in PBS thrice and blocked with blocking buffer (3% bovine gelatin + 0.1% Tween 20 in PBS) for 1 h at 37°C. Serial dilutions of the RPV-H-specific mouse hyperimmune serum/hybridoma culture supernatant/ascitic fluid in PBS were used as primary antibody and incubated at 37°C for 1 h. The plates were then washed and appropriately diluted antiserum whole Ig-HRP or antiserum IgM-HRP conjugate was added and the plates were incubated at 37°C for 1 h. The reaction was developed using O-phenylenediamine dihydrochloride and H2O2 and the reaction was stopped with 2 N H2SO4. The plates were read at 490 nm in an ELISA reader.

Supernatants collected from each hybridoma clone were subjected to an isotyping ELISA using the Boehringer Mannheim isotyping kit according to the manufacturer’s instructions.

Peptide ELISA

ELISA plates were coated overnight at +4°C with the peptides at a concentration of 25 ng per well using carbonate bicarbonate buffer (pH 9.6 ± 0.05) and blocked with blocking buffer (3% bovine gelatin + 0.1% Tween 20 in PBS) for 1 h at 37°C. Appropriate dilutions of the ascites (both RPV-H mAbs) in blocking buffer were added and incubated at 37°C for 1 h, followed by addition of appropriately diluted secondary antibody (antimouse whole Ig-HRP conjugate) incubated at 37°C for 1 h. Between each step, plates were washed three times with wash buffer (0.1% Tween 20 in PBS). As a positive control, serum from cattle vaccinated with TCRV was added and incubated at 37°C for 1 h. The reaction was developed using diluted secondary antibody (antibovine whole Ig-HRP conjugate) incubated at 37°C for 1 h, followed by addition of antimouse whole Ig-HRP conjugate incubated at 37°C for 1 h. The plates were then washed and appropriately diluted antiserum whole Ig-HRP conjugate was added and the plates were incubated at 37°C for 1 h. The reaction was developed using O-phenylenediamine dihydrochloride and H2O2 and the reaction was stopped with 2 N H2SO4. The plates were read at 490 nm in an ELISA reader.

Immunofluorescence

Vero cells grown on coverslips were infected with RPV (RBOK) and PPRV (Nig 75/1) at a m.o.i. of 10 and when the cells showed 30–40% CPE, coverslips were washed gently in PBS. Acetone-fixed cells (intracytoplasmic detection) or unfixed cells were used for immunofluorescence as described by Harlow and Lane (1988).

Hemagglutination and hemagglutination inhibition assays

Hemagglutination assay was performed according to Rosanff (1961). For HI assay, ascitic fluids were double diluted in PBS in a volume of 25 μl starting with an initial dilution of 1 in 5 in a round-bottom 96-well plate; 25 μl of 4 HA units of PPRV antigen (PPRV-Nig-75/1-infected cell extract) was added to the wells. Then the standard HI procedure was followed (Norrby, 1962).

Virus neutralization assay

RPV-H mAbs were tested for their ability to neutralize RPV (RBOK) and PPRV (Nig 75/1) infectivity in vitro on Vero cells according to the method of Barrett et al. (1989). Development of CPE was monitored by light microscopy and the titers were expressed as the reciprocal of the highest dilution of ascites, which neutralized 50% of virus infectivity.

Hemolysis (HL) and HLI

Both HL and HLI assays were performed according to the method of Norrby and Gollmar (1975).

REFERENCES


