Immunogenicity of a Bovine Rotavirus Glycoprotein Fragment

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Previous experiments demonstrated that an antigenic site responsible for virus neutralization and cell attachment was located on a 14,000-molecular-weight fragment of the major bovine rotavirus (BRV) glycoprotein (M. Sabara, J. E. Gilchrist, G. R. Hudson, and L. A. Babiuk, J. Virol. 53:58–66, 1985). However, it was necessary to investigate whether this fragment also had the ability to induce the production of neutralizing antibodies. Upon immunization of mice, the bovine serum albumin-conjugated 14,000-molecular-weight fragment, the unconjugated 14,000-molecular-weight fragment, and the native glycoprotein all induced a similar neutralizing antibody response, albeit to a lesser extent than did the infectious, whole virus. In addition, immuno-blot enzyme-linked immunosorbent assay analysis of the reactivity of anti-peptide serum versus anti-glycoprotein serum with the glycoprotein was very comparable. These results suggest that the 14,000-molecular-weight fragment may represent not only a biologically active region but also an immuno-dominant area of the glycoprotein.

The major neutralizing antigen of bovine rotavirus (BRV) is an outer surface glycoprotein with an approximate molecular weight of 38,200 in its unreduced form and 41,900 in its reduced form (5, 9). Several studies have indicated that the presence of disulfide bridges within this molecule is an important feature with respect to its ability to induce a good neutralizing antibody response (1, 9).

A more detailed characterization of the major rotavirus glycoprotein has been achieved by using a variety of monoclonal antibodies (3, 11, 12). Recently, an epitope located on a 14,000-molecular-weight fragment (14K fragment) of the BRV glycoprotein was identified and found to be involved in neutralization and cell attachment (9). However, this peptide was antigenic only in its unreduced conformation. To determine its immunogenic potential, the unreduced peptide fragment, derived from infectious virus particles, was injected into animals in a bovine serum albumin (BSA)-conjugated form and an unconjugated form. The resulting antibody responses were compared with those induced by infectious double-shelled BRV and the purified unreduced form of the glycoprotein. A further experiment was performed to determine whether the peptide could prime an immune response to infectious BRV in the event that a neutralizing antibody response could not be induced by using the peptide alone.

To prepare the various antigens to be used for immunization, BRV (isolate C486, subclone 13) (7) was propagated in MA-104 cells and purified by centrifugation as described previously (9). One milligram of purified double-shelled virus was then fractionated on a 10% preparative polyacrylamide gel. The 38.2K glycoprotein was localized in the gel by staining side strips of the gel with Coomassie blue. To extract the glycoprotein, gel strips were subjected to electrophoresion under conditions previously described (9).

The 14K peptide fragment was prepared by placing a gel strip containing the 38.2K glycoprotein into the well of a 5% stacking–20% resolving polyacrylamide gel. A gel strip (13 by 1 cm) was routinely treated with papain (Calbiochem-Behring, La Jolla, Calif.) at a concentration of 130 μg/cm of gel; since this gave the best preparation of 14K peptide (Fig. 1, lanes 1 and 2). Electrophoresis and in situ enzyme-digestion of the 38.2K glycoprotein was performed as described by Cleveland et al. (2) and prestained molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Md.) were used to visualize the time of maximum resolution between the 14.3K and 18.4K markers. Further localization of the 14K peptide was achieved by using the molecular weight markers. The peptide fragment was then electrophoresed from the gel slices, as previously described (9), and a protein determination was performed. The authenticity and purity of the peptide were confirmed by examination of its profile on polyacrylamide gels and by its reaction with monoclonal antibodies from hybridoma 11D12-6 (Fig. 1, lane C). The electrophoresed 14K peptide was then dialyzed and portion of the preparation was conjugated to BSA as follows. One milligram of peptide was first dissolved in 125 μl of 0.1 M phosphate-buffered saline (pH 7.4). The BSA solution was prepared by dissolving 1.25 mg of BSA in 600 μl of 0.1 M phosphate-buffered saline and adding dropwise 250 μl of a 2.5 M gluteraldehyde solution and the peptide solution consecutively over 15 min. The reaction mixture was gently agitated for 24 h at room temperature and then dialyzed extensively against sterile distilled water. Lysophosphatidyl of the conjugate yielded a pinkish powder which was stored in dessicant at −20°C.

Once all the antigens were prepared, groups of 10 mice (Charles River, Wilmington, Mass.) were immunized with the unconjugated peptide, the BSA-conjugated peptide, the infectious double-shelled virus, or the purified glycoprotein by the protocol outlined in Table 1. The quantities of antigen to be administered were determined on an equimolar basis. Antibody responses to the different antigens were characterized by enzyme-linked immunosorbent assay (ELISA) and immunoblot ELISA with BRV (isolate C486, subclones 12 and 13) as the antigen and by serum neutralization assays (9).

There was a significant antibody response to all the antigens used (Fig. 2, upper panel). Noteworthy is the similarity in response between the 38.2K glycoprotein and the 14K peptide fragment. It also appears that conjugation of the 14K peptide to a carrier was not necessary to induce a

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good antibody response. This may be due to the large size of the peptide fragment, thereby increasing the probability that it contained both B-cell and T-cell determinants (10).

The animals immunized with the 14K fragments were boosted at 61 days with infectious, double-shelled virus. This was done to investigate the possibility of the fragments priming an immune response. Even though, after analysis of all the sera, the animals did show a good antibody response to the peptides, they also demonstrated an additional, albeit minor, response after the infectious virus was administered (68 days). These results were encouraging since they illustrated that the 14K peptide alone was capable of inducing a respectable antibody titer. However, of more importance was that these antibodies had neutralizing ability. Sera from groups A through D possessed neutralizing antibodies (Fig. 2, lower panel), with the best response produced by animals immunized with infectious virus (group D). Total antibody titers, as measured by ELISA, and neutralizing antibody titers were similar for both the conjugated and unconjugated form of the peptide. At 68 days, after all the groups had been exposed to infectious virus, the neutralizing antibody titer increased slightly over that seen at 51 days, suggesting that each subsequent exposure further stimulates the immune response or alternatively that there may be other antigens on the infectious virus that are capable of inducing a neutralizing response. The most likely candidates for such antigens are the minor outer shell 84K protein and the major inner shell 45K protein. Several reports have indicated that both of these proteins are capable of inducing neutralizing antibodies, although to a much lesser extent than the major glycoprotein (1, 8, 9). This, in fact, is supported by the presence of antibodies to the 45K protein as illustrated by the immunoblot ELISA reactions at 68 days, even though antibodies to the 84K protein could not be detected (Fig. 3). As well, monospecific and monoclonal antibodies to the 45K

### Table 1. Immunization schedule

<table>
<thead>
<tr>
<th>Group (immunogen)</th>
<th>Dose (µg) given with the following adjuvant on the indicated day^</th>
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<tbody>
<tr>
<td></td>
<td>FC, day 8</td>
<td>F1, 31</td>
<td>F1, 44</td>
<td>F1, 61</td>
</tr>
<tr>
<td>A (14K unconjugated fragment)</td>
<td>13.6</td>
<td>13.6</td>
<td>13.6</td>
<td>0.675</td>
</tr>
<tr>
<td>B (14K conjugated fragment)</td>
<td>13.6</td>
<td>13.6</td>
<td>13.6</td>
<td>0.675</td>
</tr>
<tr>
<td>C (38.2K glycoprotein)</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>0.675</td>
</tr>
<tr>
<td>D (infectious virus)</td>
<td>0.675</td>
<td>0.675</td>
<td>0.675</td>
<td>0.675</td>
</tr>
<tr>
<td>E1 (negative control)</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
<td>0.675</td>
</tr>
<tr>
<td>E2 (negative control)</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
</tr>
</tbody>
</table>

^ Quantities shown are per mouse (10 mice per group except E1 and E2 which contained 5 mice each). FC, Freund complete adjuvant; F1, Freund incomplete adjuvant. Mice were prebled on day 1 and bled on days 37, 51, and 68.

^ All mice except five mice in group E2 were boosted with infectious virus.

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**FIG. 1.** Isolation of a BRV 14K peptide fragment. Lanes A and B illustrate the digestion patterns of the BRV glycoprotein produced with 65 and 130 µg of papain per cm of gel, respectively. Lane C illustrates the purified 14K peptide fragment. Lanes represent immunoblot ELISA reactions with monoclonal antibodies from hybridoma 11D12-6 (9). Lane D illustrates the purified 14K peptide fragment in a silver-stained polyacrylamide gel.

**FIG. 2.** Antibody titers to various antigen preparations at three different times during the immunization schedule. The upper panel shows total antibody titers as determined by ELISA with double-shelled rotavirus as the antigen. The lower panel shows neutralizing antibody titers as determined by plaque reduction assays. Group A, 14K unconjugated peptide; group B, 14K BSA-conjugated peptide; group C, purified 38.2K glycoprotein; group D, infectious BRV; group E1, animals given infectious BRV at 61 days; group E2, animals given only saline and Freund adjuvant. The immunization schedule for each group is described in more detail in Table 1.
antigen have demonstrated a low degree of neutralizing ability (1, 8).

Immunoblot ELISA reactions of sera from selected animals in each group at 37, 51, and 68 days are shown in Fig. 3. All the sera, except those obtained before immunization and the negative control group (Fig. 3, E), possessed antibodies to the major glycoprotein. Anti-peptide antibodies, although produced to a 14K peptide prepared from only one of the glycoprotein species present in BRV isolate C486, reacted with both glycoprotein species present in the protein profile of the parent isolate C486. This was significant since electrophoretic analysis of the genomic RNA from the two subclones of isolate C486 demonstrated a difference in mobility of the corresponding genes coding for this glycoprotein (7). However, it appears that despite this genetic heterogeneity, the 14K peptide is to a large extent conserved between the two glycoprotein species of BRV isolate C486 subclones 12 and 13. Another interesting observation was the similar intensity displayed by the reaction of the glycoprotein species with anti-peptide antibodies, suggesting that the 14K peptide may represent an immunodominant region of the glycoprotein.

In addition, all the sera including prebleeds and negative controls produced antibodies to a 92K protein, which has the same molecular weight as a minor BRV inner shell protein. There are several possible explanations for this phenomenon. (i) The mice had a previous rotavirus infection. The mouse rotavirus in this situation would only be cross-reactive with the 92K protein of BRV isolate C486. (ii) The 92K protein is “sticky.” Skim milk was used as a blocker of nonspecific sites (during the immunoblot ELISA procedure) instead of BSA or gelatin, which are more adhesive, and this may have enabled the 92K protein to remain somewhat exposed. Unfortunately, there was not enough mouse serum to repeat this experiment with alternative blockers; however, anti-glycoprotein serum obtained from rabbits did not give a visible reaction with the 92K protein even when skim milk was used (data not shown). This suggests that the reaction of mouse serum with the 92K protein is inherent in the serum and may account for the presence of anti-rotavirus antibodies in the control group (Fig. 2, lane E).

As shown in this study and in other studies with different viruses, only antibodies to sites representing important biological functions can neutralize virus infectivity. This consideration is, of course, the basis for designing synthetic vaccines. By first searching for such critical sites on the rotavirus glycoprotein, by using monoclonal antibodies as probes, we have identified a 14K fragment which possesses the site for virus attachment and neutralization (9). The antigenic determinants on this peptide appear to represent conformation-dependent antigenic sites (9), posing a difficult immunochemical problem with respect to synthesis of an effective immunogen; especially if these sites are discontinuous. However, this situation is not without precedent, as evidenced by the tick-borne encephalitis virus antigens (4). One approach which has been used to construct such immunogens employs surface simulation synthesis. In this concept, the spatially-adjacent residues of a protein binding site are linked directly into a single peptide that does not exist in the protein but mimics a surface region of it. This approach is currently under investigation for the rotavirus peptide discussed in this paper. In addition, potential conformational sites within this peptide, which fulfill the criteria of hydrophilicity, the presence of proline residues, size, and charge, are being synthesized and tested for this efficacy as immunogens.

LITERATURE CITED

