Canine vaccine recipients recognize an immunodominant region of the rabies virus glycoprotein

N. Johnson, K. L. Mansfield and A. R. Fooks
Rabies Research and Diagnostic Group, Veterinary Laboratories Agency, Weybridge, Addlestone, Surrey KT15 3NB, UK

To investigate the immune response to anti-rabies vaccination in the principal recipient (the domestic dog), four truncated fragments of the rabies virus glycoprotein were expressed as glutathione S-transferase fusion proteins. Immune sera from vaccinated rabbits and dogs were then used to probe for reactivity with these expressed proteins. In two rabbits and four dogs tested, the dominant antibody response to non-conformational antigenic sites appeared to be directed to a region of the glycoprotein between amino acids 222 and 332. The N-terminal fragment of the glycoprotein was also significantly antigenic. Further studies to assess whether the antibody response to the internal domain could neutralize the rabies Challenge Virus Standard (CVS) strain, using antibody depletion, suggested that this fraction did contribute to the ability of post-vaccination sera to neutralize and therefore protect against infection.

Introduction

Infection with classical rabies virus (RV) causes fatal encephalitis for which there is no treatment once symptoms develop (Hemachuda & Phuapradit, 1997). However, protection against challenge with RV can effectively be obtained through vaccination or transfer of anti-rabies immunoglobulins. Indeed, studies in transgenic mice suggest that only a deficiency in the ability to develop an antibody response allows the disease to develop with avirulent strains of RV (Hooper et al., 1998). Vaccination with whole-inactivated virus induces a strong humoral response to a range of antigens, the most significant response being that against the viral glycoprotein. This protein is expressed as a 524 amino acid polypeptide with the first 19 residues forming a signal peptide, which is cleaved as the protein enters the endoplasmic reticulum (Anilionis et al., 1981). The remaining regions of the protein can be subdivided into a 439 residue ectodomain (surface-exposed), a 22 residue transmembrane domain and a 44 residue endodomain. The three-dimensional structure of this protein is not known, but it contains between 12 and 16 conserved cysteine residues (Coll, 1995), in common with other rhabdovirus glycoproteins, and potentially forms trimers (Gaudin et al., 1992).

Monoclonal antibodies (mAbs) have been instrumental in elucidating the antigenic sites on the RV glycoprotein and these can be classified into conformational sites and linear, non-conformational sites. Two major sites dominate the former. The first, termed antigenic site II, is formed from two sites between residues 34 and 42 and residues 198 and 200 (Prehaud et al., 1988). Conversely, a single region between residues 330 and 338 forms the second site, antigenic site III, with an arginine at position 333 being critical for virus neutralization (Seif et al., 1985). This residue is also critical for neuroinvasion, possibly through its role in binding to a cellular receptor (Dietzschold et al., 1983; Coulon et al., 1989; Badrane et al., 2001). There is also evidence that these antigenic sites are structurally conserved among all rhabdoviruses (Walker & Kongsuphan, 1999), and the immunodominance of these sites is emphasized by the observation that 97% of mAbs recognized either site (Coulon et al., 1993). Mapping the epitopes of neutralizing mAbs has also identified non-conformational sites (Bunschoten et al., 1989; Dietzschold et al., 1990) and a number of critical residues (Van der Heijden et al., 1993; Ni et al., 1995; Luo et al., 1997). Alternative approaches to mapping antigenic sites have utilized cyanogen bromide (CNBr) cleavage fragments of purified glycoprotein (Dietzschold et al., 1982) and short glycoprotein peptides expressed in yeast (Lafay et al., 1996). The former study concluded that, in the rabbit, the principle regions recognized were internal sites between residues 103 and 330, whereas the latter mapped a
panel of mAbs to a short region between residues 223 and 276. This contrast suggests that different species may respond to different sites on the RV glycoprotein.

In this study we have investigated the polyclonal antibody response to non-conformational epitopes in rabbit and dog recipients of a commercially available anti-rabies vaccine. Truncated fragments of the RV glycoprotein were fused to glutathione S-transferase (GST) and used to map the response to defined regions. This approach confirmed the immunodominance of an internal region of the glycoprotein observed with mAbs. It also identified a second region close to the N terminus of the protein towards which dogs in particular direct an antibody response.

Methods

Immunizations. Three New Zealand White rabbits were inoculated intramuscularly with 0.25 ml of Rabisin (Merial Animal Health), a commercial vaccine based on the G. S.57 Wistar virus strain grown in NIL (hamster embryo) cells. Virus was inactivated with β-propiolactone and adsorbed to aluminium hydroxide. Rabbits were inoculated at 0, 21, 42 and 57 days, and given a final boost at 136 days. Exsanguination was performed on day 150. Confirmation of a positive response was obtained by testing serum from each animal with the fluorescent antibody virus neutralization assay (FAVN). Dog sera were selected from samples submitted to the Veterinary Laboratories Agency for routine testing as part of the UK Pet Travel Scheme (Fooks et al., 2002). Each had been given a single dose of Rabisin (1 ml intramuscularly) and bled 1 month after vaccination.

Fluorescent antibody virus neutralization assay (FAVN). This assay was carried out following the protocol of Cliquet et al. (1998). Briefly, sera were heat-inactivated by incubation at 56 °C for 30 min and then serially diluted in a 96-well plate. One hundred TCID 50 of rabies Challenge Virus Standard (CVS-11) was added and the plate was incubated at 37 °C for 1 h. Finally, 50 μl of baby hamster kidney (BHK) cells at 4 × 10 5 cells/ml were added in Dulbecco’s modified Eagle’s medium with penicillin (100 U/ml), streptomycin (100 μg/ml), mycostatin (25 U/ml). The plate was incubated for 48 h at 37 °C. The supernatant was then discarded and the adherent cells fixed with acetone (80% in H 2 O) for 20 min. The plate was allowed to air-dry and then stained with an anti-rabies–FITC conjugate (Centocor) at a dilution of 1:50. Fluorescence was measured at each dilution of sera and titres were calculated using the Spearman–Karber method. Results are presented as the number of International Units by comparison with an Office International des Epizooties (OIE) positive and negative standard for rabies immunoglobulin.

Construction of truncated glycoprotein fragments. DNA fragments encoding the four regions were amplified using PCR. The target for the amplification was RNA (1 μg/ml) extracted from BHK cells infected with RV (Pasteur Virus strain) (Heaton et al., 1997). Briefly, RNA was reverse-transcribed using the protocol of Heaton et al. (1997) using the upstream primer and then amplified using the combinations of primers detailed in Table 1. The product for each reaction was then cloned into plasmid pCR2.1 using the TA cloning system (Invitrogen) and transformed into competent E. coli TOP10F’ cells (Invitrogen) following the manufacturer’s instructions. Plasmids were then extracted using the Wizard plasmid preparation kit (Promega) and digested with BamHI and Sall restriction endonucleases. The digested glycoprotein fragments were purified and cloned into the pGEX-4T-3 plasmid (Pharmacia), and then transformed into competent E. coli BL21 cells (Pharmacia).

Expression of glycoprotein fragments fused to GST. To express each truncated fusion protein, transformants containing each recombinant plasmid were grown up overnight at 37 °C in LB broth containing 100 mg/ml ampicillin (Sigma). A 1 ml aliquot of each transformant was inoculated into 20 ml of fresh LB broth with ampicillin and grown for 2 h at 37 °C. Fusion protein expression was induced by the addition of 0.1 mM IPTG (Sigma) and the cultures were incubated for a further 4 h at 28 °C. Cells were harvested by sedimentation (2000 g, 5 min) and resuspended in sonication buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.2, 0.5 mM EDTA, 1 mM PMSF). Each batch of cells was sonicated three times for 15 s each on ice. Insoluble material and intact cells were removed by sedimentation (10000 g, 5 min) and the supernatant removed to a separate tube.

Immunoblotting. Protein separations were carried out by SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad) in transfer buffer (0.025 M Tris–HCl, pH 8.8, 0.192 mM glycine, 0.1%, w/v, SDS, 20% methanol) and then incubated with mAbs. It also identified a second region close to the N terminus of the protein towards which dogs in particular direct an antibody response.

<table>
<thead>
<tr>
<th>Table 1. Sequence and genomic position of primers used to construct truncated fragments of the RV glycoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>RvG1-2</td>
</tr>
<tr>
<td>RvG3-4</td>
</tr>
<tr>
<td>RvG5-6</td>
</tr>
<tr>
<td>RvG7-8</td>
</tr>
</tbody>
</table>

* Base positions of the genomic region amplified by the primers are derived from the PV genome (GenBank accession no. M13215).
† The positions of the peptides are derived from the mature PV glycoprotein with residue 1 representing the lysine at the proposed ER cleavage site.
25%, v/v, methanol). Membranes were blocked with 5% non-fat milk in PBS (pH 7.2) with 0.1% Tween 20 (PBS-T). Probing with polyclonal sera was carried out at a dilution of 1:2000 in 1% non-fat milk in PBS-T for 1 h at 37 °C. Each membrane was washed three times with PBS-T and then incubated with swine anti-rabbit horseradish peroxidase (Dako) or rabbit anti-dog–peroxidase (Sigma) conjugates as appropriate, both at a dilution of 1:2000 for 1 h at 37 °C. Membranes were washed five times and developed using enhanced chemiluminescence (Amersham Pharmacia). Protein sizes were estimated by comparison with pre-stained broad-range markers (Bio-Rad).

Results

Expression of truncated RV glycoprotein fragments

To investigate the regions of the RV glycoprotein against which immunized animals generated antibodies, truncated fragments of the Pasteur Virus (PV) strain glycoprotein were expressed as fusions with GST. Each fragment was between 100 and 120 amino acids in length and, when expressed as a fusion protein, migrated with a molecular mass of between 40 and 42 kDa (Fig. 1). Whilst GST alone was expressed as a soluble protein (Fig. 1, lane 2), each of the fusion proteins proved insoluble, with the vast majority of protein being recovered from the pellet fraction following sonication (Fig. 1, lanes 6–9). Fig. 1 also demonstrates that the fusion proteins were not all expressed at equal levels, with protein RvG7–8 being consistently expressed at a lower level. To achieve equal loading in subsequent experiments for the purposes of assessing differential antibody binding in polyclonal sera, solubilized fusion proteins were diluted with an appropriate volume of sample loading buffer. Equal amounts of protein within each sample were confirmed by Ponceau red (Sigma) staining of nitrocellulose membranes following transfer after SDS–PAGE.

Antigenic analysis of RV-vaccinated rabbit sera

Three rabbits were immunized with the commercial anti-rabies vaccine Rabisin to induce an antibody response. Post-vaccination analysis of each serum sample showed a strong response to the rabies glycoprotein as measured by a protective level of neutralizing antibodies greater than 0.5 International Units per ml (IU/ml) (see Fig. 4). Immunoblotting also detected a strong response to the mature glycoprotein (Fig. 2a, lane G). Screening of the truncated fusion proteins with each rabbit polyclonal sera (Fig. 2) showed a distinct bias in reactivity to the third fusion protein. In both rabbits, R411 (Fig. 2b, upper panel) and R413 (Fig. 2a, b, lower panel), the dominant band was RvG5–6 (lane 4), which covered a 111 amino acid fragment of the glycoprotein from alanine-222 to valine-332. R412 produced a weaker response to non-conformational epitopes, but even with this sample, the strongest response was to RvG5–6. Overall, there was little recognition of GST (faint recognition by R411, Fig. 2b, upper panel, lane 1), indicating that the response was to linear epitopes on the rabies glycoprotein. The truncated fragment RvG1–2 was also recognized by both R411 and R413, indicating that antigenic sites are present between residues 1 and 128 of the glycoprotein.

Antigenic analysis of hyperimmune dog sera

In order to confirm the observations of immunodominance of the glycoprotein region between positions 222 and 332, the truncated fusion proteins were probed with sera from six dogs that had also been immunized with the Rabisin vaccine. In contrast to the rabbit samples whose final FAVN titres were lower than 100 IU/ml, these samples were purposely selected because of the scale of their response, with titres above 1000 IU/ml. Fig. 3(a) shows that dog sera D5951 produced a strong response to the glycoprotein (indicated at approximately 65 kDa) and to other RV proteins, presumably the nucleoprotein (50 kDa) and the matrix protein (23 kDa) (Coll, 1995). Although there was some background binding to E. coli proteins, the dominant reactive band appeared to be RvG5–6 (Fig. 3a, lane 4). Fig. 3(b) shows the results of two other dog sera, D5993 (upper panel) and D6183 (lower panel). These samples were representative of the animals tested with a strong response to the N-terminal fragment (RvG1–2) and to RvG5–6. D6183 responded in a similar way to the rabbit sera (R411 and R413) with a large response focused on the fragment RvG5–6. A summary of the responses of this panel of dog sera is presented in Table 2. With the exception of D6183, and to a lesser extent D5951, all animals produced a more varied response than that observed in rabbit sera, but also showing little response to RvG7–8.

Are the antibodies directed against the region 222–332 neutralizing?

The approach taken to identifying RV glycoprotein epitope sites has relied on the identification of mutant viruses that escape neutralization with mAbs. With polyclonal sera, this is

![Fig. 1. 12% SDS–PAGE analysis of truncated glycoprotein fragments following expression in E. coli. Lane 1, GST supernatant without IPTG induction; lane 2, GST supernatant with IPTG induction; lane 3, molecular mass markers; lane 4, GST pellet without IPTG induction; lane 5, GST pellet with IPTG induction; lane 6, RvG1–2 pellet with IPTG induction; lane 7, RvG3–4 pellet with IPTG induction; lane 8, RvG5–6 pellet with IPTG induction; lane 9, RvG7–8 pellet with IPTG induction.](image-url)
not possible because of the heterogeneous nature of the response to more than one epitope. As an alternative approach to addressing this, we have taken advantage of the insolubility of the expressed protein RvG5-6 and used it to remove specific binding antibodies from sera by adsorption. Through subsequent centrifugation, immune complexes could be removed and the sera retested for neutralizing activity. Fig. 4 shows the result of this with examples of rabbit (Fig. 4a, R413) and dog (Fig. 4b, D5951) sera. Confirmation that depletion had occurred in both cases was obtained by observing the reduced detection of this protein by sera incubated with insoluble RvG5-6 for up to 60 min (Fig. 4a, b, insets). For the rabbit sera, this reduction in the detection of RvG5-6 by immunoblotting was associated with a decrease in the neutralizing titre from 3-42 to 1-97 IU/ml after 60 min depletion. The dog sera D5951 did not show a reduction in neutralizing titre after 30 min incubation with insoluble RvG5-6, although comparison with the strip blot results suggested that a significant level of depletion had not occurred at this time point. However, after 60 min depletion, the neutralizing titre dropped from 40-5 to 30-77 IU/ml in association with a noticeable drop in detection of the protein by the depleted sera. These data suggest that depletion of sera with insoluble RvG5-6 results in a small but measurable reduction in the ability of sera to neutralize RV.
Table 2. Antigenic characterization of dog sera by FAVN and immunoblotting against truncated RV glycoprotein fragments

<table>
<thead>
<tr>
<th>Sample</th>
<th>FAVN (IU/ml)</th>
<th>Glycoprotein fusion protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GST</td>
</tr>
<tr>
<td>D6183</td>
<td>1493-13</td>
<td></td>
</tr>
<tr>
<td>D6159</td>
<td>1093-50</td>
<td></td>
</tr>
<tr>
<td>D6020</td>
<td>1439-13</td>
<td></td>
</tr>
<tr>
<td>D5993</td>
<td>1439-13</td>
<td></td>
</tr>
<tr>
<td>D5951</td>
<td>1039-50</td>
<td></td>
</tr>
<tr>
<td>D5904</td>
<td>1439-13</td>
<td></td>
</tr>
</tbody>
</table>

* The results are presented as relative reactivity compared with binding to intact glycoprotein (see Fig. 3a, b).

Discussion

The antibody response following immunization to known antigenic sites on the RV glycoprotein in the principal species (man, dog and fox) that receive rabies vaccination has not been studied. The approach used in this study suggests that in two species, the rabbit and the dog, the region between alanine-222 and valine-332 (fragment RvG5-6) of the PV glycoprotein is immunodominant in relation to the rest of the protein. In a number of canine vaccine recipients, the N terminus of the protein also contained antigenic sites. Removal of the antibody component that binds to this immunodominant region reduced the capacity of polyclonal sera to neutralize RV.

These observations compare favourably with two previous studies that identified antigenic sites on the RV glycoprotein. Dietzschold et al. (1982) generated shortened fragments of the ERA strain with CNBr. Immunoprecipitation of such radio-labelled CNBr fragments with hyperimmune rabbit sera identified two regions of the glycoprotein that formed antigenic sites. The second of these partially overlaps the RvG5-6 fragment over the sequence 293–332. These two regions, and a non-precipitatable peptide from the N-terminal 50 residues, were capable of inducing neutralizing antibodies. The second study by Lafay et al. (1996) using peptide fragments of the CVS strain expressed in yeast identified a region between residues 223 and 276 that bound six of twelve non-conformationally dependent mAbs. The site is located within the core of the RvG5-6 peptide. The remaining mAbs bound to sites flanking this region. Prediction studies on this region suggest that there are multiple sites that could form antigenic determinants, although this is not matched by the limited number of predicted surface-exposed sites (N. Johnson, unpublished observations). In addition, three separate studies (van der Heijden et al., 1993; Ni et al., 1995; Luo et al., 1997) have characterized neutralizing mAbs that bind to a region between residues 248 and 268. It is possible that within polyclonal dog sera, the total binding of neutralizing antibodies to the RvG5-6 fragment could be due to binding of antibodies to this site. However, a more detailed analysis is required to assess the number of epitopes within this truncated region.

As with both previous studies (Dietzschold et al., 1982; Lafay et al., 1996) that mapped the RV glycoprotein, the C-
terminal fragment between residues 327 and 443 does not appear to contain any antigenic determinants. One possible explanation for this could be that this region is protected from immune surveillance by N-terminal domains of the glycoprotein. However, in the absence of a full secondary structure, this remains speculative, despite being a consistent finding between research groups.

Depletion studies using insoluble RvG5-6 caused a limited reduction in the neutralizing titre of both rabbit and dog sera. Insoluble material from sonicated E. coli alone caused no reduction in titre (data not shown) and this step could be used to remove non-specific binding without any reduction in RV glycoprotein detection. The remaining components that constitute the neutralizing titre within a polyclonal serum are antibodies that bind to linear epitopes at the N-terminal region, which could be induced following immunization with a peptide covering this site by Dietzschold et al. (1983). Also, antibodies that bind conformational sites such as the previously described antigenic sites II and III (Coulon et al., 1993) will make up the neutralizing component of immune sera.

It should be noted that the approach described above only identifies non-conformational antigenic regions. The relative importance of each component is impossible to compare within polyclonal sera, although the experience with neutralizing mAbs suggests that conformational sites are dominant (Lafay et al., 1996). However, the absence of studies in carnivores means that this may not be the case in animals such as the dog. Approaches that develop non-mouse mAbs could be used to address this issue (Champion et al., 2000).

The importance of investigating the antibody response in vaccine recipients lies in the information it provides in identifying alternative approaches to vaccine design. The identification of short linear epitopes allows their replication in the form of peptides, which can then be used as vaccines (Dietzschold et al., 1990). However, the ability of RV to accommodate point mutations that allow escape from neutralization, and ironically the method used to identify many epitopes, suggests that this approach may be limited. Using a larger region of the glycoprotein as the antigen, containing more than one neutralizing epitope, may overcome this problem. The major observation of this study suggests that some epitopes recognized by mAbs are probably also recognized by vaccinated dogs. This should allow the design of a large fragment of the glycoprotein, which is capable of inducing a neutralizing polyclonal response following immunization, that could protect against challenge with a divergent range of classical RV isolates.

This work was supported by a grant (SEO416) from the Department for the Environment, Food, Rural Affairs (DEFRA), UK.

References


glycoprotein of rabies virus that contains Trp251 is a linear epitope. *Virus Research* **51**, 35–41.


Received 24 April 2002; Accepted 10 July 2002