Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein

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Egtved virus, the rhabdovirus causing viral haemorrhagic septicaemia in rainbow trout, was analysed at the antigen level with a future subunit vaccine in mind. Three monoclonal antibodies to the viral G protein were characterized with respect to neutralizing activity at the cell culture level, as well as their ability to protect rainbow trout fingerlings against virus infection following passive immunization. Two antibodies showed strong protective activity in fish. Only one of these antibodies was able to neutralize viral infectivity in vitro. Reduction of disulphide bonds in the G protein abolished reactivity of this antibody in immunoblotting, whereas antigen deglycosylation did not influence the binding ability of any of the antibodies. These data suggest that the G protein contains linear as well as non-linear, carbohydrate-free epitopes, which are involved in the protection against Egtved virus. However, an indirect influence of oligosaccharide side chains on epitope formation could not be excluded, since in situ inhibition of glycosylation prevented the binding of the protecting antibodies in immunofluorescence.

Introduction

Egtved virus, sometimes referred to as VHS virus, is an enveloped RNA virus belonging to the rhabdovirus family (Lenoir & de Kinkelin, 1975). The virus is the major viral pathogen in European trout farms, being the causal agent of viral haemorrhagic septicaemia (VHS) in rainbow trout (Oncorhynchus mykiss) (Jensen, 1965). Although several attempts to develop potent vaccines based on inactivated or attenuated virus have been made (Kinkelin et al. 1984) they have not been successful. A recombinant subunit vaccine might be a potential alternative to conventional preparations, as reported by Gilmore et al. (1988).

Rhabdovirus glycoproteins have, in general, been found to induce formation of neutralizing antibodies in the infected host (Kelley et al., 1972; Wiktor et al., 1973). In the present study an initial identification of host-protecting viral determinants by the use of monoclonal antibodies (MAbs), as well as knowledge of the biochemical nature of such determinants, were the major goals.

Production of non-neutralizing MAbs to the structural proteins of Egtved virus has been reported earlier (Lorenzen et al., 1988) and recently neutralizing MAbs were also obtained in our laboratory. The neutralizing activity at cell culture level (in vitro) of three MAbs to the G protein of Egtved virus was compared to their in vivo protective ability by passive immunization of trout. Two MAbs were able to confer immunity to the fish, although only one of these MAbs could neutralize viral infectivity in vitro.

The identified epitopes on the viral glycoprotein were further characterized with respect to carbohydrate content and conformational continuity.

Methods

Cells and virus. Egtved virus strain F1 (Jensen, 1965) was cultivated on BF2 cells (Wolf et al., 1966) and purified by sucrose gradient centrifugation according to the procedure of Kinkelin (1972). The purified virus was used for generation of MAbs and as an antigen in immunoblotting. For plaque neutralization epithelioma papulosum cyprini (EPC) cell cultures (Fijan et al., 1983) and a heat-adapted clone of reference strain F1 (F25) (Kinkelin et al., 1980) was used. Cultivation was in Eagle’s MEM with 10% foetal bovine serum and antibiotics. Cell culture supernatant containing low passage isolates of Egtved virus was used for immunofluorescence, as well as challenge virus, in the passive immunization experiment.

Monoclonal antibodies. Production of mouse hybridoma cell lines that secrete MAbs specific for the structural proteins of Egtved virus has recently been described and two of the three MAbs used in the present study have already been characterized in detail, i.e. IP1D11 (here called MAb-II) and J1H3 (MAb-III) (Lorenzen et al., 1988). The hybridoma cell line secreting the third MAb, MAB-I, was selected from a subsequent fusion performed after 3 months of continued immunization, following the procedure reported earlier. A plaque neutralization test (50% PNT) (Olesen & Jørgensen, 1986) was used this time in the initial screening step, instead of the previously described ELISA. The three MAbs all belonged to the IgG1 subclass, as revealed by means of
Ouchterlony double diffusion assays in agarose gels (reagents from Serotec).

MAbs used for passive immunization were purified from hybridoma supernatants by affinity chromatography on columns (Econo, Bio-Rad) containing Protein A-agarose (Kem-En-Tec). Before application to the columns the hybridoma supernatants were adjusted to 1/65 M-NaCl, 0.83 M-glycine, 0.1 M-NaOH and pH 8.8 to 9.0 (buffer conditions suggested by Dr Claus Koch, State Serum Institute, Copenhagen, Denmark). Bound MAbs were eluted from Protein A by 0.03 M-citric acid pH 3.0. Purified MAbs for the neutralization experiments were adjusted to approximately 0.6 mg mouse Ig per ml in phosphate-buffered saline (PBS) pH 7.2. MAbs were quantified by means of a sandwich ELISA, described by C. Koch (personal communication) and quoted by Lorenzen et al. (1988).

Plaque neutralization test (50% PNT). Titration of the neutralizing activity of the MAbs at the cell culture level was performed in microtitre plates as described (Olesen & Jørgensen, 1986), with or without rainbow trout complement.

Passive immunization. Rainbow trout, 2 to 4 g each, were anaesthetized (chlorobutanol, 0.05%) and given an intraperitoneal injection of 30 µg MAb in 50 µl PBS, or PBS alone. After recovery (2 h later) the fish were exposed to virus by bath infection with 5×10⁵ TCID₅₀ per ml of water for 1.5 h. Afterwards the fish were kept in running tap water at 11 °C ± 2 °C in aquaria containing 5 l of water and 32 to 37 fish. Dead fish were collected daily. After 4 weeks the percentage of survivors was calculated.

Preparation of samples for electrophoresis. The viral glycoprotein was deglycosylated by means of an enzyme preparation containing equal activities of endoglycosidase F and glycopeptidase F (ENDO-F, Boehringer) (Plummer et al., 1984). Purified virus (approx. 30 µg protein per ml) in 15% sucrose, 150 mM-NaCl, 10 mM-EDTA, 10 mM-NaH₂PO₄ pH 7.5 and 0.025% (w/v) SDS was incubated overnight at 37 °C with or without 2 units per ml of ENDO-F. Samples were subsequently adjusted to 3% glycerol, 1% SDS and 30 µM-bromophenol blue. In samples to be reduced 25 mM-2-mercaptoethanol was also included. After boiling for 3 min, 1/20 volume 1 M-iodoacetamide was added to all samples in order to avoid reduction of unreduced samples during electrophoresis.

Electrophoresis and blotting. Sample proteins were separated by electrophoresis in SDS–10% polyacrylamide gels according to Laemmli (1970) and transferred to a 0.45 tam pore size polyvinylidene difluoride membrane (Immobilon, Millipore) in a semi-dry electroblotter (Kyhse-Andersen, 1984). Excess binding sites on the membrane were blocked recommended by the supplier. Immunological staining, using MAbs as the first antibody reagents (approx. 2 µg mouse Ig per ml) and horseradish peroxidase (HRP)-conjugated rabbit Ig to mouse Ig as the second antibody, was done as outlined before (Lorenzen et al., 1988). Lectin-mediated staining of the viral glycoprotein was performed using biotinylated wheatgerm agglutinin (WGA), at 15 µg/ml in PBS supplemented with 0.05% Tween-20, 1% BSA (PBS-T-BSA) and with or without 0.2 M-N-acetylglucosamine as the first layer. HRP-conjugated avidin (2 µg/ml in PBS-T-BSA containing 0.5 M-NaCl) was used as the second layer (both reagents from Kem-En-Tec). Incubation times were 2 h and 1 h, respectively, both at room temperature. 3-Amino-9-ethyl-carbazole was used as a colour reagent for detection of bound HRP-conjugate (Heegaard & Bjerrum, 1988). Occasionally antigen-containing membranes stained by WGA were reused for immunological staining.

Immunofluorescence microscopy of virus-infected cell cultures. Cover-glass cultures of EPC cells were infected with 1×10⁵ TCID₅₀ of Egtved virus per ml culture medium. Two h post-inoculation the virus-containing medium was removed and medium containing 0.5% methylcellulose with or without 10 µg tunicamycin (Sigma) per ml was added. Following two rinses in medium without serum the cell cultures were fixed at room temperature for 10 min with acetone, or for 30 min with 3% paraformaldehyde in PBS 44 h post-inoculation. The immunological staining procedure was as described earlier (Lorenzen et al., 1988), except that this time fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) followed the procedure given by Goding (1986).

Staining of the cellular Golgi zone was performed with FITC-conjugated WGA (Kem-En-Tec) at 20 µg per ml in PBS and incubation was overnight at 4 °C.

Results

Neutralization in vitro and in vivo

In order to identify protecting epitopes, the influence of G protein-specific MAbs on the pathogenic effects of Egtved virus was investigated both in cell cultures and in fish. Neutralizing activity of the MAbs in vitro was determined by means of the 50% PNT, whereas the in vivo protective ability was investigated by passive immunization followed by challenge with virus. Plaque neutralization titres, as well as the percentage of survivors in the immunization experiments, are given in Table 1. MAb-I was the only antibody capable of neutralizing virus in vitro. Similar results were obtained with F25 and the low passage isolates of Egtved virus used in immunofluorescence and in the challenge experiments. After boiling in the form of trout serum (Dorson & Torchy, 1979) did not influence the results (not shown). MAb-I conferred a high degree of immunity against virus infection to the fish. However, in vivo, MAb-II was also able to protect significantly against the virus challenge. MAb-III had no effect in the cell culture system nor in the fish, and this was also the case for MAbs directed against three of the other known structural proteins of Egtved virus (N, M1 and M2) (not shown).

<table>
<thead>
<tr>
<th>Group of fish*</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected MAb</td>
<td>0 (PBS)</td>
<td>0 (PBS)</td>
<td>MAb-I</td>
<td>MAb-II</td>
<td>MAb-III</td>
</tr>
<tr>
<td>50% PNT titre of MAb†</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>5×10⁵</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Challenge with virus</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Survivors (%)‡</td>
<td>93</td>
<td>2</td>
<td>86</td>
<td>50</td>
<td>9</td>
</tr>
</tbody>
</table>

* Each group included two aquaria with 32 to 37 fish in each.
† Neutralization titre was expressed as the reciprocal of the dilution of the antibody reagent reducing the plaque number by 50%.
‡ Values represent average of two aquaria.
Characterization of epitopes by immunoblotting

Competitive binding studies by means of ELISA have shown that the three Mabs recognize different determinants on the viral G protein (N. Lorenzen, unpublished). In order to acquire knowledge of the biochemical nature of the epitopes recognized by the three G-specific Mabs, the influence of chemical reduction of intramolecular disulfide bonds and of enzymic deglycosylation of the G protein on antibody binding ability was investigated by means of immunoblotting. MAb-II and -III were both able to bind to the reduced G protein (Fig. 1). However, as shown in Fig. 1 (a, lanes 1 and 2) reduction and alkylation totally prevented the binding of MAb-I. ENDO-F treatment of the antigen prior to SDS-PAGE and blotting did not affect the ability of the three Mabs to recognize the G protein, whether this was reduced or not (Fig. 1a, b and d). However, the enzyme treatment eliminated the binding ability of the lectin WGA, which confirmed the effect of ENDO-F on the G protein (Fig. 1c). The sugar specificity of WGA was verified by the absence of bound WGA on the intact G protein when incubation was performed in the presence of 0.2 M-N-acetylglucosamine (not shown).

Due to a reduction in its size, and possibly some other carbohydrate-related properties (Leach et al., 1980), the mobility of the glycoprotein was increased following ENDO-F treatment and, although migrating like BSA (67K) before deglycosylation, the carbohydrate-free G protein had an Mr of approximately 55K. From Fig. 1 (a) lane 4 and (d) lanes 2 and 4, it appears that after the ENDO-F treatment the G protein was seen as an upper, sharp and intensely stained band and a lower, faintly stained band. MAb-II obviously did not bind to the lower band, whether reduced or not (Fig. 1b, lanes 2 and 4). Whether this band dichotomy reflects a natural or a protease-induced size heterogeneity in the viral G protein molecule population remains to be seen.
Incomplete carbohydrate removal is a possible explanation, but this seems unlikely as no WGA binding could be detected.

Concerning the other structural proteins of Egtved virus, neither migration in the gel nor antibody binding ability was affected by the ENDO-F treatment (not shown).

**Effect of tunicamycin on immunofluorescence staining patterns**

The staining patterns found in virus-infected cell cultures fixed in acetone and analysed by direct double immunofluorescence with MAb-II and -III were in accordance with our previous findings obtained by indirect staining techniques (Lorenzen et al., 1988). Briefly, MAb-II intensely stained intracellular, cisternae-like structures in a juxtanuclear position (presumably related to the Golgi apparatus) and in addition a faint membrane staining was observed. MAb-III, apart from the presumed Golgi zone, also stained reticular structures throughout the cytoplasm in most of the infected cells. The direct double immunofluorescence technique used in the present study confirmed the differences in staining patterns obtained by the two G-specific MAbs (Fig. 2a, b). The _in vitro_ neutralizing MAb-I induced a Golgi zone staining corresponding to that obtained with MAb-II (Fig. 2a), although the intensity was somewhat weaker.
The lectin WGA has been shown to bind preferentially in the intracellular area related to the Golgi apparatus of eukaryotic cells and may be used as a rough indication of the intracellular localization of this organelle (Vir-\textit{tanen et al.}, 1980). The WGA-binding zone in virus-infected cells normally included the presumed Golgi-related structures stained by the G-specific MAbs (not shown). However, when the drug tunicamycin (Takatsuki \textit{et al.}, 1971), which inhibits N-linked glycosylation (Tkacz & Lampen, 1975), was added to the cell culture medium MAb-III was the only G-specific MAb that could stain at all (Table 2) and appeared unable to stain the centre of the Golgi zone (Fig. 2c, d). Furthermore, only single cells, or pairs of cells originating from the same infected cell, and not the usual plaques of infected cells were stained (Fig. 2b, d and Table 2).

Paraformaldehyde, unlike acetone, leaves the cell membrane impermeable to antibodies (Biberfeld \textit{et al.}, 1974) and thus enables antibody-mediated detection of viral proteins only on the surface of infected cells. In cell cultures fixed with paraformaldehyde each of the three G-specific MAbs stained plaques consisting of 20 to 50 infected cells (Table 2), whereas MAbs to the other structural proteins (N, M1 and M2) of Egtved virus were unable to stain at all, except for a few disrupted cells (not shown). In tunicamycin-treated cell cultures fixed in paraformaldehyde not even the G-specific MAb-III stained the infected cells, indicating the absence of detectable amounts of G protein on the cell surface.

**Discussion**

In this study MAbs against the G protein of Egtved virus have been used in identification and characterization of viral determinants that are important in antibody-mediated neutralization \textit{in vitro} (cell culture) as well as \textit{in vivo} (fish). Epitopes recognized by MAbs which were able to protect fish against Egtrved virus appeared to be free from carbohydrate structures. The results further demonstrate that a MAb which protects fish after passive immunization may be either with or without neutralizing activity at the cell culture level.

The MAb-I, which was highly neutralizing \textit{in vitro}, led to the highest degree of protection observed \textit{in vivo}. However, MAb-II, although it was totally negative in the 50% PNT, was similarly able to confer a significant protection against VHS to the fish. Whether these observations can be correlated with actual differences in the mechanisms of the protection conferred by the MAbs will require further experiments. As an example, it is possible that the trout immune system, in contrast to serum-containing cell culture medium, might in some way assist MAb-II in the neutralization of virus. However, at this time too little information about the complement system and cellular immune mechanisms in fish is available to justify further speculations on this subject. The lack of effect of MAb-III \textit{in vitro} as well as \textit{in vivo} confirms the observation of others that G-specificity of an anti-rhabdovirus antibody does not necessarily imply neutralizing or protective activity (Flamand \textit{et al.}, 1980; Lefrancois & Lyles, 1982).

To investigate whether carbohydrate was integrated in the epitopes identified by the MAbs used in the present work, oligosaccharide chains on the viral G protein were removed by means of ENDO-F. The net result of this enzyme treatment may vary, depending on the substrate, but will usually be peptide chains free from N-linked carbohydrate (Plummer \textit{et al.}, 1984; Tarentino \textit{et al.},...
N-linked oligosaccharide chains represent the only known type of carbohydrate in rhabdovirus glycoproteins (Dubois-Dalcq et al., 1984). The binding ability of the three MAbs studied was not affected by the enzyme treatment of the antigen (Fig. 1). This suggests the assumption that the MAbs recognize carbohydrate-free determinants.

However, correct glycosylation is known to influence protein folding and may thereby control the conformation of carbohydrate-free determinants (Hongo et al., 1986; Kaluza et al., 1980), e.g. by affecting formation of intramolecular disulfide bonds (Vidal et al., 1989).

MAb-I, the MAb able to neutralize Egtved virus in vitro as well as in vivo, appeared to recognize a disulfide bond-dependent epitope (Fig. 1). In order to analyse whether the oligosaccharide chains of the G protein may play an indirect part in epitope formation in situ, tunicamycin was used to block N-linked glycosylation in virus-infected cell cultures. The cultures were subsequently analysed by immunofluorescence microscopy.

Tunicamycin clearly inhibited the multiplication of virus, as only single cells became infected (Fig. 2d). Similar observations have been made with other viruses (Kuismanen et al., 1984) but may not be a general feature of rhabdoviruses, since formation of vesicular stomatitis virus infective particles has been reported also in the presence of tunicamycin (Gibson et al., 1978). As far as Egtved virus is concerned the staining patterns with the G-specific MAbs (Table 2) indicated that the drug prevented the appearance of the G protein on the cell surface by blocking the intracellular transport of the protein. As a consequence of this blocking tunicamycin probably inhibited the formation or exposure of glycoprotein determinants not present or not detectable on the protein before the Golgi stage, i.e. the determinants recognized by the protective MAbs (MAb-I and MAb-II, Table 2).

Analogous results have been reported for the glycoprotein of Uukuniemi virus (Kuismanen et al., 1984), and in the case of vesicular stomatitis virus G protein glycosylation has been found to influence formation of disulfide bonds as well as the intracellular transport (Machamer & Rose, 1988a, b).

At the moment the Egtved virus G protein is about to be cloned into a prokaryotic expression system. This work will probably clarify whether the observed influence of tunicamycin on the binding ability of the MAbs means that glycosylation is essential for formation of the epitopes in question.

The present study represents the initial steps in identification of vaccine determinants. Passive immunization followed by challenge appeared to be a valuable alternative to neutralization at cell culture level for identification of epitopes involved in antibody-mediated neutralization of viral pathogenicity. Further experiments also dealing with active immunization and including analysis of cellular immune mechanisms may have to be performed before the optimal composition of a future recombinant vaccine can be established.

We thank L. Troels, T. Ingeman and M. Jeppesen for their excellent technical assistance. Dr Claus Koch, State Serum Institute in Copenhagen, Denmark, is thanked for having kindly suggested the buffer conditions for purification of MAbs from cell culture supernatant. This work was supported by the Danish governmental biotechnology programme running from 1987 to 1990.

References


*(Received 1 September 1989; Accepted 23 October 1989)*