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M Béarzotti, Af Monnier, P Vende, J Grosclaude, P de Kinkelin, A Benmansour

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The glycoprotein of viral hemorrhagic septicemia virus (VHSV): antigenicity and role in virulence

M Béarzotti, AF Monnier, P Vende, J Grosclaude,
P de Kinkelin, A Benmansour *

INRA, unité de virologie et immunologie moléculaires, 78352 Jouy-en-Josas cedex, France

Summary — In order to study the antigenic structure of the G protein of VHSV, we produced several anti-G monoclonal antibodies (MAbs) and used 4 neutralizing MAbs (NMAbs) to select resistant (MAR) mutants. Each MAR mutant was confronted with the 4 NMAbs in a neutralization test, and also with our panel of MAbs in surface plasmon resonance (SPR) analysis to determine the extent of their relatedness. Determination of the sequence of the entire *G* gene of representative MAR mutants allowed us to map the mutations responsible for the resistant phenotypes. We identified several locations on the G protein sequence, which represent, most probably, critical positions within the binding sites of the neutralizing MAbs. In addition, the MAR mutants selected with a cross-reactive MAb exhibited a reduced pathogenicity for fish. This indicated that the regions bearing the point mutations selected with MAb C10 were probably involved in the determination of the virulent phenotype.

VHSV / glycoprotein / antigenicity / virulence / epitope mapping

Résumé — La glycoprotéine du virus de la septicémie hémorragique virale (VSHV): antigénicité et rôle dans la virulence. *Dans le but d'étudier la structure antigénique de la glycoprotéine (G) du virus de la septicémie hémorragique virale (VHSV), nous avons produit plusieurs anticorps monoclonaux (AMC) spécifiques de la glycoprotéine. Quatre AMC neutralisants ont été utilisés pour sélectionner des mutants résistant à la neutralisation. Chaque mutant a été confronté à tous les AMC neutralisants dans un test de neutralisation croisée, et à l'ensemble de nos AMC anti-G dans un test de résonance de surface (BIAcore). La détermination de la séquence du gène entier de la glycoprotéine de mutants représentatifs a permis de localiser les mutations responsables des phénotypes résistants. Nous avons ainsi identifié plusieurs régions de la glycoprotéine qui représentent vraisemblablement des positions critiques pour la fixation des AMC neutralisants. Certains mutants sélectionnés avec un AMC qui neutralise tous les sérotypes du VHSV présentent une virulence réduite pour la truite. Ce qui est une indication de l'implication des régions qui portent certaines mutations dans le déterminisme du phénotype virulent.*

virus de la septicémie hémorragique virale (VHSV) / glycoprotéine / antigénicité / virulence / cartographie des épitopes

* Correspondence and reprints

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV), also known as Egtved virus, is a fish rhabdovirus which was initially isolated from rainbow trout *(Oncorhynchus mykiss)*. It is the agent that causes the most economically serious viral disease for the fish farming industry in continental Europe (de Kinkelin *et al,* 1979).

VHSV belongs to the *Rhabdoviridae* family. It is composed of a single-stranded RNA molecule which encodes for 5 viral structural proteins: the nucleocapsid protein N; the polymerase-associated protein P; the matrix protein M; the transmembrane glycoprotein G; and the polymerase L. In addition, a 6th functional gene, which encodes for a nonstructural protein (NV), was recently characterized (Benmansour *et al,* 1994; Benmansour, unpublished results).

Neutralizing antibodies are directed against the G protein and are the most important component of the protective immune response against VHSV (Lorenzen *et al,* 1990). The gene coding for the G protein of several strains of VHSV has been cloned and sequenced (Thiry *et al,* 1991; Lorenzen *et al,* 1993; Benmansour, unpublished results). It has also been expressed in different plasmids and viral vectors, with the aim of producing a subunit recombinant vaccine (Lorenzen *et al,* 1993; Lecocq-Xhonneux *et al,* 1994). Despite its antigenicity (Lorenzen *et al,* 1993), the recombinant G protein of VHSV was shown to confer little or no protection when expressed by bacterial cells (Thiry, personal communication) and only moderate protection when expressed in insect cells (Lecocq-Xhonneux *et al,* 1994). These results are an indication that some important antigenic determinants are probably not properly folded when the protein is expressed as a recombinant protein. The resolution of these problems will necessarily require knowledge of the antigenic structure of the G protein down to the epitope level.

Three to 4 different serotypes are currently defined among the different strains of VHSV by means of cross-neutralization tests performed with polyclonal antibodies. Neutralizing mAbs have also been used to detect differences among isolates (Olesen *et al,* 1993). However, no attempts have been made so far to characterize the epitopes involved in the neutralization of VHSV.

Neutralizing MAbs (NMAbs) and NMAb resistant mutants have been widely used to map the epitopes involved in the neutralization of many viruses. The most extensively studied is probably the rabies virus (Coulon *et al,* 1983; Dietzschold *et al,* 1983; Seif *et al,* 1985; Tuffereau *et al,* 1989; Benmansour *et al,* 1991). In order to study the antigenic structure of the G protein of VHSV, we produced several anti-G monoclonal antibodies (MAbs) and used 4 NMAbs to select MAb-resistant (MAR) mutants. Each MAR mutant was confronted with the 4 NMAbs in a neutralization test, and with our panel of MABs using surface plasmon resonance (SPR) analysis to determine the extent of their relatedness. The determination of the sequence of the entire G gene of representative MAR mutants allowed us to map the mutations responsible for the resistant phenotypes. We identified several locations on the G protein sequence which probably represented critical positions within the binding sites of the neutralizing MAbs. In addition, the MAR mutants selected with MAb C10 exhibited a reduced pathogenicity for fish. This indicated that the regions bearing the point mutations selected with MAb C10 were probably involved in the determination of the virulent phenotype.

MATERIALS AND METHODS

Cells and viruses

The myeloma cell line Sp2/0 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 10 mM

sodium pyruvate. Hybridomas were grown in the same medium supplemented with 15% FCS, 0.1 mM hypoxanthine, 0.4 mM aminopterin, and 16 mM thymidine.

VHSV strains 07-71 and 23-75 and their antigenic mutants were cultured as previously described (de Kinkelin and Le Berre, 1977) on epithelioma papulosum cyprini (EPC) cells. Virions were purified from clarified supernatants by PEG precipitation followed by banding on a 15–45% sucrose gradient. Small scale purifications of the antigenic mutants for RT-PCR amplification were obtained by centrifugation through a 25% glycerol cushion.

MAb production and characterization

Balb-C mice were immunized at 4 week intervals with 3 intravenous (iv) injections of gradient purified VHSV strain 07-71 or 23-75. Two intraperitoneal booster injections alternating with 2 iv injections were made during the 4 d preceding the fusion. Spleen cells from the immunized mice were used with Sp2/O myeloma cells, according to standard procedures. Hybridomas secreting anti-VHSV antibodies were selected by an ELISA test against the whole virus and were further characterized as anti-G by a membrane fluorescence test and a plaque neutralization test. Hybridomas secreting neutralizing antibodies were cloned by limiting dilution, and raised in the peritoneal cavity of Pristane-treated Balb/C mice.

Selection of antigenic mutants

The selection of antigenic mutants was performed as previously described (Seif et al, 1985). Briefly, 10^7 pfu from 4 different cloned stocks of 07-71 or 23-75 strains were incubated 1 h at 14°C with ascitic fluid and plated onto monolayers of EPC cells. Well-separated plaques were selected and small stocks of each putative mutant were prepared on EPC cells. The stocks were confronted with the selecting MAb, and only those with 90% resistance were used for this study.

RNA isolation

The viral RNA for use in the RT-PCR amplification was extracted from purified viral particles by SDS/proteinase K digestion followed by 2 cycles of phenol and diethyl ether extraction.

RT-PCR amplification, sequencing and cloning

RT-PCR was performed on viral RNA as previously described (Benmansour et al, 1992), except that the concentration of the 4 deoxy nucleotides was reduced to 20 mM. For directional cloning of the PCR products, we used 5' and 3' primers with EcoRI or XhoI restriction sites at their 5' end. Double-restricted PCR fragments and similarly restricted pBlueScript (Stratagene) plasmids were separated on 1.5% Nusieve GTG agarose (FMC) gel. Aliquots of the restricted PCR product and plasmid in melted gel, were used directly in a ligation reaction. The ligation product was used to transform competent bacterial cells. The recombinant plasmids containing the whole G gene were purified through 2 cycles of CsCl gradient centrifugation, and washed 3 times with double-distilled water on a centricon 100 device (Amicon). Aliquots were subjected to automated cycle sequencing (ABI automated sequencer 373A and autosampler 6000) with M13 forward and reverse universal primers (ABI dye-primer protocol) or specific internal primers (ABI dye-terminator protocol). For direct consensus sequencing, RT-PCR products representing either the whole or fragments of the G gene were washed 3 times with ultrapure water on a Centricon 100 device, and aliquots were used directly in an automated cycle sequencing reaction with specific internal primers (ABI dye-terminator protocol).

Virulence test

Pathogenicity tests were performed in virus-free rainbow trout juveniles from the Spring strain (INRA, France) maintained in flow-through aquaria at 10°C. In a first series of infection trials, groups of 40 trout, 1 350 degree-days old and weighing 2–3 g each were injected intra-muscularly (im) with 1 000 pfu of wild type (wt) viruses or MAR mutants in 20 ml of cell culture medium. In a second series of trials, groups of 30 trout of the same origin but 400 degrees-days older, received 10, 100, or 1 000 pfu of wt virus or MAR mutant c10 by the im route. In addition, 2 groups of 40 fish were waterborne-infected by immersion for 3 h

in a suspension of wt virus or MAR c10 at a concentration of 5 x 10⁴ pfu/ml. Mock-infected control groups were included in each series and for each route of infection. The course of infection was monitored on the basis of daily records of mortality and clinical signs over 30 d.

Sequence analysis

The sequences were assembled and analyzed with the Genetics Computer Group package (Devereux *et al*, 1984) run on a Sun/Unix minicomputer station.

BIAcore analysis

The BIAcore instrument (Pharmacia) is a biosensor-based system for real time interaction analysis. One of the reactants is immobilized on a dextran layer present on the sensor chip while the other is introduced in a solution that flows over the surface. To immobilize the MAbs on the dextran layer, a rabbit anti mouse Fc (RAMFc) sensor chip was used. Ascitic fluids diluted in Hanks balanced salt (HBS) were injected at a flow rate of 5 μl/min. The unoccupied sites on the sensor chip were blocked with a nonspecific MAb. VHSV (wild type or mutant) at a concentration of 50 μg/ml in HBS-10 mM EDTA was then injected at a flow rate of 15 μl/min. When necessary, second and third specific MAbs were injected sequentially. Wash and block steps were included after each introduction of a reactant.
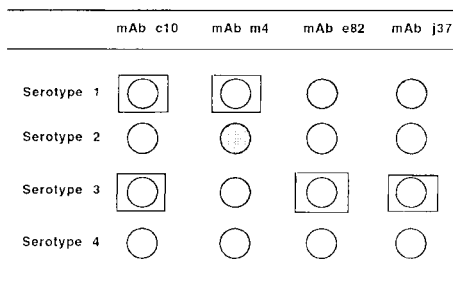
RESULTS AND DISCUSSION

Characterization of neutralizing MAbs

We selected 2 neutralizing MAbs (m4 and c10) from the mouse immunized with strain 07-71 (serotype 1) and 2 neutralizing MAbs (j37 and e82) from the mouse immunized with strain 23-75 (serotype 3). MAbs m4, e82 and j37 were type specific (they only neutralized strains from one serotype) while
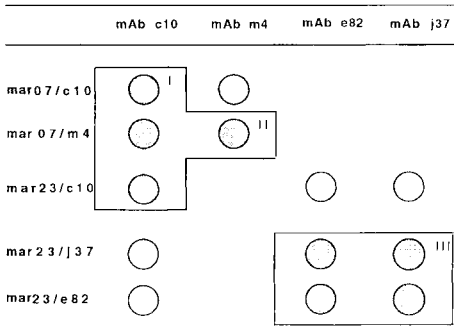
MAb c10 was cross-reactive and neutralized strains from all serotypes (fig 1).

MAb m4 was used to select antigenic mutants from each of 4 different cloned stocks of strain 07-71. Similarly, MAbs e82 and j37 were used to select antigenic mutants from the same number of different stocks of strain 23-75. MAb c10 was used to select antigenic mutants from both strains 07-71 and 23-75. The mutant frequency was found to be equivalent for both strains and ranged from 4 x 10⁻⁴ to 1 x 10⁻⁵. This mutant frequency is similar to those reported for other rhabdoviruses (Coulon *et al*, 1983; Seif *et al*, 1985; Tuffereau *et al*, 1989). From each round of selection, we chose at least 5 well-separated plaques, which were used to prepare small stocks of mutant viruses. The titers of the stocks of MAR mutant ranged from 3 x 10⁸ to 1 x 10⁹ pfu/ml and were not significantly different from titers of the parental strains. This indicated that the mutants had the same replication and maturation efficiency in EPC cells as the parental strains. As expected, a 100% escape to neutralization was recorded when 200 pfu of each stock of MAR mutant was confronted with the corresponding selecting MAb.

The MAR mutants were cross-reacted with all the selecting MAbs in a plaque neu-

Fig 1. MAbs neutralizing specificity. White circles: 100% neutralization; black circles: 0% neutralization; boxes: virus and MAb used for selection.

Fig 2. Characterization of escape mutants by cross-neutralization. White circles: 100% neutralization; black circles: 100% escape.

tralization test. According to their pattern of neutralization, they were divided into 3 classes (fig 2). The first class comprised the MAR mutants, which escaped neutralization with MAb c10. All of them retained full sensitivity to the neutralizing activity of MAb m4. The second class comprised the MAR mutants which escaped neutralization with MAb m4. In contrast to the MAR mutants of the first class, they also escaped neutralization with MAb c10. This indicated that MAbs c10 and m4 belong to a group with related specificity. MAb C10 was probably directed to an epitope which overlapped

with the epitope covered by MAb m4. The reverse was not true, since mutants selected with MAb c10 were still neutralized with MAb m4. The third class comprised the MAR mutants selected from strain 23-75 with MAbs e82 or j37. The MAR mutants selected with MAb e82 escaped neutralization with MAb j37. Conversely the MAR mutants selected with MAb j37 escaped neutralization with MAb e82. This was a strong indication that MAbs e82 and j37 belonged to the same specificity group and were probably directed to the same or to a largely overlapping epitope.

MAbs reactivity with virion-bound glycoprotein (SPR analysis)

An RAMFc immunoglobulin antibody immobilized on the sensor chip was used to attach anti-G-VHSV MAbs to the solid phase of a BIAcore instrument (Pharmacia). Of the 8 MAbs (table I) attached to the RMAFc, only MAb m4 was able to efficiently trap strain 07-71 (data not shown). It was interesting to note that escape mutants to m4 were no longer trapped. Thus, the resistance to neutralization by these mutants was probably due to a loss in the MAb binding. An unexpected result was the inability of mAb m4

Table I. List of MAbs.

MAbs	Virus	Isotype	Neutr	Fluo	Wb
m4	07–71	IgG2a	++	+/–	–
c10	07–71	IgG2a	++	+/–	+/–
a17	07–71	IgG3	--	++	++
a3	07–71	IgG2b	–	++	++
i16	07–71	IgG1	–	++	++
i10	07–71	IgG1	–	++	++
e82	23–75	nd	++	+/–	–
j37	23–75	nd	++	+/–	–

Neutr : plaque neutralization test ; Fluo : indirect fluorescence test ; Wb : Western Blot test.

to trap escape mutants to c10, although these mutants were still neutralized by m4. Trapping is, however, more demanding than neutralization in terms of binding.

With VHSV trapped to the sensor chip *via* m4, we tested different combinations of MAbs as the second and third position binders (table II). All the MAbs were able to bind the virus in the second position, except i10 and i16. Thus, i10 and i16 were probably directed towards cryptotopes. With m4 fixed in the second position, c10, a3 and a17 were still able to bind the virus, which implies that the respective epitopes were distinct from the m4 epitope. With c10 fixed in the second position, a3 and a17 were still able to bind, while m4 lost this ability. Thus, the c10 epitope overlapped the m4 epitope, and a3 and a17 were distinct from the c10 epitope. With a17 fixed in the second position, a3 was able to bind in the third position. The reverse was not true since a17 partially lost its ability to bind when a3 was fixed in the second position. Thus, the a3 and a17 epitopes were partially overlapping.

The SPR determination confirmed the relationship of the epitopes m4 and c10 already observed in cross-neutralization. It permitted the identification of 2 non-neutralizing epitopes, a17 and a3, which were partially overlapping and distinct from epitopes c10 and m4. It also permitted the characterization of the i10 and i16 epitopes as cryptotopes.

Mapping of mutations in antigenic mutants

In order to map the mutations in our antigenic mutants, we cloned the entire glycoprotein gene of 1 or 2 independent MAR mutants representative of each selecting MAb on pBlueScript plasmids and then subjected the purified plasmids to automated sequencing with universal and specific primers. Once a mutation was spotted on a MAR mutant, the region of interest from 2 to 3 additional MAR mutants, selected independently with the same MAb, was amplified by RT-PCR, and partially sequenced with the appropriate primers.

One MAR mutant selected with MAb c10 (MAR c10-3) was found to carry 3 point mutations in 3 distantly separated regions of the glycoprotein gene (table III). The first mutation (AAG to AGG) converted Lys140 into Arg. The second mutation (AAG to AGG) changed Lys161 into Arg. The third mutation (ATT to ACT) changed Ile433 into

Table II. Surface plasmon resonance epitope mapping.

MAb1(m4)/bl/VHSV/bl/MAb2/bl/MAb3

MAb 2				MAb 3			
	None	*m4*	*c10*	*a17*	*a3*	*i16*	*i10*
m4	+		+	+	+	−	−
c10	+	−		+	+	−	−
a17	+	+	+		+	−	−
a3	+	+	+	+/−		−	−
i16	−						
i10	−						

Table III. MAR mutants selected from strain 07-71 (serotype 1).

Selected MAb	Number	Mutated codon	aa change
MAb c10	1	AGG → AAG	Lys140 → Arg
		ATT → ACT	Ile 433 → Thr
	1	AGG → AAG	Lys140 → Arg
		AAG → AGG	Lys161 → Arg
		ATT → ACT	Ile 433 → Thr
	1	AGC → AAC	Ser139 → Asn
	1	AGC → ATC	Ser139 → Ile
MAb m4	1	ACA → GCA	Thr254 → Ala
	1	ACA → ATA	Thr254 → Ile
	2	GAA → GAT	Glu259 → Asp

Thr. As the simultaneous occurrence of 3 mutations is a statistically rare event, we determined the sequence of the *G* gene of one of the 07-71 cloned stock used for selection and found it conform to the original wild type sequence (Thiry *et al,* 1991). Partial direct sequencing of RT-PCR products amplified from viral RNA extracted from these 2 MAR mutants confirmed the presence of the 3 concomitant mutations, and ruled out their possible generation through PCR or cloning artifacts. Thus, their occurrence seemed to be specifically selected under the neutralizing pressure of MAb c10. A second MAR mutant selected independently with MAb c10 (MAR c10-2) was found to carry 2 point mutations already found on MAR c10-3: Lys140 to Arg and Ile433 to Thr. A 3rd and 4th independent MAR mutant selected with MAb c10 (MAR c10-11 and MAR c10-12) were found to have only one mutation located on codon 139AGC (Ser), which was changed into either AAC (Asn) or ATC (Ile).

Two MAR mutants selected with MAb m4 bore 2 alternate mutations which are located on the same codon (table III). Depending on the viral stock used for selection, the wild type codon ACA (Thr254) was changed into either GCA (Ala), or ATA (Ile). Two other independent MAR mutants selected with MAb m4 had the same mutation on codon 259 (GAA, Glu), which was changed into GAC (Asp).

A MAR mutant selected with MAb c10 from strain 23-75 (table IV) displayed a mutation located on codon 139AGC (Ser), that was changed into AAC (Asn) similarly to MAR c10-11. This finding was a confirmation of the importance of region 139-140 for the integrity of the c10 epitope.

Of the 7 MAR mutants selected with MAb e82 or j37, all displayed a mutation at the same nucleotide position (table IV). The wild type codon ACT (Thr23) was changed into either AAT (Ile) in 2 mutants, or ATT (Asp) in 5 mutants. These findings were a direct confirmation of the prediction made from the cross-neutralization experiments, that MAb e82 and j37 were probably directed to the same epitope.
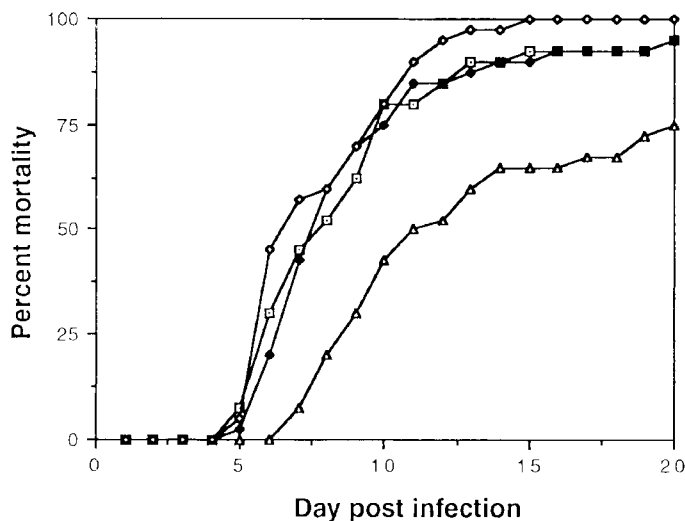
Table IV. MAR mutants selected from strain 23-75 (serotype 3).

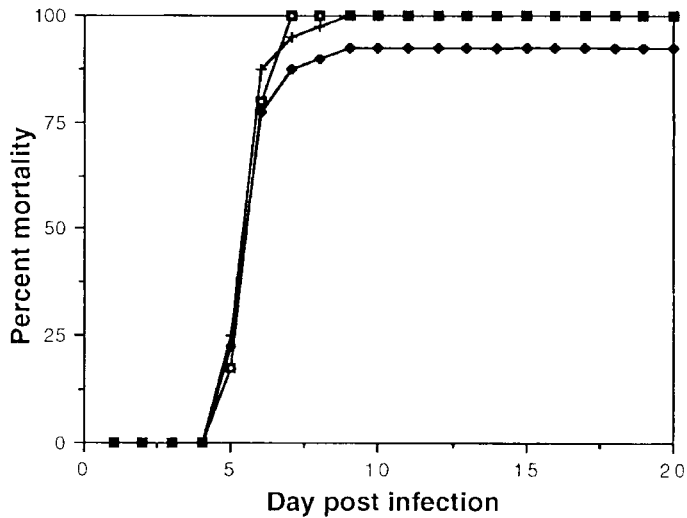| Selecting MAb	Number	Mutated codon	aa change
MAb c10	1	AGC → AAC	Ser139 → Asn
MAb e82	2	ACT → AAT	Thr23 → Ile
	2	ACT → ATT	Thr23 → Asn
MAb j37	3	ACT → ATT	Thr23 → Asn

Virulence test

To test the effect of the observed mutations on virulence, we performed a series of infection trials. In the first set of experiments, wild type and mutant viruses were injected at 1 000 pfu to juvenile trout. Under these conditions, strain 07-71 provoked symptoms characteristic of viral hemorrhagic septicemia, and a 100% mortality (fig 3). Mortality first appeared at d 5 and rose to a maximum over an 11 d period. The MAR mutants m4-1 (Ile at position 254) or m4-2 (Ala at position 254) behaved similarly to the wild type, although the maximum mortality levels were slightly lower (90%). The MAR mutants c10-11 or c10-12 (a single mutation at position 139) were also similar to the wild type (data not shown). In contrast, the onset of the disease due to the MAR mutants c10-2 or c10-3 (2 or 3 mutations) was delayed to d 7, and mortality levels rose more slowly over a 14 d period. The maximum mortality level obtained with these MAR mutants was also significantly lower (70%) than that obtained with the wild type

Fig 3. Virulence test, im injection, strain 07-71. White diamonds: strain 07-71 wt; black diamonds: mar m4-2; squares: mar m4-1; triangles: mar c10-2.

Fig 4. Virulence test, im injection strain 23-75. White squares: strain 23-75 wt; crosses: mar e82; black diamonds: mar j37.

(fig 3). Under the same experimental conditions, strain 23-75 was much more virulent, as the maximum mortality rate of 100% was recorded only 3 d after the onset of the disease. MAR mutants e82 (Gln at position 23) and j37 (Ile at position 23) were not significantly different from the wild type, as they gave similar mortality curves (fig 4).

In experiments where the fish were infected by the water-borne route, the wild type virus gave mortality scores ranging from 30 to 90% depending on the age of the fish, while MAR c10-2 gave mortality scores ranging from 0 to 60% (data not shown). Thus, MAR c10-2 was consistently less virulent than the wild type virus. This decrease in virulence was most probably related to the double mutation in the glycoprotein.

The epitope covered by MAb c10 appeared to be rather complex, since we have found patterns associating from 1 to 3 mutations. These patterns suggest that 3 different regions of the glycoprotein were participating in the structure of the c10 epitope. However, positions 139 or 140 seemed to be dominant, since it was rep-

resented in all the escape mutants to c10. The companion mutations at positions 161 and 433 were then probably co-selected with mutation 140. Region 254-259, which represented the m4 epitope, also participated in the integrity of the c10 epitope, since the escape mutants to m4 were also resistant to c10.

The region formed by the association of positions 140 and 433 seemed to play an important role in determination of the virulence status. The importance of this region was further substantiated by the finding that an attenuated variant of strain 07-71, which was developed in our laboratory through progressive adaptation to be able to grow at 25°C, had 2 mutations at positions 135 and 431, very close to those found in the attenuated escape mutants. Another indication of the importance of this region for virulence came from the sequence determination of the glycoprotein of an American strain of VHSV. This strain is avirulent for trout, and among the 39 amino acid differences with strain 07-71, it was striking that it displayed different amino acids at positions 139 and 431.

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