

## A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout

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Viral haemorrhagic septicaemia (VHS) is a fish rhabdovirus infection of world-wide importance. Control policies have been established but the disease still causes heavy losses in fish farming. The development of a recombinant subunit vaccine was initiated to produce a safe and effective vaccine to protect fish against VHS. The VHS virus (VHSV) glycoprotein, which induces neutralizing antibodies in rainbow trout, was chosen for expression in insect cells using a baculovirus vector. The  $M_r$  of the recombinant protein estimated by SDS-PAGE was slightly lower than that of the native viral protein. The recombinant protein displayed different degrees of

glycosylation and was recognized in ELISA by neutralizing antibodies. It was transported to the plasma membrane of insect cells where its ability to induce membrane fusion was preserved. The efficacy of the recombinant protein as a vaccine was compared with those of an inactivated and an attenuated vaccine. When injected intraperitoneally into rainbow trout, the baculovirus-encoded protein was shown (i) to induce the synthesis of VHSV-neutralizing antibodies and (ii) to confer protection against virus challenge. Immunization performed by immersion failed. This is the first report of a recombinant vaccine that protects fish against VHSV.

### Introduction

Viral haemorrhagic septicaemia (VHS) is a rhabdovirus infection of cold water fish such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), grayling (*Thymallus thymallus*), whitefish (*Coregonus* sp.), pike (*Esox lucius*) and turbot (*Scophthalmus maximus*). It is prevalent in continental Europe where it is a matter of concern because of its economic consequences to rainbow trout farming in both fresh and sea water. Recently VHS virus (VHSV) has spread to the North Western Pacific coast of America where rainbow trout (Brunson *et al.*, 1989), chinook salmon (*O. tshawytscha*) (Hopper, 1989) and Pacific cod (*Gadus macrocephalus*) (Meyers *et al.*, 1992) were found to be asymptomatic virus carriers. VHS is thus becoming a fish disease of world-wide importance.

Until now, the spread of VHSV has been restricted through the establishment of a control policy. Most of the time, the results were rather poor, in so far as this method was applied to heavily infested zones where VHSV is harboured by farmed and wild fish.

For over 10 years it has been established that fish that are naturally protected against overt VHS possess virus-

neutralizing antibodies (NAb) (de Kinkelin *et al.*, 1977). Thus immunoprophylaxis appeared to be a possible means of controlling VHS. Several vaccination studies were performed after the mid-1970s as previously reviewed (de Kinkelin, 1988). Briefly, effective anti-VHS protection could be obtained either by the injection of inactivated virus or by the water route delivery of attenuated virus variants (Jørgensen, 1976; de Kinkelin *et al.*, 1980; de Kinkelin & Bearzotti-Le Berre, 1981). The former vaccine was safe but was not suitable for delivery to a large number of juvenile fish which are the main target of VHSV infection. The latter vaccines were easy to deliver but retained some degree of virulence which from a regulatory point of view prohibited their use, in as much as the mechanisms of attenuation were not clarified (Bernard *et al.*, 1983, 1985).

Recombinant subunit vaccine technology therefore appeared to be a suitable alternative for producing a safe and effective vaccine against VHS. It has been shown that the VHSV epitopes responsible for inducing protection are located on the surface glycoprotein (G), as is the case for other rhabdoviruses (Wiktor *et al.*, 1973; Kelley *et al.*, 1972; Engelking & Leong, 1989*a, b*), and that antibodies to the G protein are able to abolish virus

infectivity (de Kinkelin *et al.*, 1984; Lorenzen *et al.*, 1990). A cDNA copy of the mRNA coding for G protein of VHSV has been cloned (Thiry *et al.*, 1990) and sequenced (Thiry *et al.*, 1991). The VHSV G protein was also expressed in *Escherichia coli*, as a fusion protein with the bacterial enzyme TrpE but this protein failed to induce either consistent protection or the synthesis of neutralizing antibodies whatever the route of delivery (unpublished results).

Prior to and in parallel with the above work, other investigations were carried out on the molecular biology of infectious haematopoietic necrosis virus (IHNV) (Koener *et al.*, 1987), another fish rhabdovirus, which is the causative agent of a severe systemic infection of salmonid fish species that resembles VHS clinically. IHNV was originally found in the North Pacific Ring but has recently spread to Continental Europe (Hattenberger-Baudouy *et al.*, 1989). These experiments led to the construction of a recombinant vaccine in which a fragment of the G protein of IHNV was fused with the *E. coli* enzyme TrpE (Gilmore *et al.*, 1988). Fingerling trout immunized by immersion in crude bacterial extract were protected against virus challenge. More recently, the immunodominant domain of the IHNV glycoprotein has been localized to the carboxy-terminal region (Xu *et al.*, 1991).

Concerning VHS, it was demonstrated very recently (Lorenzen *et al.*, 1993) that the injection of rainbow trout with an almost complete VHSV G protein produced in *E. coli* could elicit specific anti-G antibodies. The recombinant protein had to be carefully renatured before injection. The serum of some of the immunized fish possessed a heat-labile neutralizing activity against VHSV.

In contrast with the previous findings, our negative results with the recombinant G protein of VHSV produced in *E. coli* prompted us to shift from expression in bacteria to expression in eukaryotic cells, a system more likely to result in a properly glycosylated protein. According to the existing evidence from other animal rhabdovirus research the glycosylation of G protein is likely to be crucial for the induction of a neutralizing antibody response (Gibson *et al.*, 1981; Machamer & Rose, 1988; Prehaud *et al.*, 1989).

The present paper describes how the cDNA of the gene of VHSV G protein was cloned in a baculovirus vector (Luckow & Summers, 1988) which mediated a high level of expression. The potency of the recombinant vaccine was compared with those of a live-attenuated vaccine and an inactivated virus vaccine. When injected into rainbow trout, the baculovirus-encoded protein was shown (i) to induce the synthesis of VHSV-neutralizing antibodies and (ii) to confer protection against virus challenge.

## Methods

**Virus and cells.** *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant baculovirus stocks were grown and assayed in confluent monolayers or suspensions of *Spodoptera frugiperda* cells (Sf9) in Ex-Cell 400 medium (JRH Biosciences) according to the procedures described by Summers & Smith (1988).

The *epithelioma papulosum cyprini* (EPC) cell line (Fijan *et al.*, 1983) was grown at 30 °C in Stoker's medium (Gibco BRL) buffered with 0.16 M-Tris-HCl at pH 7.4 and supplemented with 10% fetal calf serum (FCS), 10% tryptose phosphate and antibiotics. For virus production, the pH was adjusted to 7.6 and the FCS content was reduced to 2%.

VHSV 07.71, a French isolate from rainbow trout, belonging to serotype 1, was propagated in EPC cells at 15 °C as previously described (Fijan *et al.*, 1983) whereas 07(25)111, an attenuated variant of 07.71 (Bernard *et al.*, 1985) was propagated at 22 °C.

Inactivated VHSV vaccine was prepared by u.v. irradiation of a suspension of 50 µg/ml purified 07.71 virus with an over-killing factor of 10. Virus was purified by sucrose gradient centrifugation according to de Kinkelin (1972).

**Construction of AcNPV recombinant transfer vector.** DNA manipulations were carried out essentially following the procedures described by Sambrook *et al.* (1989). The transfer vector pJVP10Z (Vialard *et al.*, 1990), kindly provided by Dr C. D. Richardson (Biotechnology Research Institute, National Research Council, Montreal, Canada), containing the polyhedrin gene and the *E. coli* β-galactosidase gene was digested with *NheI* prior to insertion of the G gene of VHSV isolate 07.71 under the control of the AcNPV polyhedrin promoter. The VHSV G gene was synthesized by PCR, according to Saiki *et al.* (1988), from plasmid pSHVG1 (Thiry *et al.*, 1991). The following amplimers were used in order to introduce *NheI* sites near the initiator codon and stop codon: 5' GG GCT AGC ATG GAA TGG AAC ACT 3' 5' GG GCT AGC TCA GAC CGT CTG ACT 3'. The correct orientation of the G protein coding region in the derived recombinant transfer vector, pBacSHVG, was verified by restriction endonuclease mapping.

**Transfection and selection of recombinant viruses.** Sf9 cells were transfected with a mixture of the recombinant plasmid pBacSHVG (10 µg) and wild-type AcNPV DNA (1 µg) by calcium phosphate precipitation. Recombinant viruses were selected following three rounds of purification of plaques that were polyhedron-negative and stained blue as described by Summers & Smith (1988).

**Recombinant glycoprotein production.** Sf9 cell suspensions in exponential growth phase were infected, according to the procedures described by Summers & Smith (1988), by selected recombinant baculovirus at an m.o.i. of 1. The cells were harvested 4 days post-infection by centrifugation and cell pellets were stored at -70 °C until use. Negative controls were prepared in the same manner with wild-type baculovirus or uninfected Sf9 cells.

**Immunoblotting.** The VHSV G protein-specific monoclonal antibody (MAb) used (A17) was isolated by Bearzotti & de Kinkelin (1987) and purchased from Sanofi Aquatic Animal Health. Immunoblot analyses were carried out under denaturing conditions. Samples for SDS-PAGE were boiled for 5 min in dissociation buffer containing 2% SDS and 5% 2-mercaptoethanol, subjected to electrophoresis (Laemmli, 1970) and electrotransferred to PVDF membrane (Immobilon; Millipore). The blot was incubated for 1 h at 37 °C or overnight at 4 °C with blocking solution (5% non-fat dried milk in 10 mM-PBS pH 7.3), then transferred to a blocking solution containing 5 µg/ml of MAb A17 and incubated for 3 h at 37 °C. After several washes, bound antibodies were detected with a peroxidase-conjugated anti-mouse immunoglobulin (Gamma). The horseradish peroxidase colour development reagent

from Bio-Rad Laboratories was used as chromogenic substrate for peroxidase activity detection.

**Deglycosylation assay.** Proteins were denatured for 5 min at 100 °C with 1% SDS and 0.5% 2-mercaptoethanol before being diluted tenfold in PBS containing 20 mM-EDTA and 0.6% Triton X-100. Samples were incubated for 18 h at 37 °C with 5 U/ml of a mixed endoglycosidase F/N-glycosidase F preparation (Boehringer Mannheim) and analysed by immunoblotting as described above.

**ELISA.** Microtitre wells (Nunc; Immunoplate Maxisorp) were coated by incubation overnight at 4 °C with 100 µl of MAb A17 diluted to 2 µg/ml in carbonate buffer pH 9.6 (15 mM-Na<sub>2</sub>CO<sub>3</sub>, 35 mM-NaHCO<sub>3</sub>, 0.2 g/l NaN<sub>3</sub>). After repeated washing with PBS containing 0.1% Tween 20 (PBS-T), remaining sites were blocked by incubation with 1% BSA in PBS-T for 45 min at 37 °C. Twofold serial dilutions of antigen (50 µl/well) were then incubated for 3 h at 37 °C in blocking solution with 50 µl of peroxidase-conjugated MAb L7 (Sanofi Aquatic Animal Health) according to Mourton *et al.* (1990). MAbs L7 and A17 are both VHSV G protein-specific but L7 is able to neutralize VHSV *in vitro* whereas A17 is not (Bearzotti & de Kinkelin, 1987).

For antigen preparation, insect cells (10<sup>7</sup> cells/ml) and purified virus (5 µg/ml) were disrupted in PBS with 50 mM-octyl-glucopyranoside (OG; Boehringer Mannheim) and incubated for 30 min on ice before assay. An anti-protease mixture (1 mM-EDTA, 1 mM-PMSF, 0.5 µg/ml leupeptin and 0.7 µg/ml pepstatin; Boehringer Mannheim) was also added.

The chromogenic substrate for peroxidase, *o*-phenylenediamine dihydrochloride (Sigma) was used in appropriate buffer (66.7 mM-Na<sub>2</sub>HPO<sub>4</sub>, 34.7 mM-citric acid pH 4.5) at a concentration of 1 mg/ml with 1 µl/ml of 30% H<sub>2</sub>O<sub>2</sub>. The reaction was stopped after 15 min with 50 µl per well of 4 M-H<sub>2</sub>SO<sub>4</sub> and the A<sub>492</sub> determined.

This assay allowed the determination of the approximate concentration of the G protein in samples intended for administration to fish. ELISA values generated by a known quantity of purified viral proteins were compared with those of lysed recombinant baculovirus-infected insect cells in the linear part of the curves. G protein was considered to account for 16% of total viral protein according to de Kinkelin *et al.* (1980).

#### Rainbow trout fry immunization trials

In the first series of trials, the immunity raised in trout by our recombinant vaccine was compared with that induced by inactivated VHSV and attenuated VHSV, both acting as positive vaccine controls. A second series of trials was designed to check the reliability of protection due to injection of the recombinant vaccine and the effectiveness of its water-route delivery. The trials on animals were performed at 10 to 11 °C in a contained laboratory and the effluent water was sterilized by chlorination.

(i) **First series of trials.** Virus-free fry from the INRA's strain of rainbow trout (an extensively cross-bred population representative of French strains of rainbow trout), 1400 degree days old and weighing 2 to 3 g, were used. Each fish immunized with the recombinant vaccine received 1 × 10<sup>5</sup> baculovirus-infected Sf9 cells in PBS via an intra-peritoneal (i.p.) injection whereas the fish from the control group received the same dose of Sf9 cells alone. The fish immunized with inactivated virus were injected i.p. with 160 ng virus in PBS and the controls with PBS only. All injected products were mixed 1:1 (v/v) with Suvaxyn, an oil-in-water emulsion used as an adjuvant, kindly provided by Dr L. Hilgers (Solvay Duphar Animal Health). Lastly, the attenuated virus was administered by immersion of the fish in water containing 5 × 10<sup>5</sup> p.f.u. virus/ml for 25 min, with bubbling air. The fish in the control group were similarly immersed in an aqueous suspension of disrupted EPC cells (10<sup>8</sup> cells/ml).

Forty-nine days post-immunization, 10 fish/group were bled after severing the caudal peduncle. Blood samples (50 µl) were immediately

diluted 1:10 in sterile PBS and stored at 4 °C for 16 h before further processing. Following blood sampling, each group of fish was divided into two smaller groups of approximately equal size and waterborne challenge was performed by immersing the fish for 3 h in a static aqueous suspension of VHSV isolate 07.71 at a concentration of 5 × 10<sup>4</sup> p.f.u./ml. The mortality in these groups was recorded daily for 30 days post-challenge and the diagnosis of VHS was made on the basis of clinical signs of disease or in the case of asymptomatic infection, by virological examination.

Statistics were calculated according to the  $\chi^2$  table with the Yates correction (Table 1). The relative percentage survival (RPS; Johnson *et al.*, 1982) was also determined by the formula RPS = [1 - (% loss of immunized fish/% loss of controls)] × 100.

(ii) **Second series of trials.** This series of trials was conducted from mid-September to mid-November using 1000 degree days old fish from the INRA's spring spawning trout strain (mean weight 1.8 g). The recombinant vaccine was either injected as in the first series or administered by immersion. In the latter instance 50 fish were immersed for 15 min with bubbling air in 200 ml of an aqueous suspension of 4 × 10<sup>7</sup> recombinant baculovirus-infected cells disrupted by one freezing and thawing cycle. The mock-immunized group was similarly treated with a suspension of Sf9 cells. The immunization trial and challenge were the same as for the first series (Table 1).

**Serum neutralization test (SNT).** The SNT in the presence of trout complement (Dorson & Torchy, 1979) was performed by plaque assay in microplates (Olesen & Jørgensen, 1986) using 07.71 as the viral antigen. For that purpose, the clotted, PBS-diluted blood samples were centrifuged for 20 min at 2000 g at 4 °C and the supernatants were collected, heated at 45 °C for 30 min and cooled to 4 °C. SNTs were conducted with twofold serial dilutions of sera from 1/50 to 1/400 resulting from the mixing (v/v) of 25 µl of sera diluted 1/25 to 1/200 with 25 µl of virus suspension containing 6 × 10<sup>3</sup> p.f.u./ml and containing trout complement. Incubation was performed at 4 °C overnight and the plaque assay was performed by inoculating EPC cell monolayers grown in 96-well culture plates with 10 µl of each virus-serum mixture (two wells/dilution). After 1 h of incubation at 15 °C, 60 µl/well of Stoker's medium with carboxymethylcellulose was added and plates were incubated for 72 h at 15 °C. The infected monolayers were then fixed and stained with 0.5% crystal violet in 10% formalin, microplaques were counted and the neutralizing antibody titre was determined as the reciprocal value of the highest serum dilution causing 50% reduction of the average number of plaques counted in negative control cultures with normal trout serum. Each microplate encompassed 10 assays plus one positive and one negative control serum.

## Results

### Recombinant glycoprotein expression

Sf9 cells were infected with the wild-type and the recombinant baculovirus and analysed by immunoblotting since the recombinant G protein was masked for the most part by insect cell proteins in Coomassie blue-stained gels (not shown). Using the VHSV G protein-specific MAb A17, several bands corresponding to M<sub>r</sub>s ranging from 52K to 63K were revealed only in recombinant virus-infected cells (Fig. 1, lane 4). The glycoprotein expressed in insect cells exhibited a lower M<sub>r</sub> in SDS-PAGE than did the native G protein since the M<sub>r</sub> of the G of purified VHSV was about 66K (lane

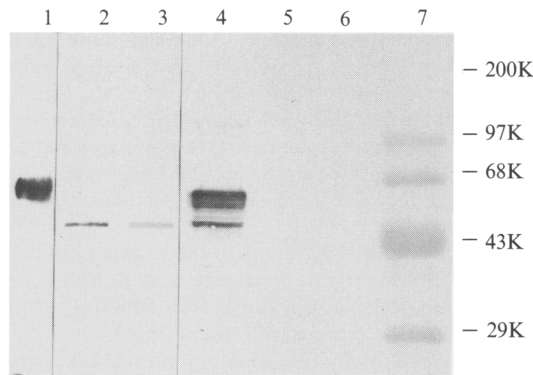


Fig. 1. Characterization of baculovirus-expressed VHSV G protein in immunoblotting by MAb to G protein. Lane 1, purified VHSV particles (1  $\mu$ g); lane 2, deglycosylated purified VHSV (0.1  $\mu$ g); lane 3, deglycosylated recombinant baculovirus-derived G protein ( $10^4$  infected Sf9 cells); lane 4, recombinant baculovirus-infected Sf9 cells ( $5 \times 10^4$  cells); lane 5, wild-type baculovirus-infected Sf9 cells ( $5 \times 10^4$ ); lane 6, uninfected Sf9 cells ( $5 \times 10^4$ ); lane 7, prestained  $M_r$  markers (Gibco BRL).

1). After deglycosylation with endoglycosidase F and *N*-glycosidase F, the native and recombinant G proteins showed the same relative mobility in the gel (lanes 2 and 3). The different bands present in the infected cells suggest different levels of glycosylation. Their relative intensity varied during the course of infection to the detriment of the highly glycosylated form (data not shown). The level of expression increased further after 4 days post-infection but infected cells began to disrupt.

### ELISA

As expected, G-specific MAbs did not react with wild-type baculovirus-infected cells, but recombinant samples were recognized by L7 MAb (Fig. 2). Saturation or steric hindrance probably interfered in the first dilutions. Another anti-G neutralizing MAb, C10 (Bearzotti & de Kinkelin, 1987), was also shown to bind to the recombinant protein (data not shown).

As shown in Fig. 2,  $10^5$  cells were estimated to contain approximately 32 ng of recombinant G protein. Indeed,  $3.1 \times 10^4$  recombinant infected cells produced the same signal as 63 ng of purified virus (approximately 10 ng of G protein). Similar values were obtained for different preparations of the recombinant G protein.

Total cell lysates, prepared by OG treatment, were compared by ELISA with the supernatants of a 100 000 *g* centrifugation of the same extracts. Similar  $A_{492}$  values were obtained for both samples (data not shown). This assay therefore detected only the soluble fraction of the recombinant proteins. ELISA was not performed on the insoluble pellet.

The same samples were then analysed by immunoblotting (data not shown). In this case, the response of

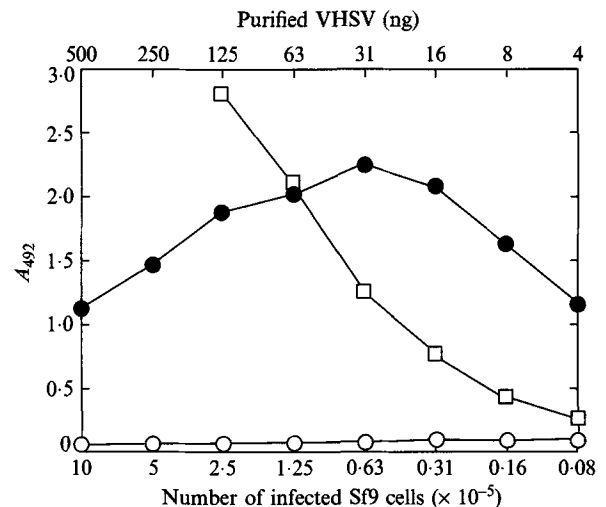


Fig. 2. Characterization of baculovirus-expressed VHSV G protein in ELISA with anti-G protein MAb. Symbols: purified VHSV ( $\square$ ); Sf9 cells infected with wild-type baculovirus ( $\circ$ ); Sf9 cells infected with VHSV G recombinant baculovirus ( $\bullet$ ).

the total cell lysate was higher than that of the 100 000 *g* supernatant, suggesting that SDS and 2-mercaptoethanol treatment solubilized the recombinant G protein more efficiently than OG treatment.

If OG treatment did not solubilize all the synthesized recombinant glycoproteins and the ELISA recognized only the soluble fraction of these, the value of 32 ng G protein per  $10^5$  Sf9 cells was probably an underestimate. However, this assay allowed the estimation of the minimum quantity of correctly folded G protein that was administered to fish and allowed the comparison of several vaccine preparations.

### Fusion activity

When Sf9 cells were infected with the recombinant baculovirus, multinucleate syncytia spontaneously appeared from 48 h post-infection (Fig. 3). This was never observed with wild-type virus or mock-infected cells. The pH of the Ex-Cell 400 medium was 6.3 during the infection course and fusion was inhibited when the pH of the culture medium was adjusted to greater than 6.6 (not shown). Syncytia were more readily observed on monolayers than in suspension culture.

### Immunization trials

Vaccination with attenuated VHSV caused the death of 13 fish. VHSV multiplication could be shown in nine of these fish. The administration of the different products, and especially that of the recombinant vaccine, was safe (Table 1). The delivery of this vaccine by i.p. injection was followed by the production of neutralizing anti-

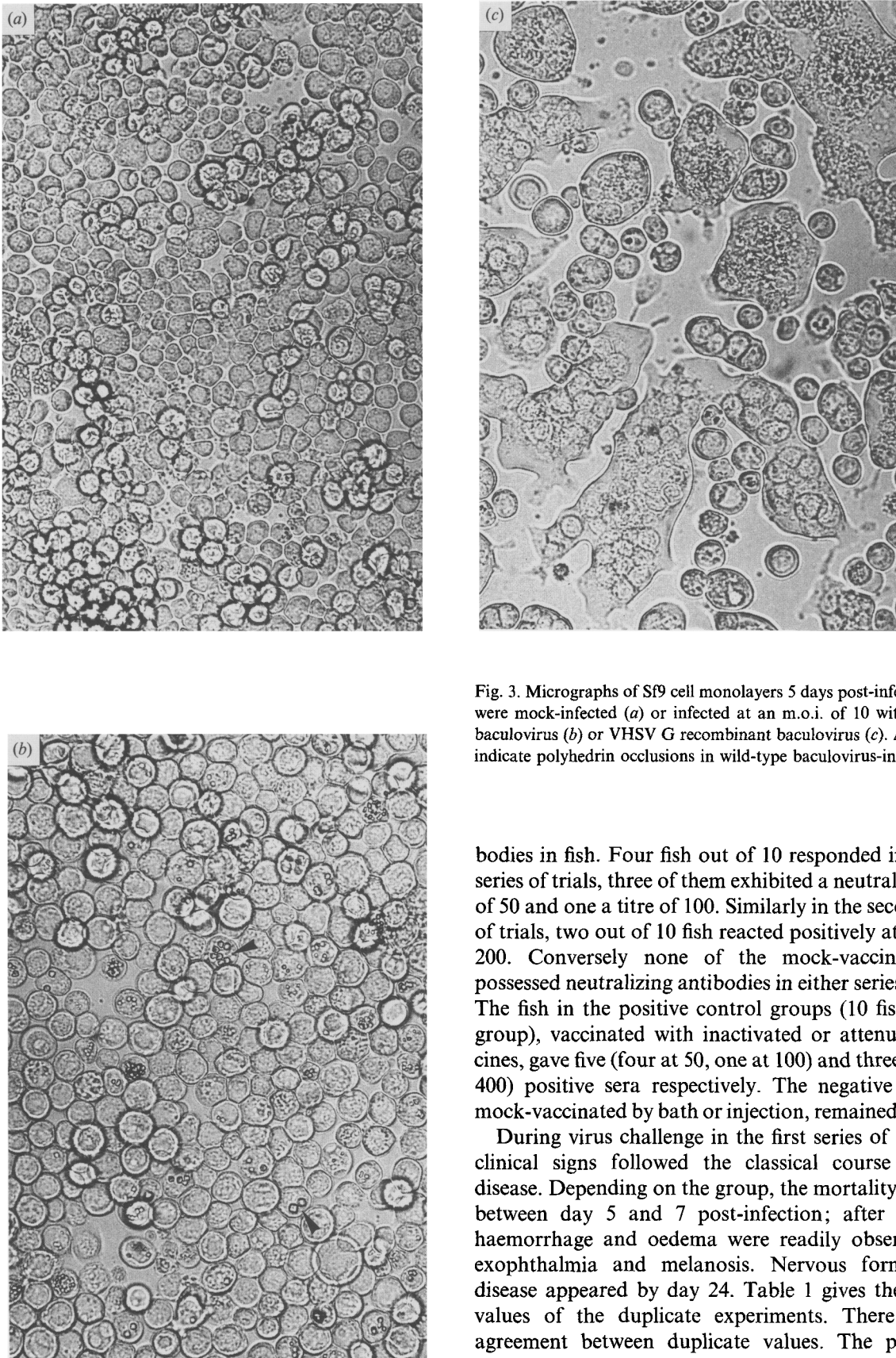


Fig. 3. Micrographs of Sf9 cell monolayers 5 days post-infection. Cells were mock-infected (a) or infected at an m.o.i. of 10 with wild-type baculovirus (b) or VHSV G recombinant baculovirus (c). Arrowheads indicate polyhedrin occlusions in wild-type baculovirus-infected cells.

bodies in fish. Four fish out of 10 responded in the first series of trials, three of them exhibited a neutralizing titre of 50 and one a titre of 100. Similarly in the second series of trials, two out of 10 fish reacted positively at a titre of 200. Conversely none of the mock-vaccinated fish possessed neutralizing antibodies in either series of trials. The fish in the positive control groups (10 fish in each group), vaccinated with inactivated or attenuated vaccines, gave five (four at 50, one at 100) and three (50, 100, 400) positive sera respectively. The negative controls, mock-vaccinated by bath or injection, remained negative.

During virus challenge in the first series of trials, the clinical signs followed the classical course of VHS disease. Depending on the group, the mortality occurred between day 5 and 7 post-infection; after this time haemorrhage and oedema were readily observed with exophthalmia and melanosis. Nervous form of the disease appeared by day 24. Table 1 gives the survival values of the duplicate experiments. There is good agreement between duplicate values. The percentage survival for the recombinant, inactivated and attenuated

Table 1. Results of immunization trials of rainbow trout fry against VHS with vaccine preparations of recombinant baculovirus, inactivated VHSV and attenuated VHSV

Series	Experimental group	Fish immunized	Positive SNT*	Fish challenged	Challenge survivors	Survival (%)	$\chi^2$	RPS
1	Recombinant vaccine (injection)	105	4/10	46+47	31+33	68.8	4.2 $P < 0.05$	33.9
1	Control (injection)	105	0/10	44+45	22+25	52.8		
1	Inactivated vaccine (injection)	103	5/10	45+45	30+33	70	25.7 $P < 0.005$	56.5
1	Control (injection)	103	0/10	45+45	13+15	31.1		
1	Attenuated vaccine (bath)	93	3/10	40+40	32+26	72.5	22.3 $P < 0.005$	57.6
1	Control (bath)	101	0/10	46+45	17+15	35.2		
2	Recombinant vaccine (injection)	94	2/10	42+42	39+41	95.2	11.8 $P < 0.005$	80.2
2	Control (injection)	110	0/10	49+50	36+39	75.8		
2	Recombinant vaccine (bath)	50	ND†	48	28	58.3	0.001 $P > 0.1$	-4.3
2	Control (bath)	50	ND	50	30	60		

\* Number of sacrificed fish out of 10, responding in the seroneutralization test at titres  $\geq 50$ .

† ND, Not done.

vaccine groups and their controls were 68.8, 52.8; 70, 31.1; and 72.5, 35.2, respectively. According to the  $\chi^2$  analysis, the differences in the mortalities of vaccinated and non-vaccinated groups were statistically significant ( $P < 0.05$ ).

In the second series of trials (Table 1), the course of immunization and virus challenge was similar overall to that recorded in the first series but the mortality rates remained moderate as we observed frequently with challenges performed in the autumn. They finally resulted in 95.2% survival in the group of fish injected with the recombinant vaccine and 75.8% in the mock-vaccinated fish group. In contrast no significant difference in survival between water route recombinant vaccine-treated and mock-treated fish was found.

## Discussion

The  $M_r$  of the recombinant VHSV G protein expressed in insect cells never reached that of the natural viral G protein. It displayed a range of  $M_r$ s corresponding to different degrees of glycosylation (Fig. 1). Such differences in electrophoretic mobility have been observed with other recombinant viral glycoproteins expressed in insect cells (Prehaud *et al.*, 1989; Bailey *et al.*, 1989; Kuroda *et al.*, 1990; Matsuura *et al.*, 1992; Tuchiya *et al.*, 1992). They appear to be due to differences in the complexities and types of glycan attached to proteins made in the different cell types. Sf9 cells have the capacity to trim *N*-glycans to trimannosyl cores and to

process these further by the addition of fucose (Kuroda *et al.*, 1990), but they are known to lack sialyl transferase activities (Prehaud *et al.*, 1989).

Correct glycosylation is known to influence protein folding. Carbohydrate chains protect the G protein of vesicular stomatitis virus (VSV) from forming aberrant interchain disulphide bonds (Machamer & Rose, 1988). The size of oligosaccharides present can be crucial for attaining the proper conformation (Gibson *et al.*, 1981). But in spite of differences in glycosylation, the recombinant VHSV G protein seemed to be correctly folded since the presence of neutralizing epitopes could be detected by ELISA with neutralizing monoclonal antibodies L7 and C10. Both antibodies are known to confer passive protection of rainbow trout against VHSV challenge (P. de Kinkelin & F. Lecocq-Xhonneux, unpublished results).

Formation of syncytia with cells infected by VHSV G recombinant baculovirus proved that the recombinant protein was anchored in the plasma membrane. Immunofluorescence analysis on intact cells confirmed the location of the glycoprotein on the cell surface (data not shown). The formation of syncytia was also observed when Sf9 cells expressed VSV G protein (Bailey *et al.*, 1989) and rabies virus G protein (Tuchiya *et al.*, 1992). The fusion was shown to be pH-dependent and was inhibited when the pH of the medium exceeded 6.2 and 6.0 respectively. Recombinant VHSV G thus seemed to induce membrane fusion at a slightly higher pH than the other rhabdoviral glycoproteins.

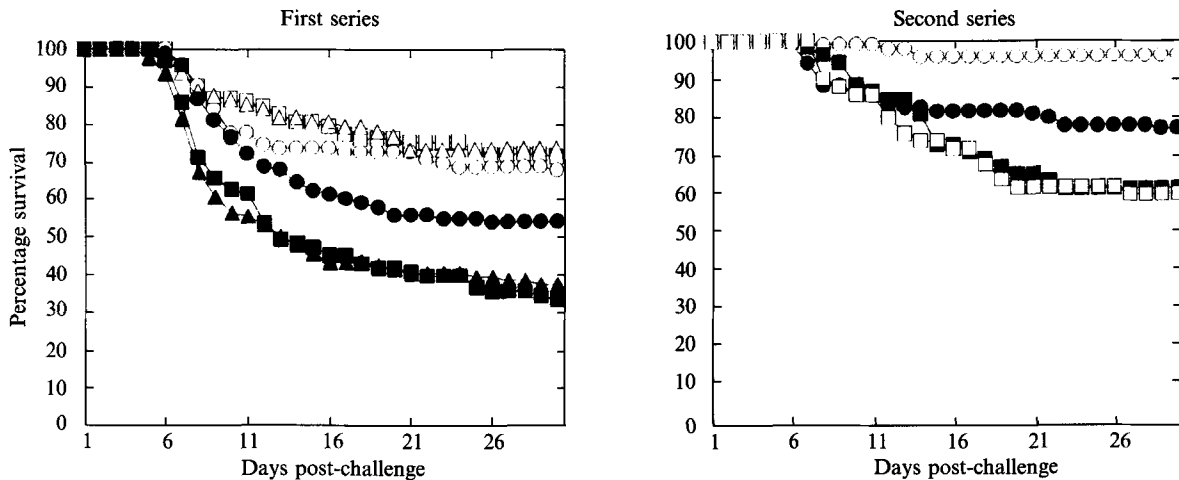


Fig. 4. Survival rate of fish challenged with VHSV. First series of trials: fish were immunized 49 days before challenge, either by i.p. injection with recombinant baculovirus-infected Sf9 cells (○), mock-infected Sf9 cells (●), inactivated VHSV (□) or PBS (■), or by balneation with attenuated VHSV (△) or disrupted EPC cells (▲). Second series of trials: fish were injected i.p. with recombinant baculovirus-infected cells (○) or mock-infected Sf9 cells (●), or immersed with the same products (◇, ◆) respectively. Points have been plotted as an average of the duplicates.

The protein was transported to the cell surface in a functional conformation since it could mediate the fusion of the cellular membranes at low pH. Indeed, the glycoproteins of rhabdoviruses are known to trigger fusion between the viral envelope and the membrane of the endocytosis vesicles at low pH, leading to the transfer of the viral ribonucleoprotein into the cytoplasm (Matlin *et al.*, 1982; Superti *et al.*, 1984). The low pH allows these proteins to adopt a different conformation probably leading to the exposure of hydrophobic regions at the protein surface and thus allowing membrane fusion (Crimmins *et al.*, 1983; Gaudin *et al.*, 1991).

All these results suggested that the VHSV G protein expressed in insect cells was a good candidate for vaccination trials. Nevertheless, only a fraction of the synthesized G protein seemed to be correctly processed and exported to the cell surface. Only a small amount of VHSV recombinant glycoprotein could be solubilized in non-ionic detergents such as Triton X-100, CHAPS or OG (data not shown). The bulk of the protein was probably in an aggregated form within intracellular membranes or associated with cellular proteins. This is in agreement with cytoplasmic inclusions seen by immunofluorescence (data not shown).

Even though the degree of protection is rather low, the i.p. injection of the baculovirus-encoded recombinant VHSV G protein provided the same level of protection against challenge as the classical vaccines (Fig. 4). This is the first report of protection against this viral disease provided by a recombinant antigen.

The protective response was accompanied by the synthesis of neutralizing antibodies. These were not heat-labile since they were resistant to the decomplexation

procedure (30 min at 45 °C). Nevertheless, some of the sera tested were negative in SNT. They were probably below the threshold of sensitivity or came from fish that did not respond to the vaccination. This variability in SNT response is recognized (de Kinkelin *et al.*, 1984; Olesen *et al.*, 1991).

Not all the vaccinated fish were protected against challenge with VHSV but the survival rates were significantly higher than in mock-immunized controls. The two immunization trials showed differences in survival rates with the same antigen (68.8% and 95.2%, 52.8% and 75.8%; Table 1). Differences in mortality were often observed either between different batches of fish or depending on the season at which the challenge was performed. We have observed over many years that mortality can vary between 20 and 95%. This results in significant variability of the RPS factor. It thus seems inappropriate to evaluate the efficacy of vaccines on the basis of this ratio alone. In this work, the RPS provided by the recombinant vaccine was shown to vary from 33.9 to 80.2% depending on the experiment (Table 1).

Differences in survival rates between vaccinated and non-vaccinated groups were higher for classical vaccines than for the recombinant one. The i.p. administration of non-infected Sf9 cells induced some protective response but no NAb synthesis. In previous experiments, it was also observed that the injection of *E. coli* or yeast crude extracts could provide a limited but significant level of protection against the VHSV challenge in the absence of neutralizing antibodies (unpublished results). Similarly, injection of fish with PBS or the supernatant from bacterial cultures was shown to increase the level of natural antibodies (Michel *et al.*, 1990), and it was also

demonstrated that such antibodies could interfere *in vitro* with virus multiplication (Gonzales *et al.*, 1989). It thus seems possible that these antibodies might play a role in the resistance to viral infection. Moreover, we cannot exclude an effect of the Suvaxyn adjuvant, although this oil-in-water emulsion would act rather as a carrier of antigen than as an immunostimulating adjuvant.

Nevertheless, the immunogenicity of the recombinant vaccine was demonstrated and in the first series the survival rate of vaccinated fish (68.8%) was clearly higher than that of controls (52.8%). To assess the efficacy of the vaccine, its effects should be compared with untreated controls. Since these controls are lacking from this set of trials, the survival rate of the control set of immersed fish (35.2%) would probably be the more suitable for this comparison. In this case, the  $\chi^2$  value would reach 19.6 ( $P < 0.005$ ) and the RPS factor 51.8.

The recombinant vaccine was effective by i.p. injection. This administration route guaranteed that a known dose of vaccine had been delivered to the fish. A 10-fold dilution of the recombinant vaccine failed to protect fish (data not shown). A more practical administration method, immersion, was tested and neither NAb synthesis nor protection was obtained. A possible explanation could be that the product did not penetrate into the fish. Indeed, the disruption of infected cells by one freeze-thaw cycle released large aggregates probably unable to migrate through the epithelia of the fish body surface. Moreover the concentration of antigen was low ( $< 100$  ng/ml) as compared with the successful IHN bath vaccination (Gilmore *et al.*, 1988). In this case, the concentration of the IHN G protein was estimated to be greater than 100  $\mu$ g/ml.

A recent vaccination assay *per os* was performed (data not shown) but failed in NAb synthesis and protective response as was previously found to be the case for inactivated and attenuated vaccines administered via this route.

The recombinant VHSV G protein expressed in Sf9 cells was recognized by anti-G neutralizing MAbs and was transported to the cytoplasmic membrane surface where the fusogenic activity was preserved. In immunization trials, the recombinant protein could induce NAb synthesis and protection in rainbow trout when administered by the i.p. route, but failed by more practical administration methods like balneation and *per os*. Nevertheless, this is the first report of a recombinant vaccine against VHSV and the potential commercial future of this product is in the process of being studied.

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