Recombinant protein fragments from haemorrhagic septicaemia rhabdovirus stimulate trout leukocyte anamnestic responses *in vitro*

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This work shows that viral protein fragments are capable of stimulating fish anamnestic immunological responses in leukocytes from the rainbow trout (*Oncorhynchus mykiss*, W.). Recombinant protein fragments of glycoprotein and nucleoprotein from the rhabdovirus causing viral haemorrhagic septicaemia of trout (VHSV), were cloned and expressed in *Escherichia coli*, *Yersinia ruckeri* (a trout pathogen) and *Saccharomyces cerevisiae*. The recombinant protein fragments stimulated anamnestic responses in leukocyte cultures derived from the anterior kidney of survivors of VHSV infection but not from uninfected trout. Two types of stimulatory anamnestic responses were detected, (i) a stimulation of lymphoproliferation as measured by thymidine incorporation assays and (ii) an increase in number, spreading and size of cells as determined by fibrin-clot and/or flow cytometry techniques. The evidence presented suggests that both adherent and non-adherent trout cell populations are needed for the immunological response to VHSV in this primitive vertebrate. The possible use of *in vitro* lymphoproliferation assays as a preliminary screening method for candidate fish vaccines prior to their testing *in vivo* is discussed.

Introduction

The mechanisms of the teleost fish anamnestic immune response to viruses (Jørgensen, 1982; De Kinkelin, 1988) remain largely unknown (Estepa & Coll, 1991*a*; Estepa *et al.*, 1991*b*). Teleost fish are some of the most primitive vertebrates to possess an adaptive immune system (Stet & Egberts, 1991). Although functional and morphological characteristics suggest the existence of both B and T lymphocytes, the properties of specific lymphocytes are not yet clear. Little is known of the fish histocompatibility system and only one major class of IgM-like immunoglobulin has been detected (reviewed in Sanchez & Coll, 1989). The rainbow trout (*Oncorhynchus mykiss*, W.) and the rhabdovirus causing viral haemorrhagic septicaemia (VHSV) constitute a model system to study the possible existence of antiviral anamnestic cellular responses in these primitive vertebrates.

The rhabodovirus VHSV causes up to 30% of the annual losses in the European production of trout. The virus possesses two membrane proteins, the glycoprotein (G; 65K) and the matrix protein (M_2; 20K), and several internal proteins, the polymerase, (L; 200K), a second matrix protein, (M_1; 24K), the phosphorylated nucleoprotein (N; 38K) and the nucleoprotein (Nx; 34K) immunologically related to N but mostly found associated with free nucleocapsids (Basurco *et al.*, 1991). The internal proteins, L and N/Nx and the viral RNA form the viral ribonucleoprotein.

Studies on the induction of protective anti-VHSV immunity *in vivo* have focused on the response to the external glycoprotein, G, because neutralizing antibody to VHSV shows exclusive specificity for this protein (Bernard *et al.*, 1983; Lorenzen *et al.*, 1990). Additionally, glycoprotein G stimulates trout leukocyte cultures from uninfected fish (Estepa *et al.*, 1991*a*), VHSV-immunized fish (Estepa *et al.*, 1991*a*), and survivors of VHSV infection (Estepa & Coll, 1991*b*). The internal nucleoprotein N/Nx was also found to have a stimulatory effect on leukocyte cultures from trout that had survived VHSV infection (Estepa & Coll, 1991*b*), suggesting that it also was important for the *in vivo* immunological response, as it has been demonstrated for a second salmonid rhabdovirus, the infectious haematopoietic necrosis virus (IHNV; Engelking & Leong, 1989; Engelking *et al.*, 1991; Gilmore *et al.*, 1988), for mammalian rhabdoviruses such as rabies virus (Ertl *et al.*, 1989) and for many other viruses.

An adaptive immune response to VHSV has been demonstrated (Jørgensen, 1982; De Kinkelin & Bearzotti, 1981; De Kinkelin, 1988; Basurco & Coll, 1992). However, because only about 50% of the VHSV infection survivors showed an increase in antiviral antibody titres following a second exposure to VHSV

(Olesen *et al.*, 1991), the present studies were focused on the possible cellular immune response. To study this we obtained VHSV glycoprotein G and nucleoprotein N and/or their fragments cloned in bacteria (*Escherichia coli* and *Yersinia ruckeri*) or yeast (*Saccharomyces cerevisiae*). Both proliferative responses (as measured by thymidine incorporation) and cellular morphological responses (as measured by fibrin-clot and flow cytometry techniques) were determined using leukocyte cultures from trout surviving the VHSV infection or from uninfected trout.

Methods

Virus purification. Five VHSV isolates from Spain (Basurco, 1990) and the VHSV-07.71 isolate from France (De Kinkelin & Bearzotti, 1981) were obtained from rainbow trout and grown in epithelial papillosum cyprini (EPC) cells. Viruses were purified by concentration
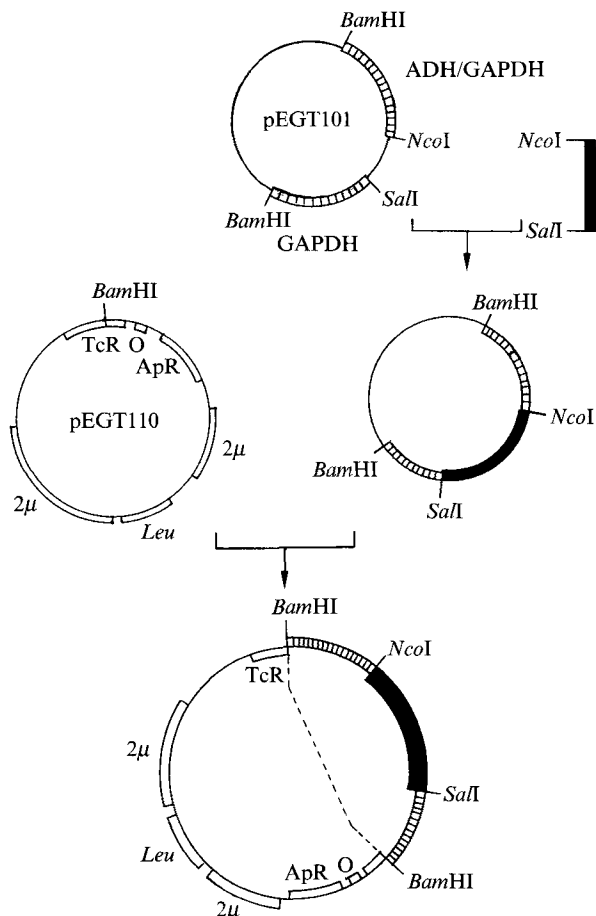

Fig. 1. Plasmid construction for cloning and expression of VHSV protein fragments in *S. cerevisiae*. The PCR-generated VHSV DNA fragments with *NcoI/SalI* restriction sites were inserted into unique restriction sites in the plasmid pEGT101. The *Bam*HI cassette was then excised and introduced into the *Bam*HI site of the pEGT110 yeast plasmid. *Leu*, leucine gene for selection of yeast transformants; ApR, ampicillin resistance; TcR, tetracycline resistance for selection in *E. coli*; 2 μ, yeast plasmid; O, replication origin.

and ultracentrifugation as previously described (Basurco & Coll, 1989 *a, b*; Basurco *et al.*, 1991). Viral proteins (N1 and G1, Table 1) were purified by PAGE under denaturing conditions and electroeluted (Estepa *et al.*, 1991 *b*).

Recombinant VHSV protein expression in bacteria. The N2 protein was cloned and expressed in *E. coli* as a fusion protein with β-galactosidase under the control of the *lac* promoter (Bernard *et al.*, 1990). The G7 protein was cloned in *E. coli* (Thiry *et al.*, 1991 *a, b*), using the plasmid pBT (Boehringer Mannheim). Both recombinant protein extracts (RPE) were used as a supernatant after disruption of the bacteria with a French press. Other G protein fragments were expressed using the pATH plasmid (Dieckman & Tzagaloff, 1985) as fusion proteins with anthranylate synthetase (TrpE) and their expression was induced with 3-(3-indolyl) acrylic acid. The G34 fusion protein was expressed with a signal peptide (eight amino acids) from a lipoprotein (lpp) of *E. coli* under the control of the *lac* promoter using the pINIIIc plasmid (Lunn *et al.*, 1986) and was targeted to the outer membrane of the bacterium (not shown). Recombinant protein expression was examined by comparing the RPE by PAGE in the absence or presence of the inducers and by immunoblotting with anti-VHSV polyclonal antibodies (PAbs) and/or monoclonal antibodies (MAbs).

To combine recombinant VHSV proteins with the properties of one of the best known vaccines in salmonids, that against the trout pathogen *Y. ruckeri* (Cipriano & Ruppenthol, 1987), the recombinant plasmids encoding G25, G27 and G34 (Table 1) were used to transform *Y. ruckeri* strain 09 by electroporation (Conchas & Carmiel, 1990). The resulting transformants were then plated in rich medium, the plasmid promoters were induced and VHSV recombinant protein expression was demonstrated by immunoblotting with anti-VHSV PAbs and/or MAbs. Cultures of *Y. ruckeri* transformants were centrifuged and the bacteria were lysed by incubation for 1 h at pH 9·5. The pH was adjusted to 7·0 and the solution incubated with 0·37% formaldehyde for 24 h. The cell debris was pelleted, resuspended in 0·15 M-sodium chloride in 0·05 M-sodium phosphate pH 7·4 and kept frozen until use.

Recombinant VHSV protein expression in yeast. The N (Bernard *et al.*, 1990) and G (Thiry *et al.*, 1991 *a*) cDNA sequences were amplified by PCR using specific amplimers. Amplimer 1 hybridized with the cDNA region corresponding to the N-terminal part of the protein and introduced an initiating methionine codon (ATG) contained within a *NcoI* (CCATGG) restriction enzyme site. The C-terminal part contained a stop codon (TGA) followed by a *SalI* site in amplimer 2. The PCR-generated DNA was recovered after agarose gel electrophoresis, digested with *NcoI* and *SalI*, and introduced into *NcoI/SalI*-linearized pEGT101 plasmid (Fig. 1). The plasmid pEGT101 (Eurogentec) is a pBR322 derivative harbouring a *Bam*HI expression cassette with the alcohol dehydrogenase/galactose phosphate dehydrogenase (ADH/GAPDH) hybrid promoter upstream of the *NcoI/SalI* restriction sites, followed by a GAPDH terminator. The *Bam*HI expression cassette was then excised and introduced into the unique *Bam*HI site placed in the tetracycline resistance (TcR) gene of the shuttle vector pEGT110 (described in Fig. 1). The resulting construct was used to transform *S. cerevisiae* strain DCO4 (*leu⁻*) by electroporation and a few recombinant clones were selected for studying the expression of VHSV proteins by immunoblotting with PAbs and/or MAbs (Thiry *et al.*, 1991 *a, b*). The complete G3 protein was expressed with a low yield. The fragment G4 devoid of the signal peptide and the membrane anchor domain could be obtained at ⩾ 80% purity in the pellet from the RPE after centrifugation because of its cytoplasmic aggregation. A mild treatment was applied to the pellets to solubilize the protein partially (0·15 M-NaOH for 5 s followed by neutralization with Tris–HCl pH 7).

Characterization of the VHSV recombinant proteins. RPE from bacteria or yeast were kept frozen at −20 °C until used, then analysed

Table 1. *ELISA recognition of purified and recombinant viral proteins*

Protein	Source	Plasmid	M_r* ($\times 10^{-3}$)	Anti-N 2D5	Anti-N 2C9	Anti-N 3E7	Anti-G† 1H10	Anti-G† 1F10
Whole virus	Whole virus			+	+	+	+	+
N1(aa 1–404)	Whole virus		38	+	+	+	−	−
N2(aa 1–404)	E. coli	pUC13	35	+	+	−	−	−
N3(aa 1–404)	S. cerevisiae	pEGT110	35	+	+	+	−	−
G1(aa 1–507)	Whole virus‡		65	−	−	−	+	+
G7(aa 1–507)	E. coli	pBT	53	−	−	−	−	−
G25(aa 86–418)	Y. ruckeri	pATH	40	−	−	−	ND§	+
G27(aa 86–277)	Y. ruckeri	pATH	21	−	−	−	−	±
G34(aa 86–277)	Y. ruckeri	pINIII	25	−	−	−	+	+
G3(aa 1–507)	S. cerevisiae	pEGT110	57	−	−	−	−	±
G4(aa 9–443)	S. cerevisiae	pEGT110	45	−	−	−	+	+

* M_r of the VHSV moiety of the fusion proteins.
† MAb 1H10 was neutralizing and 1F10 non-neutralizing (Sanz & Coll, 1992*b*).
‡ The viral protein was isolated by electroelution after SDS–PAGE of whole virus.
§ ND, not done.

by SDS–PAGE in the presence of 2-mercaptoethanol. The gels were stained with Coomassie blue and/or silver nitrate (Bio-Rad silver staining kit) or subjected to immunoblotting with anti-VHSV PAbs or MAbs (Sanz *et al.*, 1993). Protein content was measured by A_{280} determination, using ε values of 1 for protein extracts, of 1·6 for G protein and of 0·4 for N protein, calculated by the PHYSCHEM program of the PC gene package (Intelligenetics). RPE were used for experiments to reduce the effects of protein denaturation in purified preparations and because of their potential practical use as fish vaccines.

The identity of the VHSV recombinant proteins was confirmed by ELISA (Sanz *et al.*, 1993) using a panel of anti-VHSV MAbs (Basurco *et al.*, 1991; Sanz & Coll, 1992*a*, *b*). Briefly, 0·1 to 0·5 µg of viral proteins or RPE was bound per well (96-well plates; Dynatech) and reacted with the MAbs against VHSV. Plates coated with non-RPE from the parental strains were used as controls. Horseradish peroxidase-conjugated, rabbit anti-mouse immunoglobulin (Nordic) was used to develop the reaction between the 50- to 6250-fold diluted mouse ascites containing the MAbs and the protein-coated solid phase. An ascites pool from VHSV-immunized mice was used as a positive control (dilution of 1000-fold). Development with *o*-phenylenediamine was as previously described (Sanz & Coll, 1992*a*). The results of the ELISA were classified as positive (+) when the A_{492} in the RPE-coated wells was at least twofold greater than the A_{492} obtained with non-RPE-coated wells. Otherwise they were considered negative or equivocal (±; Table 1).

Immunization and challenge of fingerling trout with VHSV. Experiments were performed in 30 l tanks using dechlorinated water at 10 to 14 °C and biological filters (trout weighing between 0·5 and 2 g, 36 trout per tank, when possible duplicate tanks were used for each sample). Phytohaemagglutinin (PHA-M; Flow Laboratories), a strong *in vitro* immunostimulator (Estepa & Coll, 1992), was added as an adjuvant for bath-immunization. To immunize trout with each of the five isolates of VHSV attenuated in EPC cell culture (10^5 TCID$_{50}$/ml), each of the RPE (0·1 µg/ml of protein from G25, G27, G34, N3, G4, or N3+G4 RPE and 1 µg/ml of PHA) or each of the controls (0·1 µg/ml of RPE protein from bacteria or yeast and 1 µg/ml of PHA), the water volume in the tank was lowered to 2 l and cooled to 8 to 10 °C. Antigens were then added to the water and the trout exposed for 2 h (De Kinkelin &

Bearzotti, 1981) with strong aeration. The tanks were then refilled with water and the flow through the filters was restored. One month after the immunizations, all the trout in each tank were challenged with 10^6 TCID$_{50}$ VHSV-144/ml (about three times the LD$_{50}$) in 2 l for 2 h. Each tank was then filled with water and the flow through the filters was restored. The dead trout were removed from each tank daily, frozen at −40 °C and when later tested for VHSV by ELISA (Sanz & Coll, 1992) they were all found to be positive. Mortality was calculated for each tank of 36 immunized trout and expressed as a percentage (Johnson *et al.*, 1982).

Production of trout survivors of VHSV. About 400 trout weighing between 0·5 and 2 g were infected for 2 h at 12 to 14 °C with 10^6 TCID$_{50}$/ml of VHSV attenuated by 10 passages on EPC cells (Basurco & Coll, 1992). After 1 month, survival was between 10 and 30 % (four experiments). The trout surviving the infection were challenged 1 to 3 months later with VHSV isolated on EPC cells from infected trout (10^6 TCID$_{50}$/ml, for 2 h at 10 to 11 °C). After 1 month, between 50 and 80 % (four experiments) of the trout had survived this second infection (similar results were obtained by De Kinkelin, 1988), they showed no signs of haemorrhagic septicaemia and were used 12 to 14 months after the last VHSV challenge (100 to 200 g per trout).

Cell culture of trout kidney leukocytes. Leukocytes from individual trout kidneys were obtained as described before (Estepa *et al.*, 1991*b*; Estepa & Coll, 1992; Estepa & Coll, 1993*a*, *b*). The cell culture medium consisted of RPMI-1640 (Dutch modification, 290 mOsm/kg) with 2 mM-L-glutamine, 1 mM-sodium pyruvate, 1·2 µg/ml amphotericin, 50 µg/ml gentamicin, 20 mM-HEPES, 50 µM-2-mercaptoethanol, 10 % pretested fetal calf serum and 0·5 % pooled rainbow trout serum. After preparation of the cell suspension, 100 µl ($2·4 \times 10^4$ round cells) was pipetted into each well of a 96-well plate (Costar). The RPE diluted in sterile water were pipetted into each well in a maximum volume of 10 µl. The plates were then sealed in a 20×12 cm plastic bag, gassed with 5 % CO_2 in air, resealed and incubated at 20 °C for 10 days. The kidney cells were then fractionated into adherent and non-adherent cells (Estepa *et al.*, 1992*b*).

Colony and cellular survival assays. Thrombin (Miles) was added to the bottom of the wells to a final concentration of 2 to 4 NIH U/ml. Fibrinogen (A. B. Kabi) was included at 0·2 mg/ml in the cell

suspension (prepared as indicated above) before pipetting into each well. Clots formed within about 30 s after pipetting. Harvesting, fixing and staining were performed as previously described (Estepa & Coll, 1991 a, b). The cells were counted and classified with the aid of an eyepiece at 400 × (Estepa & Coll, 1992, 1993 a, b). Four clots were made for each point and one field was counted for each clot. Averages and standard deviations were calculated from the data obtained in three experiments.

Lymphoproliferation assays. One μCi of [^{3}H-*Me*]thymidine (60 Ci/ mmol; Amersham) was added in 25 μl of culture medium to 8-day-old liquid cultures which were then incubated for 2 additional days (Kaattari *et al.*, 1986). The cells were harvested onto glass fibre filters (Skatron), dried, placed in vials with aqueous scintillant (NCS; Amersham) and scintillation-counted (Beckman; Model EL 3800). Results from each fish were averaged from duplicates. Assuming a normal distribution, significant differences between treatments were defined at the 95% confidence level (average + two standard deviations).

Flow cytometry analysis. Liquid cultures were aspirated into a plastic tube with a Pasteur pipette, washed by centrifugation and resuspended in PBS containing 1% BSA and 0·1% sodium azide at 4 °C (DeLuca *et al.*, 1983). The samples were analysed in a Beckton-Dickinson FACScan using the program LYSYS II 1.0. Gate settings were adjusted to exclude cell aggregates and small particles. A total of 25000 cells was assayed for each sample.

Results

Characterization of the RPE

E. coli N2 RPE contained inclusion bodies with ≤ 10% of the fusion protein as determined by PAGE. Yeast N3 RPE contained 30 to 50% N by PAGE. MAbs 2D5 and 2C9 recognizing the purified VHSV N protein by ELISA also recognize N2 and N3 (Table 1). However, MAb 3E7 did not recognize N2 most probably owing to its altered conformation in an inclusion body and/or in the fusion protein.

E. coli G7 RPE contained ≤ 5% fusion protein by PAGE. Although it possessed the full G protein sequence, it was not recognized by any of the anti-G MAbs assayed (Table 1).

Several fragments of the glycoprotein G were expressed in *Y. ruckeri* as fusion proteins with TrpE (G25 and G27) or with a signal peptide (G34) from *E. coli* (Table 1). Before formalin treatment, these RPE contained ≤ 5% fusion proteins by PAGE. After formalin treatment the non-neutralizing MAb 1F10 recognized both G25 and G34 but reacted weakly with G27 and only the G34 protein was recognized by both neutralizing (1H10) and non-neutralizing (1F10) anti-G MAbs (Table 1).

Yeast G3 and G4 RPE contained monomeric proteins (by PAGE in the absence of 2-mercaptoethanol) of 57K and 45K with about 20 and 80% purity (by PAGE in the presence of 2-mercaptoethanol), respectively. Both G3 and G4 were recognized by non-neutralizing anti-G

MAb 1F10 but only G4 was recognized by neutralizing anti-G MAb 1H10 (Table 1).

Protection against VHSV challenge after immunization with RPE

To estimate the best possible protection under the experimental conditions used, bath-immunizations using cell culture-attenuated VHSV were performed. The percentage survival obtained by immunizing fingerling trout with five different VHSV isolates attenuated by passaging in the EPC cell line and challenged with one of them (VHSV-144) varied between 23 and 80% depending on the isolate (data not shown). Because of the low yield and/or lack of recognition by the MAbs (Table 1), the N2 and the G7 proteins made in *E. coli* were not further
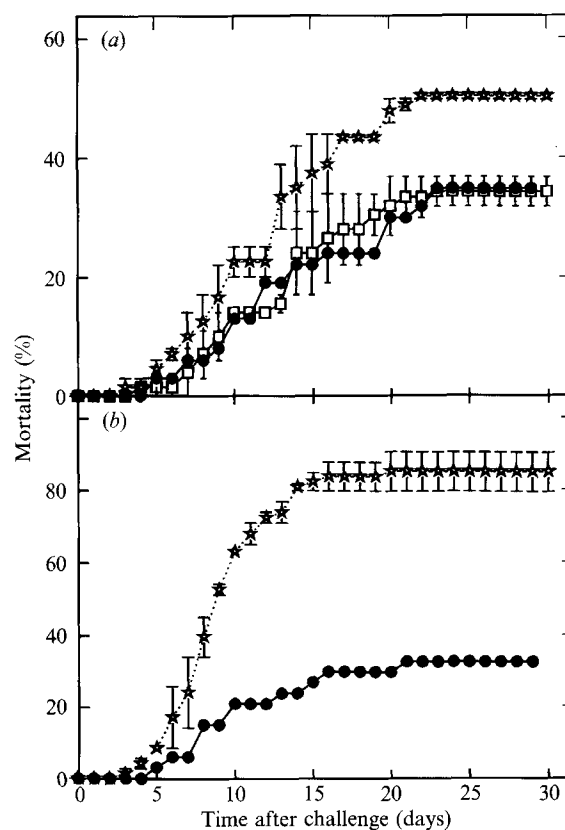

Fig. 2. Percentage mortality of fingerling trout immunized with *S. cerevisiae* RPE after challenge with virulent VHSV. Thirty-six trout each of 0·5 to 2 g body weight/tank were bath-immunized with the yeast G4 or N3 RPE in the presence of PHA and then challenged with VHSV in two experiments (*a* and *b*). Some of the results were averaged from two tanks per experiment and standard deviations are represented. Symbols: ●, fingerling trout immunized with 0·1 μg/ml of *S. cerevisiae* RPE G4 and 1 μg/ml of PHA; □, fingerling trout immunized with 0·1 μg/ml of yeast RPE N3 and 1 μg/ml of PHA; ☆, control trout, immunized with 0·1 μg/ml of yeast non-RPE and 1 μg/ml of PHA. By using trout immunized with G4 + N3 results similar to those shown in experiment (*a*) were obtained.

Table 2. *Thymidine incorporation into trout kidney leukocytes cultured in the presence of RPE from* Y. ruckeri

Trout number*	RPE				
	None	CY†	G25	G27	G34
1	0·3±0·1‡	0·1±0·1	0·1±0·1	0·2±0·1	0·1±0·1
2	0·4±0·1	0·5±0·3	0·4±0·1	0·4±0·2	0·6±0·2
3	0·2±0·1	0·3±0·1	**4·2±0·9**§	0·2±0·1	**3·2±1·2**
4	ND‖	0·9±0·1	**6·3±0·1**	**11·1±6·2**	**6·4±3·1**
5	0·6±0·1	1·0±0·2	**1·7±1·1**	0·9±0·4	**2·6±0·3**

* Trout numbers 1 and 2 were uninfected, numbers 3 to 5 were VHSV infection survivors.
† CY represents control non-RPE from *Y. ruckeri*.
‡ Results are shown as c.p.m. × 10^{-3}±S.D. of duplicates.
§ Results in bold typeface are ≥ CY+2×S.D.
‖ ND, not done.

tested *in vivo*. The survival rate of trout immunized with either the G25 or the G27 obtained in *Y. ruckeri* was about 7%. A survival rate of 15% was obtained by immunizing with the G34 from *Y. ruckeri* (data not shown). The best protection results were obtained by bath-immunization with recombinant proteins from *S. cerevisiae*. A maximum survival rate of 61% (experiment number 2) or 31% (experiment number 1) was obtained using G4 (Fig. 2). Survival of 32% and 23% was also obtained by bath-immunization with *S. cerevisiae* N3 and N3+G4 RPE, respectively (Fig. 2 and results not shown).

Lymphoproliferation assays

To optimize the concentrations of *Y. ruckeri* RPE for the lymphoproliferation experiments, RPE containing ≤ 5% of glycoprotein G fragments (G25, G27 and G34) were assayed with final total protein concentrations in cell culture from 0·4 to 4000 μg/ml, in leukocytes from trout that were uninfected or the survivors of VHSV infection. The optimal thymidine incorporation (maximal in survivors of VHSV infection and minimal in uninfected trout) was obtained with total protein concentrations between 100 and 4000 μg/ml. The G25 and G34 RPE stimulated lymphoproliferation with respect to non-RPE-containing leukocyte cultures in the three survivors of VHSV infection assayed (trout 3 to 5, Table 2) whereas G27 stimulated only those of trout 4. None of the *Y. ruckeri* RPE stimulated lymphoproliferation in uninfected trout (trout 1 and 2, Table 2).

In order to optimize the concentrations of yeast RPE for the lymphoproliferation experiments, RPE containing about 80% of G4 glycoprotein G fragment were tested at different final protein concentrations from 0·2 to 50 μg/ml, in uninfected trout or in survivors of VHSV infection. The optimal thymidine incorporation was obtained with 10 to 50 μg of protein/ml. The thymidine

Table 3. *Thymidine incorporation into trout kidney leukocytes cultured in the presence of RPE from* E. coli

Trout number*	RPE			
	None	CE†	N2	G7
6	3·4±0·8‡	2·0±1·0	ND§	1·0±0·1
7	1·0±0·3	1·0±0·5	2·0±0·3	1·0±0·3
8	11·6±0·6	10·7±1·0	ND	6·7±0·3
9	2·0±1·0	2·0±1·0	**9·0±1·0**‖	3·0±3·0
10	0·5±0·1	1·0±0·1	**2·0±0·1**	1·0±0·3
11	ND	6·4±1·7	**19·0±8·3**	4·8±1·8
12	7·0±0·2	6·4±0·3	**11·6±2·4**	**8·3±0·1**

* Trout numbers 6 to 8 were uninfected, numbers 9 to 12 were VHSV infection survivors.
† CE represents control non-RPE from *E. coli*.
‡ Results are shown as c.p.m. × 10^{-3}±S.D. of duplicates.
§ ND, not done.
‖ Results in bold typeface are ≥ CE+2×S.D.

incorporation obtained by using leukocytes from an uninfected trout was low at most of the concentrations tested (data not shown). In a series of experiments using several trout, lymphoproliferation was measured in the presence of RPE from *E. coli* or yeast containing either N or G proteins at 50 μg/ml. The *E. coli* N2 RPE stimulated lymphoproliferation from four (Table 3, trout 9 to 12) and G7 RPE from one (trout 12, Table 3) of the four survivors of VHSV infection used. None of the *E. coli* RPE stimulated lymphoproliferation in uninfected trout (Table 3, trout 6 to 8). The yeast N3, G3 or G4 RPE stimulated lymphoproliferation with at least a twofold increase in thymidine incorporation in three of the five survivors of VHSV infection tested (Table 4, trout 9 to 14). Individual trout responded differently; for instance, all five survivors of VHSV infection tested responded to at least one of the yeast RPE (Table 4), some responded only to N3 and G3 (Table 4, trout 9, 11 and 12), some only to G4 (Table 4, trout 10 and 14) and some responded to N3, G3 and G4 (Table 4, trout 11 and 12).

Table 4. *Thymidine incorporation into trout kidney leukocytes cultured in the presence of RPE from* S. cerevisiae

Trout number*	RPE				
	None	CY†	N3	G3	G4
6	3·4±0·8‡	7·0±5·0	8·0±0·3	2·0±0·1	14·0±3·0
7	1·0±0·3	2·0±0·1	1·0±0·5	ND§	ND
8	11·6±0·6	19·3±4·8	4·5±2·9	1·8±0·1	7·4±2·3
13	0·4±0·1	1·4±0·2	0·7±0·1	1·7±0·5	0·8±0·1
9	2·0±1·0	4·0±0·2	10·0±3·0‖	33·0±12·0	2·6±0·6
10	0·5±0·1	0·9±0·2	0·8±0·1	7·0±0·1	31·0±3·0
11	6·4±1·7	5·4±0·2	10·5±2·1	6·0±2·4	5·9±0·2
12	7·0±2·2	7·0±0·4	18·6±8·1	13·0±8·4	18·4±1·4
14	0·6±0·1	2·1±0·5	1·4±0·5	2·7±0·6	9·7±3·0

* Trout numbers 6, 7, 8 and 13 were uninfected, numbers 9 to 12 and 14 were VHSV infection survivors.
† CY represents control non-RPE from *S. cerevisiae*.
‡ Results are shown as c.p.m. $\times 10^{-3} \pm$ s.D. of duplicates.
§ ND, not done.
‖ Results in bold typeface are $\geqslant$ CY $+2 \times$ s.D.

Trout 12 was also a good responder to *E. coli* N2 and G7 RPE (Table 3). The leukocyte cultures from the uninfected control trout did not respond to the yeast RPE (Table 4, trout 6 to 8 and 13).

Culture in fibrin-clots

Culture of trout leukocytes by the fibrin-clot technique was used to investigate the morphology of the cells involved in the cellular responses and/or the possibility of stimulation of colony formation by the addition of the RPE. No stimulation of colonies was induced by the addition of any of the RPE assayed, in contrast with the colony stimulation obtained by the addition of PHA. Adherent cells were the cells most stimulated by VHSV, purified N1 and G1 (data not shown) and also by recombinant N2, N3, G7, G3 and G4 in trout leukocyte cultures obtained from uninfected trout as well as from survivors of infection (Fig. 3). The counts of adherent cells were higher in the cultures obtained from survivors of VHSV infection than in the cultures obtained from uninfected trout in cultures containing G7 or G3 (three
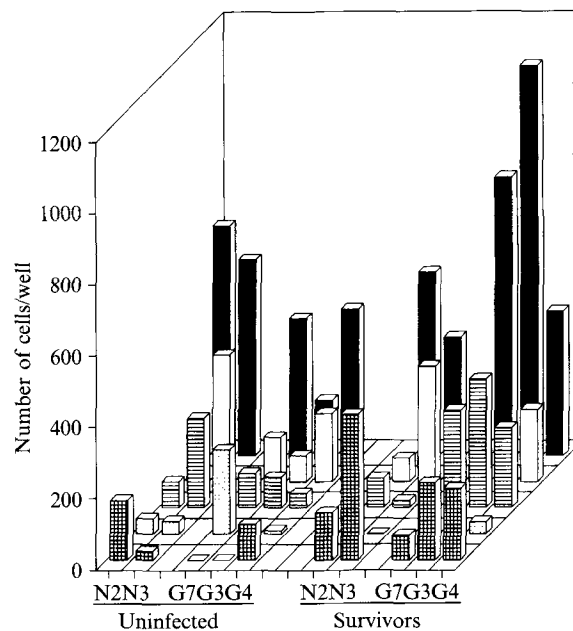
Table 5. *Stimulation of fractionated leukocytes from survivors of VHSV infection by the addition of RPE or PHA*

Kidney cells	Stimulus			
	None	N3	G4	PHA
All	0·5±0·1*	1·4±0·3†	9·7±3·0	26·2±8·3
Adherent	0·5±0·3	0·7±0·1	0·6±0·1	0·9±0·1
Non-adherent	0·4±0·3	0·8±0·1	0·8±0·2	1·3±0·1

* Results of thymidine incorporation assays are given in c.p.m. $\times 10^{-3} \pm$ s.D. of duplicates from three trout.
† Results in bold typeface are $\geqslant$ 'None' $+2 \times$ s.D.

Fig. 3. Morphological cell types stimulated in trout leukocyte cultures with purified VHSV proteins and RPE. Cells ($2·4 \times 10^4$/well in 100 µl) from uninfected trout or survivors of VHSV infection were cultured in the presence of 50 µg/ml of RPE from *E. coli* or *S. cerevisiae*. Averages from three trout are represented, standard deviations have been omitted for clarity. The counts obtained in control cultures made in the presence of non-RPE from *E. coli* or *S. cerevisiae* have been subtracted from the values shown. N2, N3, G7, G3 and G4 as shown in Table 1. Cell types were classified as previously described (Estepa & Coll, 1992). Symbols: ⊞, eccentric-nucleated cells; ◨, large-nucleated cells; ▤, multinucleated cells; □, lymphocytes; ■, adherent cells (macrophages and melanomacrophages).

experiments). The addition of recombinant N3 or G4 proteins also increased the average diameter of the adherent cells to 55 ± 15 µm (30 determinations).
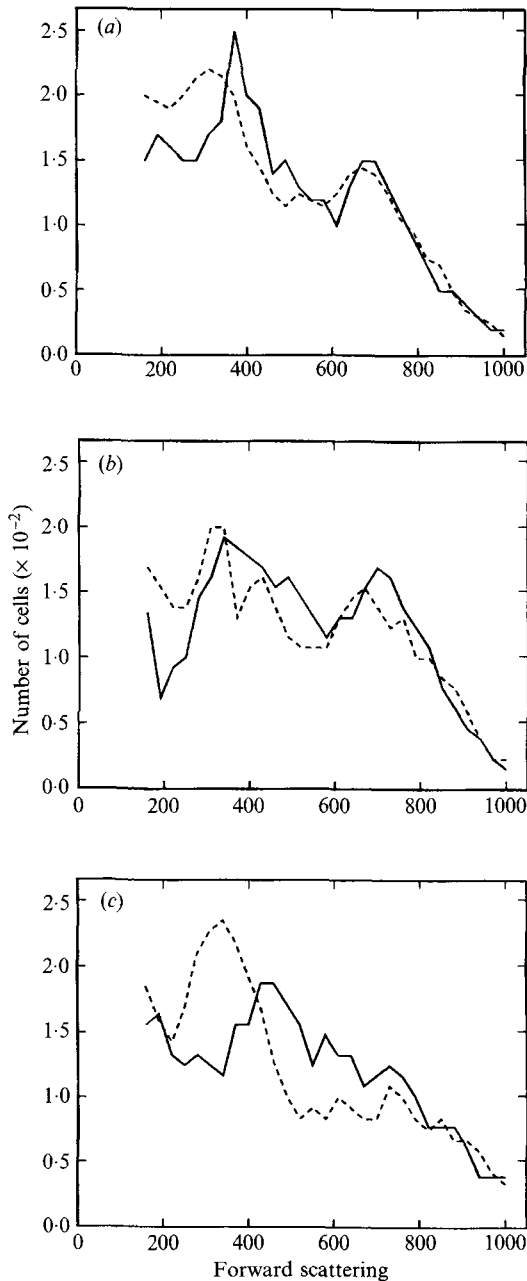
Fig. 4. Size profiles of leukocytes from uninfected trout and survivors of VHSV infection after culture in the presence of purified VHSV or *S. cerevisiae* RPE. Culture was for 2 weeks in the presence of 5 µg/ml of heat-killed, purified VHSV (*a*), 50 µg/ml of N3 (*c*) or G4 (*b*) RPE from *S. cerevisiae*. The profiles of cells cultured in the presence of non-RPE from yeast were similar whether uninfected trout or survivors of VHSV infection were used as donors (not shown). The total number of cells analysed was 25000 per assay. Leukocytes from uninfected trout, ‒‒‒; leukocytes from trout surviving VHSV infection, ——.

Lymphocytes were only apparent in cultures made in the presence of N2, N3 and G4. In general, eccentric-nucleated, large-nucleated and multinucleated cells appeared to reach greater numbers in VHSV survivors than in uninfected-trout cell cultures.

Fractionation of kidney into adherent and non-adherent cell populations

The kidneys from three survivors of VHSV infection were fractionated into adherent (33 %) and non-adherent (66 %) cells. Thymidine incorporation in the presence of RPE or PHA was about five- to 10-fold lower for the isolated adherent or non-adherent leukocyte populations than for the whole kidney (Table 5).

Flow cytometry analysis

To confirm the specific (uninfected/survivors) increase in size in the RPE-stimulated cultures, leukocytes from both types of trout were cultured in the presence of heat-killed VHSV (100 °C for 2 min), N3 and G4 yeast RPE and analysed by flow cytometry. In two different experiments the average size of the cells in trout leukocyte populations increased in the cultures from survivors of VHSV infection relative to those from uninfected trout. No differences were found between the types of leukocyte when cultured in the presence of non-RPE from yeast (data not shown). Small- and large-cell populations could be differentiated by their flow cytometry profiles. Protein G4 slightly stimulated the increase in size of both cell populations whereas protein N3 or whole denatured VHSV increased the size of only the smaller cell population (Fig. 4).

Discussion

This is the first report of the stimulation of immunological anamnestic responses (lymphoproliferative and cellular) in trout leukocytes from survivors of VHSV infection cultured with recombinant VHSV protein fragments. We also report preliminary observations on the partial protection of trout against VHSV infection by the use of recombinant protein fragments and report cloning and expression of VHSV proteins in *Y. ruckeri* or *S. cerevisiae* for the first time.

The stimulation indices for trout induced by the RPE were variable, because of the genetically heterogeneous population of fish used (Estepa & Coll, 1992; Kaatari *et al.*, 1986). Survivors of VHSV infection responded differently to each of the RPE and not every trout responded to all the RPE tested. However, the RPE did not elicit lymphoproliferative responses in the leukocytes of unchallenged trout (Tables 2, 3 and 4).

The minimum sequence of the glycoprotein G found to stimulate anamnestic lymphoproliferation was from amino acid residues 86 to 277 (G34). This region contains the most hydrophilic peak between two cysteine residues (amino acids 98 to 140), no potential glycosylation signals and it is recognized by neutralizing MAb 1H10 (Table 1). A total of six cysteine residues suggests that

this region is highly important in the conformation of the protein. Since conformation and hydrophilicity, but not glycosylation, seem to be involved in the neutralizing epitope(s) of G (Lorenzen *et al.*, 1993), it is possible that this region contains one of the neutralizing epitope(s).

Both lymphoproliferation (as estimated by thymidine incorporation) and spreading/size of trout leukocytes (as estimated by fibrin-clot culture and flow cytometry) were induced by N3 or G4 *S. cerevisiae* RPE. At least two cell populations seem to respond to G4 as suggested by the flow cytometry data (Fig. 4) but further studies will have to wait for the development of trout lymphocyte markers. On the other hand, the cell fractionation studies suggest that both adherent and non-adherent cell populations were needed for lymphoproliferation since there was no lymphoproliferation of these two populations when isolated (Table 5).

The cell types appearing in the fibrin-clot cultures in the presence of RPE showed the same morphologies as those reported for polyclonal mitogen-stimulated cultures (Estepa & Coll, 1991*b*) but there were no colonies (Estepa & Coll, 1992). The morphology does not appear to be antigen-specific, but all the cell types appeared to reach greater numbers in VHSV-infection survivors than in the uninfected trout (Fig. 3). Adherent cells were the most abundant type of cells in all these cultures. The greatest differences between uninfected and survivor trout were found in cultures containing G7 and G3 RPE. The morphology of the adherent cells stimulated by N3 or G4 (black bars in Fig. 3) was also similar to the cells stimulated by purified N and G VHSV proteins in leukocyte cultures from trout immunized against VHSV by injection (Estepa *et al.*, 1991*b*) or from survivors of VHSV infection (Estepa & Coll, 1991*b*). These adherent cells have been identified as macrophages and melanomacrophages because of their granular cytoplasm, erythrocyte phagocytosis (Estepa, 1992), acridine orange staining pattern (Estepa & Coll, 1992), staining with anti-trout immunoglobulin MAb (Estepa & Coll, 1991*b*), increase in size upon activation (Estepa *et al.*, 1991*b*), help in colony formation (Estepa & Coll, 1993*a*), susceptibility to VHSV (Estepa & Coll, 1991*a, b*; Estepa *et al.*, 1992*b*) and membrane presentation of VHSV epitopes after VHSV infection (Estepa *et al.*, 1992*a, b*). The role of macrophages/melanomacrophages in fish (Estepa & Coll, 1993*b*) is not yet fully understood.

The RPE obtained in *E. coli* as inclusion bodies were difficult to handle and characterize (as also found by Lorenzen *et al.*, 1993) because of their low yield and their minimal reaction with anti-VHSV protein MAbs (Table 1). This could be due to alterations in their conformation because of the formation of inclusion bodies and/or because of the presence of the fusion proteins needed for expression. Even though some of these problems could

be solved by cloning and expressing the VHSV proteins in *Y. ruckeri*, the percentage survival they induced *in vivo* was very low (most probably due to their low yield). Yeast RPE N3 or G4 were obtained at a higher yield and purity, were recognized by anti-VHSV MAbs, and when tested in bath-vaccine formulations were the only RPE that induced a level of protection (23 to 61 % survival) that although low was similar to the one obtained with attenuated VHSV (23 to 80 % survival) or reported by others (Bernard *et al.*, 1983; De Kinkelin & Bearzotti, 1981; Jørgensen, 1976, 1982; Engelking & Leong, 1989). Under the conditions used, addition of *S. cerevisiae* N3 RPE did not enhance the survival rate obtained with G4 in contrast to the results obtained with *E. coli*-expressed IHNV N RPE (Oberg *et al.*, 1991; Xu *et al.*, 1991). The data reported here, however, are of a preliminary nature since many other factors influence the outcome of immunization, for instance age/size of trout at immunization (De Kinkelin & Bearzotti, 1981), water temperature (Hetrick *et al.*, 1979), adjuvants (Estepa & Coll, 1991*b*), VHSV serotypes, VHSV virulence, and/or dosage. Further experiments to optimize the RPS obtained in this work are needed as this was not the focus of the present work.

The best specific proliferation (two- to fivefold increments) was obtained by the RPE from *S. cerevisiae* (N3 and G4) and *Y. ruckeri* (G25 and G34), the RPE that were recognized by the anti-VHSV MAb panel and induced the highest percentage survival *in vivo*. Also, the RPE (N2, G7, G27) that were non-stimulatory to the leukocyte cultures were only weakly recognized or not recognized at all by the anti-VHSV MAb (Table 1) and they had low *in vivo* percentage survival values (G27). These data suggest that the use of *in vitro* lymphoproliferation assays and recognition by anti-VHSV MAb may be useful as preliminary screening methods for candidate fish immune response modifiers or vaccines prior to their *in vivo* testing.

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