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Different Peptides from Hemorrhagic Septicemia Rhabdoviral Proteins Stimulate Leucocyte Proliferation with Individual Fish Variation

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Trout leucocytes from most of the survivors of viral hemorrhagic septicemia virus (VHSV) infections were capable of *in vitro* proliferation (T-like response) when cultured in the presence of short synthetic peptides designed from the G and the N cDNA-derived protein sequences of VHSV, a virus with substantial economic impact in trout farms. In contrast, no significant proliferative responses were obtained for the above-mentioned peptides from leucocytes obtained from either noninfected or genetically VHSV-resistant trout. However, since the anamnestic recognition of particular peptides (epitopes) of the G and the N protein by trout leucocytes varies largely within the outbred trout population, larger VHSV protein fragments were also tested. The finding that recombinant G and N fragments carrying multiple epitopes are recognized by the majority of the individual trout surviving VHSV infections and with higher stimulation indexes suggests that recombinant viral proteins could be used as vaccines given the outbred nature of the fish.

INTRODUCTION

Understanding the determinants of fish immunity to viruses could be one of the first steps toward effective prevention of their infections. The lack of a vaccine against fish rhabdoviruses makes these and related infections the most damaging in the international salmoniculture industry and emphasizes the importance of obtaining a noninfectious viral hemorrhagic septicemia virus (VHSV) vaccine from genetic manipulation (Estepa *et al.*, 1994; Lecocq-Xhonneux *et al.*, 1994; Lorenzen *et al.*, 1993) and/or synthetic peptide methods (Leong and Fryer, 1993).

The glycoproteins of mammalian rhabdoviruses (Coll, 1995) are the targets of neutralizing antibody (Ab) responses and are also implicated in cellular immunity at the proliferative (MacFarland *et al.*, 1984) and at the cytotoxic (Browning *et al.*, 1990) levels. Although neutralizing Abs to VHSV show exclusive specificity for the G (Bernard *et al.*, 1983; Engelking and Leong, 1989; Gilmore *et al.*, 1988; Lorenzen *et al.*, 1990; Olesen *et al.*, 1991), immunization with the N has also shown protection against *in vivo* challenge with VHSV (Estepa *et al.*, 1994), as occurs with infectious hematopoietic necrosis virus (IHNV), another fish rhabdovirus (Oberg *et al.*, 1991), and with mammalian rhabdoviruses (Lafon, 1993). Furthermore, both G and N are expressed in the membrane of infected cells as demonstrated by flow cytometry using monoclonal antibodies (MAbs) (Estepa *et al.*, 1992).

In VHSV infections it is known that: (a) most the VHSV survivors of a first infection also survive a second virus challenge (Basurco and Coll, 1992; DeKinkelin, 1988); (b) only 54% of survivors of the VHSV infection had detectable neutralizing Abs (Olesen *et al.*, 1991); (c) no anamnestic Ab responses follow a second virus challenge; and (d) recent attempts to immunize trout fingerlings with *Escherichia coli*-made VHSV proteins have failed (Estepa *et al.*, 1994; Lorenzen *et al.*, 1993) despite the production of trout anti-VHSV neutralizing Abs (Lorenzen *et al.*, 1993).

Salmonid trout have one of the earliest adaptive immune systems with largely unknown responses to major mortality-causing diseases such as rhabdoviral infections (Basurco and Coll, 1992; Estepa *et al.*, 1991). Their immune system appears to have lymphocytes, B-like cells, T-like cells or at least T-cell-receptor-containing cells (Partula *et al.*, 1994), antigen presenting cells (Vallejo *et al.*, 1991), restricted specific cytotoxic cells (Verlhac *et al.*, 1990), noncharacterized histocompatibility groups (Stet and Egberts, 1991), and only one class of IgM-like immunoglobulins (Sanchez *et al.*, 1989). Most of the studies of fish immune proliferative responses to viruses to date have been performed with polyclonal mitogens (Estepa and Coll, 1992b), whole virus (Chilmonczyk, 1978), isolated viral proteins (Estepa, 1992; Estepa and Coll, 1992a), or recombinant viral protein fragments (Estepa *et al.*, 1994). In mammalian/virus models proliferative responses occur after the presentation of a limited number of short viral pro-

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tein fragments in the membrane of the host infected cells, a mechanism that is reinforced in the anamnestic responses (Kontsekova *et al.*, 1992; Kutubuddin *et al.*, 1992). It is not known, however, whether and to what individual extent viral region(s) limitations exist in T-like responses in fish. The present studies focused on examining this possibility.

MATERIALS AND METHODS

Viruses

The VHSV 07.71 isolated in France (Le Berre *et al.*, 1977) from rainbow trout *Onchorynchus mykiss* (Walbaum) was grown in epithelial papillosum cyprine (EPC) cells and purified as described (Basurco *et al.*, 1991).

Peptides from G and N proteins of VHSV

A series of 15-mer peptides overlapping by 5 amino acids (aa) and spanning the G (Thiry *et al.*, 1991) and the N (Bernard *et al.*, 1990) cDNA-derived protein sequences of VHSV 07.71 were chemically synthesized (Chiron Mimotopes, Victoria, Australia). Each of the 15-mer peptides was numbered by the amino-terminal position in the protein sequence of its eighth aa. The first peptide of the G was 26, in order to exclude most of the signal peptide (aa 3–23), because it was cleaved in the mature protein, and the first peptide of the N was 9 due to synthesis requirements. The synthetic free peptides diluted in 5 m*M* HEPES, pH 7, were pipetted into each well in 10 μl (final concentration 1–4 μM) (Ertl *et al.*, 1989). Irrelevant peptides pA (TWKEYNHNLQLDDGTC), pB (PYRRDCVTTTVENED), pC (LANETGYRIVDSTDCNRD), pD (KTLRNRYYEDRDSYFC), pE (RAVKRGVVNLVKYGRC), pF (MAGKNQSQKKKKSTAC), and pG (RQQPRGGQAKKKKPEKC) were a gift from Dr. Pena at CISA, Valdeolmos.

Purified and recombinant VHSV proteins

G, N, and Nx VHSV proteins were purified by electroelution of preparative gel electrophoresis of purified VHSV after SDS and β-mercaptoethanol denaturation as described (Estepa and Coll, 1992a). Fragments G4 (aa 9–443) and N3 (aa 1–404) were cloned and expressed in the yeast *Saccharomyces cerevisae* DC04 as reported previously (Estepa *et al.*, 1994; Thiry *et al.*, 1991).

Production of trout survivors of VHSV infection

Outbred trout (0.5–2 g per trout) were infected for 2 hr at 12–14° with 10^6 $TCID_{50}$/ml of VHSV attenuated by 10 passages on EPC cells (Basurco and Coll, 1992). Under these conditions, trout survival was 10 to 30% (n = 3, n = number of experiments). The trout surviving the infection were challenged, 2 months later, with VHSV isolated on EPC cells from infected trout (10^6 $TCID_{50}$/ml, 2 hr, 10–11°). Between 5 and 24% (n = 3) of the initial number of trout survived both infections (DeKinkelin, 1988), showed no signs of VHS, and have been used 4–6 months after the last VHSV challenge (Enzmann and Konrad, 1993). Three different batches of trout (T) were prepared over 3 years; the first batch was assayed when trout weighed 200 g each (T1, T2, T3, T4, and T5), and the second (T16 to T23) and third (T24, T25, and T26) batches at 50–100 g of body weight per trout. As a control for manipulation errors, T16 to T19 were assayed in the absence of trout serum. Control noninfected trout were maintained in parallel aquaria (T6, T12, T13, T14, and T15).

T7 to T11 were VHSV genetically resistant trout (about 200 g of body weight each), obtained from an INRA (Jouy-en-Josas, France) genetic selection program which started in 1984 (Dorson and Torchy, 1993). The brothers of the descendants of several male trout selected for their resistance to VHSV challenge were bred for four generations by selecting each VHSV-resistant population. The trout used had never been exposed to VHSV and belonged to a population 95% resistant to VHSV but not to IPNV nor to IHNV infections as assessed by waterborne challenge of their brothers (gift of Dr. M. Dorson, Jouy-en-Josas).

Preparation of leucocytes from trout kidney

Leucocytes from the trout kidney (the mammalian equivalent of bone marrow) were obtained as described (Estepa and Coll, 1992b) from individual trout to avoid any mixed leucocyte reactions (Stet and Egberts, 1991). The cell culture medium was RPMI 1640 (Dutch modification, 290 mOsm/kg) with 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 1.2 μg/ml amphotericin, 50 μg/ml gentamicin, 20 m*M* HEPES, 50 μM mercaptoethanol, 10% pretested fetal calf serum, and 0.5% pretested pooled rainbow trout serum. The best combination of cell concentration, fetal calf serum, and pooled rainbow trout serum was selected to give the maximum polyclonal stimulation with phytohemagglutinin (PHA) under the conditions used. The stimulation obtained was completely dependent on the presence of trout serum. Leucocytes ($3–10 \times 10^4$ cells) in 100 μl were pipetted into each well of a 96-well plate (Costar, The Netherlands) containing 10 μl of the synthetic peptides. The plates were then sealed in a plastic bag (Vaessen, Schoemaker Indtal, S.A., Barcelona, Spain) gassed with 5% CO_2 in air, resealed, and incubated at 20°.

Leucocyte proliferation assays

The regular mammalian lymphoproliferation assay was adapted to trout kidney cells (Estepa *et al.*, 1994) with longer (optimal) incubation times because of the

lower temperature requirement (not shown). Because the proliferating cell populations are not totally characterized we have chosen to use the term leucocytes throughout the paper. One microcurie of [*methyl*-^{3}H]thymidine (60 Ci/mmol, Amershan, The Netherlands) was added in 25 μl of culture medium per well to 7-day-old cultures (Estepa, 1992; Kutubuddin *et al.*, 1992). The cells were harvested 2 days later with distilled water onto glass fiber filters (Printed Filtermat A, 1450-421) with a 96-well cell harvester (Tomtec, Orange, CT). The filters were then dried, enclosed in a bag, placed in scintillation Optiphase Hisafe II liquid (LKB, Loughtorough, England), and counted on a 1450 Microbeta scintillation counter (Wallac, Oy, Turku, Finland, and Pharmacia Iberica S.A.). Results were averaged from three to four 96-well plates per trout. Positive [^{3}H]thymidine incorporation controls were included (2 wells per plate, 6–8 per trout) by adding 1 μg/ml of PHA (Flow Lab, Ayrshire, UK). Background levels were included in cultures containing no peptides (2 wells per plate, 6–8 per trout). Average background levels ± standard deviations in cpm were 260 ± 56 (T1), 100 ± 20 (T2), 712 ± 54 (T3), 190 ± 120 (T4), 246 ± 75 (T5), 394 ± 203 (T20), 205 ± 55 (T21), 219 ± 97 (T22), 407 ± 73 (T23), 435 ± 114 (T24), 737 ± 203 (T25), and 279 ± 50 (T26) for survivors of VHSV infection (Figs. 1, 2, 3, and 4) and 250 ± 86 (T6) for noninfected control trout. Stimulation index (SI) was calculated by the formula cpm incorporated in the presence of peptide/background. Immunodominant peptides in each trout were defined for ≥99% confidence levels (≥mean SI of backgrounds + 4X standard deviations) by assuming a normal distribution of the SI values. For most trout SI < 3 were nonsignificant.

Assay of anti-VHSV trout serum antibodies

Trout serum from the survivors of VHSV infections was obtained at the time of the leucocyte assays. Trout antibodies anti-VHSV, -G4, or -N3 were measured by ELISA using plates coated with 2 μg of protein per well, 1G7 MAb anti-trout IgM (Sanchez *et al.*, 1991), and peroxidase-labeled rabbit anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). Other details were as described previously (Sanz and Coll, 1992). Neutralizing antibodies were estimated by incubating threefold serial dilutions of trout serum with 100 plaque-forming units of VHSV in the presence of 10% fresh trout serum (source of complement) at 4° overnight. The next day the mixtures were added to EPC monolayers in 96-well plates, adsorbed during 1 hr at 4°, and washed with cell culture medium. After overnight incubation at 14°, the cultures were fixed with cold methanol for 10 min and dried. Staining of the VHSV foci was with MAb 2C9 (anti-N), peroxidase-labeled rabbit anti-mouse IgG, and diamino-benzidine (DAB), as described (Sanz and Coll, 1992).

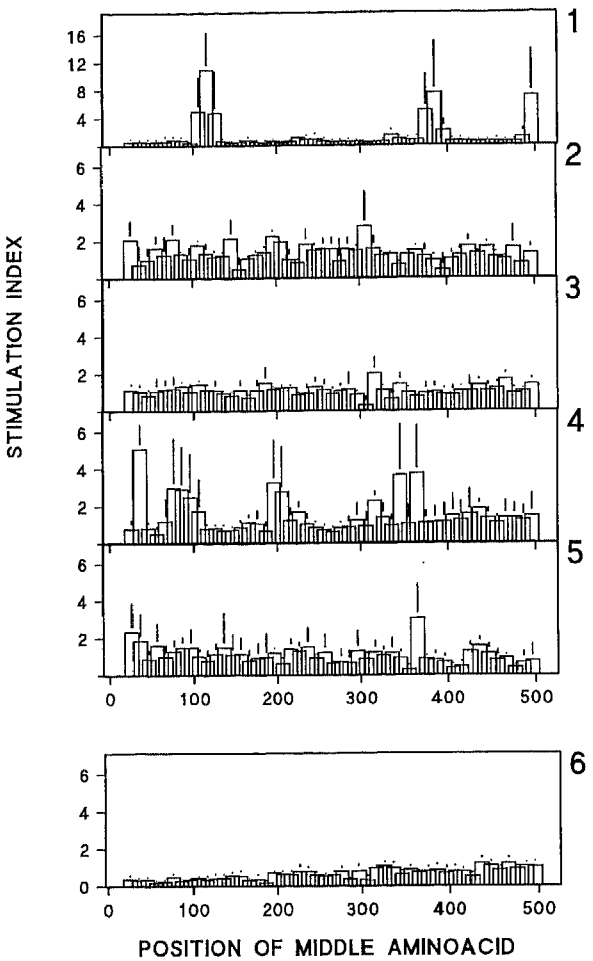

FIG. 1. SI induced by G peptides in leucocytes from the first batch (T1 to T5) of trout survivors of VHSV infection. Vertical bars represent the averages from tetraplicates + standard deviations of the SI for each peptide. *Y* axis values (SI) in T1 are higher than in the rest of the trout. Trout 6 was a control noninfected trout; similarly, in the four other noninfected trout tested (T12 to T15), none of the SI values were significant (not shown).

RESULTS

Trout leucocyte proliferation induced by G and N 15-mer peptides

Each individual trout survivor of VHSV infection showed a different profile of peptide response independent of the batch of survivors used (Figs. 1, 2, 3, and 4). The immunodominant G peptides were 106, 116, 126, 376, 386, and 496 (T1); 306 (T2); 36, 76, 86, 96, 196, 206, 346, and 366 (T4); 366 (T5); 66, 306, and 426 (T20); 456 (T21); 446 (T22); 36, 186, and 306 (T23); 246 and 306 (T24); and 306 (T25). The immunodominant N peptides were 9, 109, and 119 (T1); 49 and 229 (T4); 39, 279, and 399 (T20); 19, 79, 379, and 399 (T21); and 129, 169, 239, and 369 (T23). Some trout, T1, T23, and T24, were high responders both in number of peptides and in magnitude of SI, but others, T3 and T26, were low responders. Most

of the SI were ≤7, except those of T1, T23, and T24 obtained with the G peptides, which were higher than the rest (SI ≥ 11). Specificity of the response was shown by the fact that most of the G or N peptides tested (except the peptides mentioned above) did not stimulate leucocyte proliferation in any of the survivors of VHSV infection tested. As a further test for response specificity, no discrete responses were obtained to particular peptides when trout serum was omitted from the medium in leucocyte cultures from survivors of VHSV infection (T16 to T19, $n = 4$).

Specificity of the response was further tested on noninfected trout and these gave a nonsignificant proliferative response ($n = 5$) for the peptides tested (Figs. 1 and 3

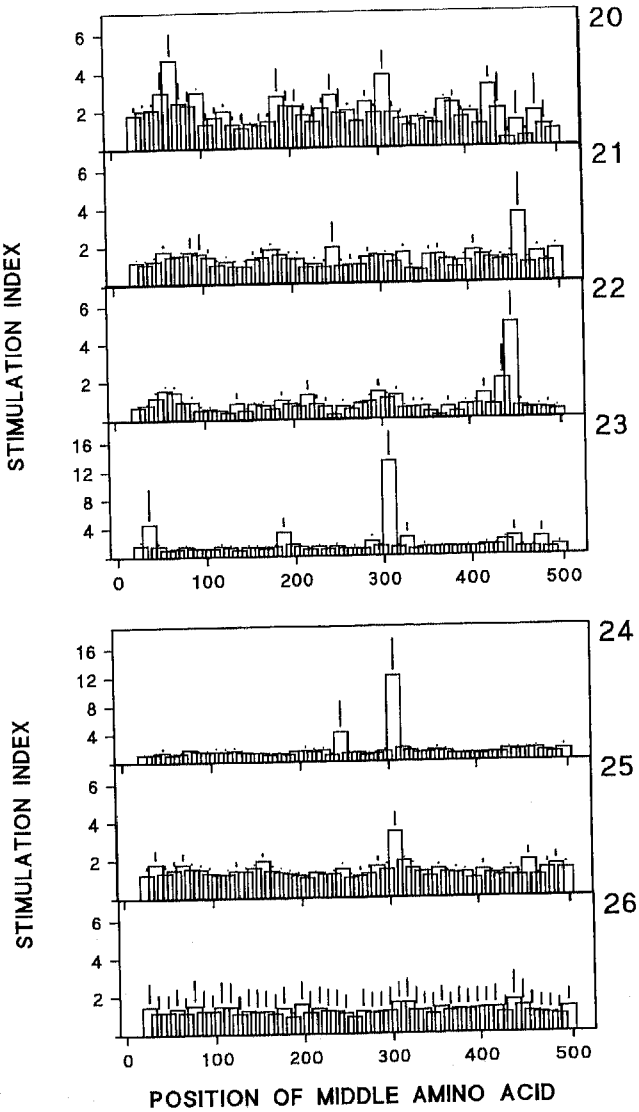

FIG. 2. SI induced by G peptides in leucocytes from the second (T20–T23) and third (T24–T26) batches of trout survivors of VHSV infection. Vertical bars represent the averages from triplicates + standard deviations of the SI from each peptide. *Y* axis values (SI) in T23 and T24 are higher than in the rest of the trout.

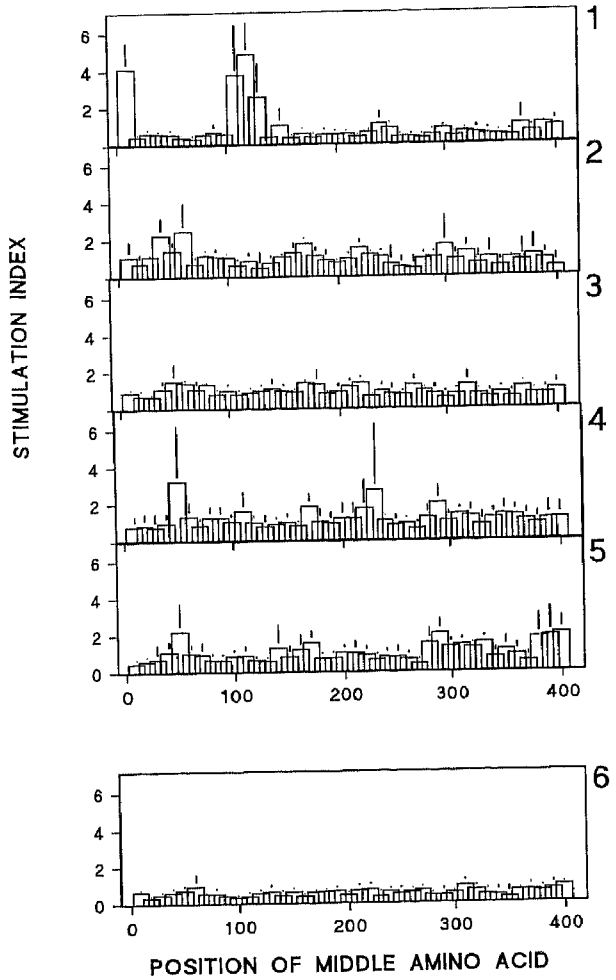

FIG. 3. SI induced by N peptides in leucocytes from the first batch (T1 to T5) of trout survivors of VHSV infection. Vertical bars represent the averages from tetraplicates + standard deviations of the SI for each peptide. Trout 6 was a control noninfected trout; similarly, in the four other noninfected trout tested (T12 to T15), none of the SI values were significant (not shown).

show T6 as a representative result). In further contrast to the data obtained with leucocytes from survivors of VHSV infection, for the proliferation data obtained with leucocytes from genetically VHSV-resistant trout ($n = 5$), the SI was not significant for the peptides tested (not shown). Although it was not possible to detect any mitogenic effect of the peptides over leucocytes from noninfected trout ($n = 5$) or from trout genetically resistant to VHSV ($n = 5$) (not shown), it cannot be excluded that some positive results could, perhaps, be found by using a larger number of trout, which would probably correspond to the natural survival of 10–30% after a first VHSV infection (DeKinkelin, 1988).

The high standard deviations observed among some of the highest SI induced by the peptides (Figs. 1, 2, 3, and 4) are most probably due to the relatively low cell concentrations required for these assays in trout. Lower

standard deviations were observed when larger protein fragments and/or PHA were used under the same cell culture conditions (not shown), suggesting the existence of a larger number of cellular clones responding to polyclonal mitogens and of a limited number of viral peptide responding cells in the assay. The level of SI obtained is about the order of magnitude obtained with other salmonids in related experiments (Tatner, 1990).

Anamnestic trout leucocyte proliferation induced by purified VHSV and recombinant G and N proteins

Because of the individual trout variability and the relatively low SI of the responses obtained by using short viral peptides, viral protein fragments were assayed to test the influence of the simultaneous presence of all the epitopes that could possibly stimulate anammestic leucocyte proliferation.

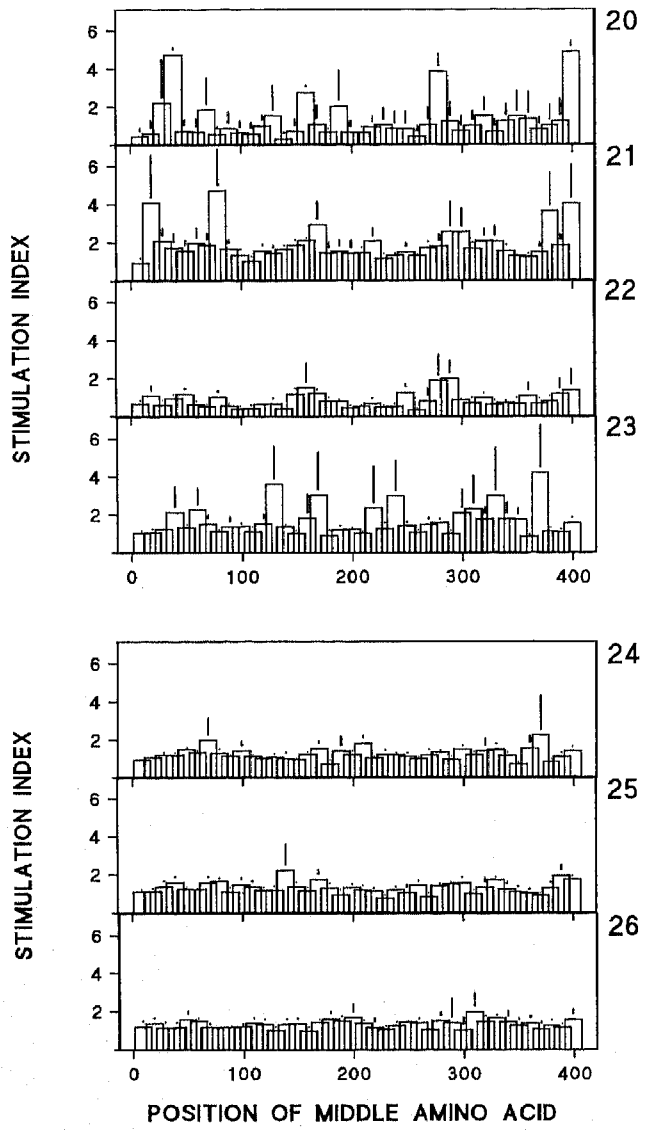

FIG. 4. SI induced by N peptides in leucocytes from the second (T20–T23) and third (T24–T26) batches of trout survivors of VHSV infection. Vertical bars represent the averages from triplicates + standard deviations of the SI from each peptide.

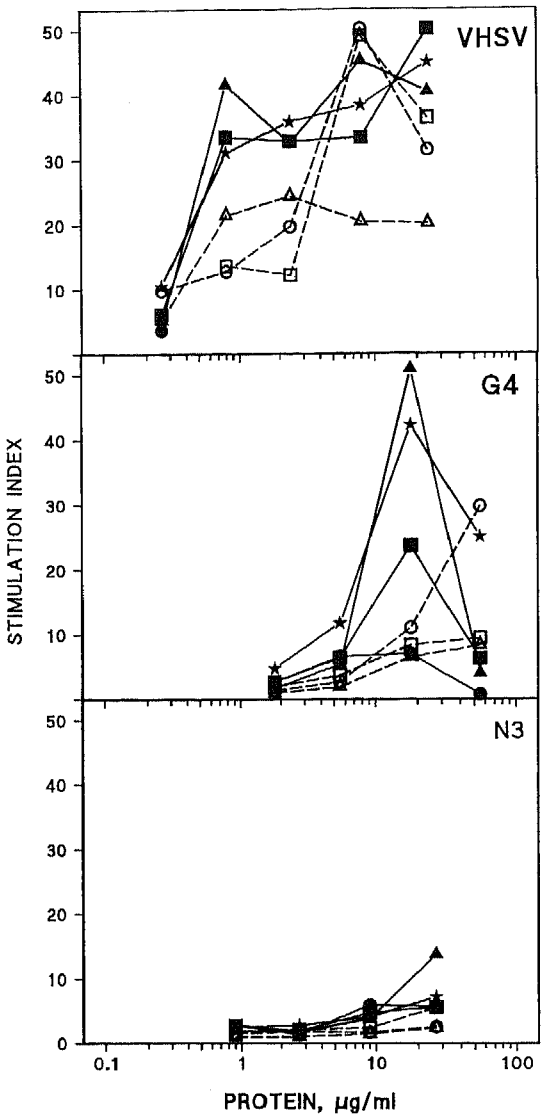

FIG. 5. SI of leucocytes from trout survivors of VHSV infection induced by purified VHSV, by G4, and by N3. Trout survivors of VHSV infection were the same donors as in Figs. 2 and 4. Trout from the second batch were ●, T20; ■, T21; ▲, T22; and ★, T23. Trout from the third batch were □, T24; △, T25; and ○, T26.

By using purified VHSV as an immunostimulant, a maximal SI of 35–40 was obtained for the second batch of trout (T21, T22, and T23) in the range 1 μg of VHSV protein per milliliter compared to a maximal SI of about 25–50 for the third batch of trout (T24, T25, and T26) in the range 10 μg of VHSV protein per milliliter (Fig. 5). Every individual trout tested ($n = 6$) showed a significant stimulation of anamnestic leucocyte proliferation by VHSV.

TABLE 1

Titers of Anti-VHSV Trout Serum Antibodies

	ELISA			
Trout	Anti-VHSV	Anti-G4	Anti-N3	Neutralizing
T3	—	—	—	—
T4	75	100	—	—
T5	75	250	—	—
T21	50	250	100	—
T22	—	—	200	—
T23	25	500	100	360
T24	25	225	200	—
T25	—	—	100	—
T26	25	—	100	—
TSNI	—	—	—	—
TSN	1350	450	1350	>2160

Note. The trout serum were assayed by three-fold serial dilutions. The ELISA titer was defined as the inverse of the dilution needed to obtain an A492 nm of 0.7. Neutralization titer was defined as the inverse of the dilution to reduce to half the number of DAB-positive foci. —, titer <25 or not detectable; TSNI, trout serum from a noninfected pool of five trout (negative control); TSN, trout serum from a VHSV survivor trout with a high neutralization titer from Jouy-en-Josas (positive control).

In contrast to the results obtained with the peptides, a significant SI was obtained for every individual trout tested ($n = 7$) with recombinant G or N viral proteins. About 20 μg of G4 per milliliter have to be used to achieve a maximal SI value. Trout to trout variation of the level of SI induced by G4 was between 5 and 50, with the trout from the second batch showing the highest SI of all the trout tested (Fig. 5). In contrast to the results obtained with G4, the highest level of SI induced by N3 recorded was only between 5 and 15.

Simultaneous assays of seven irrelevant peptides (see Materials and Methods) at different concentrations (0.1 to 40 μM) did not yield any significant SI in the trout tested (T20 to T26) (not shown). Again, noninfected trout ($n = 5$) were not capable of significant stimulation of leucocyte proliferation with the VHSV G or N proteins tested (not shown).

Anti-VHSV antibodies in the serum of survivors of VHSV infection

To preliminarily study a possible correlation between T-like and B epitopes, anti-VHSV, -G4 and -N3 antibodies were estimated in the same trout that was used for the proliferation experiments.

Controls were obtained by using pooled serum from five noninfected trout (undetectable antibody titers) and a serum from a 3-year-old trout surviving multiple VHSV infections with a pretested 50,000 neutralization titer by a 100 PFU plaque assay (Jouy-en-Josas). Table 1 shows that anti-VHSV titers in the trout population tested varied from undetectable values to 75, anti-G4 titers from undetectable to 500, and anti-N3 titers from undetectable to 200. Of the nine trout tested, the three that had undetectable antibody titers against VHSV also had undetectable antibody anti-G4 titers but two of them showed positive anti-N3 titers (T22 and T25). T3 had no detectable antibody titers of any kind. The highest anti-G4 antibody titer was recorded in T23, which was also the only trout of those tested who showed a detectable antibody neutralization titer.

DISCUSSION

The leucocytes from most of the survivors of VHSV infection in an outbred trout population proliferated *in vitro* (T-like response) when cultured in the presence of different peptides from the G and the N proteins from the VHSV.

This first analysis of individual apparent selection of viral peptides for leucocyte proliferation, not yet described by pepscan assay in fish, showed a high trout to trout variation both in the extent of SI of each peptide and in the kind of peptides stimulating each individual trout. Thus, the VHSV survivors of an outbred trout population (5–24% final survival) could be divided into viral peptide high SI responders (T1, T23, and T24), medium SI responders, and low SI or nonresponders (T3 and T26). The low percentage of high responders 4–6 months after infection and the low and highly variable anti-VHSV antibody titers (Table 1) could be explained because the highest trout anti-VHS antibody responses only last for about 2 months after infection (Enzmann and Konrad, 1993). Most probably a higher percentage of high responders would be present in field surveys in which natural survivors of VHSV infections are continuously exposed to VHSV. On the other hand, by taking into account all the data generated from individual survivors of VHSV infection ($n = 12$), there do not appear to be immunodominant regions in the G or the N proteins, perhaps except for that defined by the G peptide 306 ($n = 5$). On the contrary, the results obtained with rabies (MacFarland *et al.*, 1984) or with vesicular stomatitis virus (VSV) (Burkhart *et al.*, 1994) defined three similar T-helper regions for both viruses (Fig. 6); however, in both of these cases inbred mice populations were used.

The individually restricted selection of viral peptides that stimulates trout leucocyte proliferation suggests the existence of an individual peptide-presenting capacity, similar to that in higher vertebrates (Kutubuddin *et al.*, 1992; MacFarland *et al.*, 1984). Although the existence of processing and membrane presentation of small peptides has been demonstrated for fish external proteins (Vallejo *et al.*, 1991) and because the peptides added could, alternatively, stimulate the release of some factor(s) responsible for the observed leucocyte prolifera-

tions (Rogel-Gaillard *et al.*, 1993), only further experimentation could demonstrate that a similar mechanism is being used in the currently observed leucocyte proliferations. Formal proof that viral peptides could maintain trout leucocyte anamnestic proliferation will have to await the development of peptide-responding clones and the study of their T-cell receptor characteristics (Partula *et al.*, 1994).

On the other hand, the individual variation of the peptides that induce leucocyte proliferation (even when taking into account that different peptides could have different concentration-dependent responses, that different trout could have different trout serum optima for their leucocyte proliferation, that the size of the trout population assayed is small, and that not every possible peptide has been tested) suggested that viral proteins containing all or some of the regions defined by the peptides (epitopes) should be assayed. As foreseen by the results of the pepscan and of the mammalians, in which proteins having more epitopes induce more cellular clones to proliferate, the recombinant G4 stimulated leucocyte proliferation in every trout and with a higher SI than the synthetic peptides. However, no correlation was found between the highest G peptide responders (T1, T23, and T24) (Figs. 1 and 2) and the highest G4 responders (T21, T22, T23, and T26) (Fig. 5), suggesting that, among other possibilities, conformation might also be important in stimulating trout leucocyte proliferation. The finding that recombinant G4 and N3 carrying multiple epitopes are highly recognized by all the individual trout of an outbred population appears to be quite advantageous if vaccines are to be prepared containing the trout to trout variable immunodominant T-like epitopes in addition to the B epitopes.

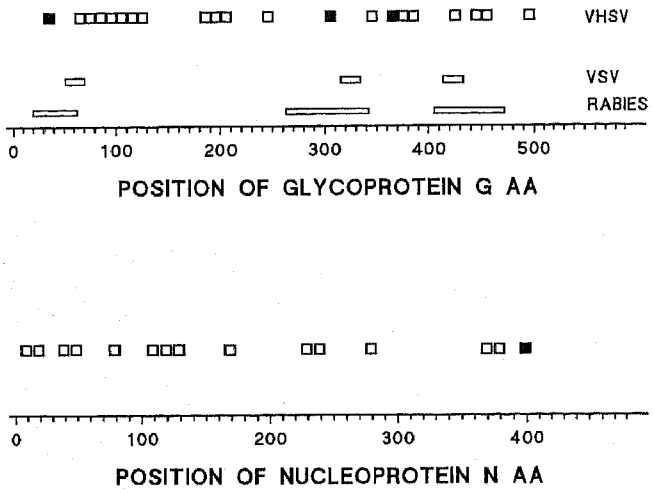

FIG. 6. Comparison of immunodominant regions in VHSV/trout and rables/VSV/mice. □, regions of VHSV defined by the peptides showing the highest (immunodominant) leucocyte proliferation in at least one of the survivors of VHSV infection tested have been plotted against their aa position. ■, regions of VHSV showing immunodominance in two outbred trout (G peptides 36 and 366 and N peptide 399) or in five outbred trout (G peptide 306). ▭, G from rabies regions showing lymphoproliferation in inbred mice (MacFarland *et al.*, 1984). ▭, VSV T-helper epitopes of G from VSV in inbred mice (Burkhart *et al.*, 1994).

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