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Mapping of the G and N regions of viral haemorrhagic septicaemia virus (VHSV) inducing lymphoproliferation by pepscan

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In this study we have used leucocyte proliferation assays in the presence of synthetic peptides designed from G and N cDNA-derived protein sequences of the viral haemorrhagic septicaemia virus (VHSV) to localize the viral protein region(s) involved in the stimulation of trout immune responses.

The VHSV 07.71 which was 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 previously (Basurco *et al,* 1991).

A series of 15-mer peptides overlapping by 5 amino acids and spanning the G (Thiry *et al,* 1991) and 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 were numbered by the amino terminal position of its 8th amino acid (aa) in the protein sequence. The first peptide of G was number 26, which excluded most of the signal peptide (aa 3-23) because it was cleaved in the mature protein. The first peptide of N was number 9 because of synthesis requirements. The peptides were diluted in 5 mM Hepes, and 10 µl were pipetted into each well (final concentration 1–4 µM).

The VHSV proteins were purified as described by Estepa and Coll (1992). Fragments were cloned and expressed as previously described (Thiry *et al,* 1991; Estepa *et al,* 1994). G4 (aa 9-443) and N3 (aa 1-404) were expressed in the yeast *Sacharomyces cerevisae* DC04.

About 400 trout (0.5–2 g per trout) were incubated for 2 h at 12–14°C with 10^6 $TCID_{50}$/ml of VHSV that had been attenuated by 10 passages on EPC cells (Basurco and Coll, 1992). Trout survival a month after the exposure was 10–30% ($n = 4$; number of experiments). The trout that survived the initial infection were challenged 1 to 3 months later with VHSV isolated on EPC cells from infected trout (10^6 $TCID_{50}$/ml, 2 h, 10–11°C). After a month, 5–24% ($n = 4$) of the initial trout population survived both infections. These fish showed no signs of

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VHS and were used 2–4 months after the last VHSV challenge (50–100 g per trout).

Leucocytes were taken from the kidney and were cultured as described previously (Estepa *et al,* 1991; Estepa and Coll, 1992). One μCi [Methyl ^{3}H]thymidine (60 Ci/mmol, Amersham, the Netherlands) was added to 25 μl of culture medium per well to the cultures on day 8 (Chilmonczyk, 1978). The cells were harvested 2 d later with distilled water onto glass fiber filters (Printed Filtermat A, 1450-421) with a 96-well cell harvester (Tomtec, Orange, CT, USA). The filters were then dried, enclosed in a bag, placed in scintillation Optiphase Hisafe II liquid (LKB, Loughtorough, UK) and counted on a 1450 Microbeta scintillation counter (Wallac, Oy, Turku, Finland and Pharmacia Iberica SA). The results were averaged from 3–4 plates per trout. Positive controls were obtained in duplicates (2 wells per plate, 6–8 total measurements per trout) containing 2 μg/ml of phytohemaglutinin (PHA, Flow Lab, Ayrshire, UK); background incorporation was also obtained in duplicate control cultures (2 per plate) containing no peptides. The stimulation index (SI) was calculated as counts per minute incoporated in the presence of peptide/background.

Each trout surviving VHSV infection showed a different profile of peptide responses induced by glycoprotein G peptides (fig 1). The immunodominant peptides were peptides 106, 116, 126, 376, 386 and 496 for trout 1; 196 and 306 for trout 2; 316 and 466 for trout 3; 36, 76, 86, 96, 196, 206, 346 and 366 for trout 4; and 26 and 366 for trout 5. Some trout were high responders (both in number of peptides and magnitude of SI) such as trout 1 and 4, but other trout were low responders, such as trout 2 and 3. The highest SI values were ≤ 5, except for trout 1 which had higher SI than the others (highest SI ≥ 11). The specificity of the response was tested on non-infected trout and no significant proliferative response (SI ≤ 1, $n = 5$) was found for any of the peptides tested (fig 1 shows trout 6 as a representative result). This confirms the data previously obtained for recombinant G fragments (Estepa *et al,* 1994).

Each trout surviving VHSV infection showed a different profile of peptide responses induced by nucleoprotein N peptides (fig 2). Peptide 49, however, was immunodominant in 3 of the 5 trout assayed. The immunodominant peptides were peptides 9, 109, 119 and 129 for trout 2; 39, 59, 169 and 299 for trout 2; 49 for trout 3; 49, 229 and 289 for trout 4; and 49, 289, 379, 389 and 399 for trout 5. Some trout were high responders (both in number of peptides and magnitude of SI) such as trout 1 and 4. Some trout were low responders like trout 3. The highest SI values ranged from 3 to 4 except for those of trout 1 which were higher than the rest (≥ 4). Specificity of the response was further tested on non-infected trout and no significant proliferative response (SI ≤ 1, $n = 5$) was found for any of the peptides tested (fig 1, trout 6). This confirms the data previously obtained for recombinant nucleoprotein N (Estepa *et al,* 1994).

Since there was a great deal of individual variability in the responses obtained using short viral peptides, longer viral protein fragments were tested in order to cover all the possible regions that might stimulate leucocyte proliferation. The SI ranged from 2.3 to 8.3 with the VHSV infection survivors. Table I shows that the highest SI were obtained for recombinant G4, which contained most of the peptides showing leucocyte proliferation.

This first analysis of apparent selection of viral peptides, not yet described by pepscan assay in fish, showed a high trout-to-trout variation in the observed responses, in both the kind of peptides stimulating each trout and the extent of the stimulation. These results suggested the need to assay for viral proteins containing all or some of those regions. This possibility appears to be quite advantageous if vaccines are going to be prepared containing the trout-to-trout vari-

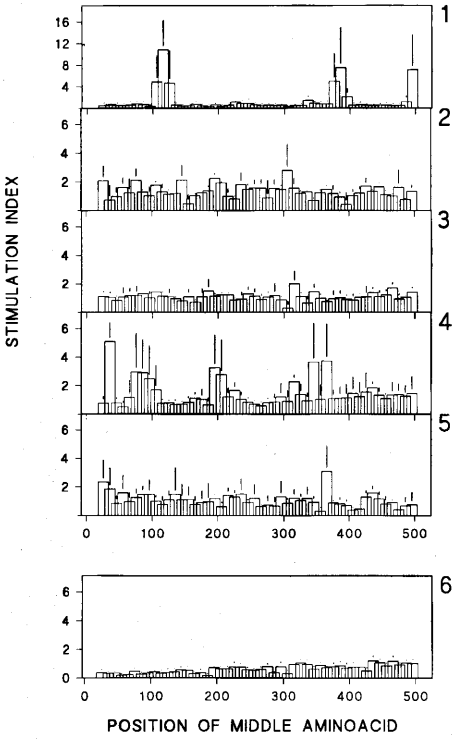

Fig 1. SI of trout leucocytes from survivors of VHSV infection induced by glycoprotein G peptides. Vertical bars represent the averages from tetraplicates ± standard deviations from 5 trout survivors of VHSV infection (numbers 1–5). Note that the *Y* axis values (SI) in trout 1 are higher than for the other trout. Trout number 6 was a non-infected control; similarly 4 other non-infected trout tested showed SI ≤ 1 for all the peptides tested (not shown). Backgrounds in counts per min (cpm) were: 260 ± 56 for trout 1; 100 ± 20 for trout 2; 712 ± 54 for trout 3; 190 ± 120 for trout 4; 246 ± 75 for trout 5; and 250 ± 86 for trout 6.

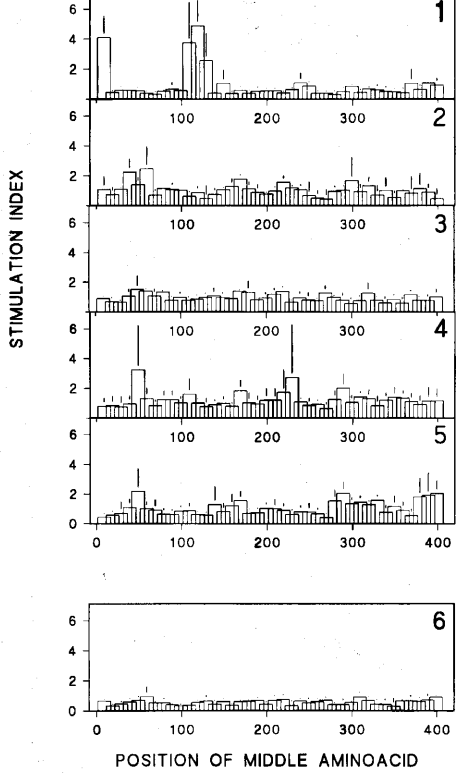

Fig 2. SI of trout leucocytes from survivors of VHSV infection induced by nucleoprotein N peptides. Vertical bars represent the averages from tetraplicates ± standard deviations from 5 trout survivors of VHSV infection (numbers 1–5). Trout number 6 was a non-infected control; similarly 4 other non-infected trout tested showed SI ≤ 1 for all the peptides tested (not shown). Backgrounds in counts per min (cpm) were: 260 ± 56 for trout 1; 100 ± 20 for trout 2; 712 ± 54 for trout 3; 190 ± 120 for trout 4; 246 ± 75 for trout 5; and 250 ± 86 for trout 6.

Table I. Stimulation index (SI) of trout leucocytes from non-infected/survivors of VHSV infection induced by purified or recombinant viral proteins.

Protein	*aa*	*SI* (n)	
		Non-infected	*VHSV survivors*
G4	9–443	0.98 ± 0.7 (3)	8.30 ± 4.4 (5)
N	1–404	1.90 ± 1.0 (2)	4.20 ± 2.8 (3)
Nx	1–404	1.00 ± 0.1 (2)	4.30 ± 1.6 (3)
N3	1–404	0.58 ± 0.3 (4)	2.30 ± 0.3 (3)

Each recombinant extract was assayed at several concentrations to give an optimal SI. *(n)* = number of trout assayed in each duplicate. G4, N, Nx and N3, 50 µg/ml (Estepa *et al*, 1994). N and Nx (Basurco *et al*, 1991) were purified from VHSV by PAGE and electroelution. Non-recombinant protein extracts from yeast were added at the same final protein concentration as the recombinant protein extracts to estimate thymidine incorporation in control cultures (background). Backgrounds were 645 ± 240 cpm for yeast (G4 and N3) and 750 ± 315 cpm for control (N, Nx). SI = cpm in the presence of viral proteins/background.

able immunodominant regions (Estepa *et al*, 1994; Lecocq-Xhonneux *et al*, 1994). As foreseen by the synthetic peptide approach (fig 3), the recombinant glycoprotein G fragment obtained in yeast without the signal peptide and transmembrane domain (G4) was capable of stimulating leucocyte proliferation with a higher SI than the synthetic peptides. The SI of the anamnestic leucocyte proliferative response to the peptides was generally lower than those elicited with the longer recombinant fragments of G (fig

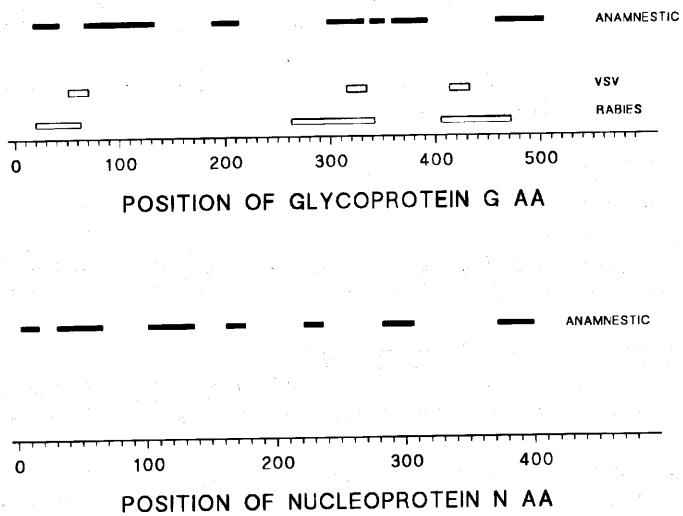

Fig 3. Glycoprotein G and nucleoprotein N regions stimulating the highest leucocyte proliferations in the trout tested. Regions defined by the peptides showing immunodominance in at least one of the trout tested have been plotted against their aa position for anamnestic response (■, survivors of VHSV infection). □, rabies glycoprotein G regions showing lymphoproliferation in mice (data from MacFarlan *et al*, 1984) and T-helper epitopes of VSV in inbred mice (data from Burkhart, 1994).

3). This was as expected from mammalian model results where large protein molecules should produce a higher SI because they have more epitopes and are, therefore, capable of inducing the proliferation of more cellular clones.

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