VIRAL IMMUNOLOGY
Volume 6, Number 4, 1993
Mary Ann Liebert, Inc., Publishers
Pp. 237–243

Enhancement of Fish Mortality by Rhabdovirus Infection after Immunization with a Viral Nucleoprotein Peptide

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ABSTRACT

A similar sequence to a mouse immunodominant CTL peptide (SYVLQGN, single-letter amino acid code, conserved amino acids underlined) identified in the nucleoproteins of several strains of vesicular stomatitis virus (VSV) (37) was found in the nucleoproteins of viral hemorrhagic septicemia virus (VHSV) of salmonid fish (GYVYQGL in VHSV 07.71 and GYVYQGS in VHSV Makah) and not in the nucleoproteins of other rhabdoviruses. The *in vivo* immunization of fingerling salmonid fish (rainbow trout *Onchorynchus mykiss*, W) with this VHSV peptide and their subsequent challenge with VHSV resulted in the enhancement rather than in the reduction of fingerling trout mortality. Possible implications for the development of subunit vaccines against VHSV are discussed.

Rhabdoviruses cause up to 30% annual losses in salmonid farms in Europe and North America (4). Neutralizing antibody to viral hemorrhagic septicemia virus (VHSV), a rhabdovirus affecting trout, shows exclusive specificity for the glycoprotein G as in other rhabdoviruses (4,9,21,39). Furthermore, glycoprotein G stimulated leukocyte cultures from noninfected (11), VHSV-immunized (11), and survivors of VHSV infection (10) trout. However, nucleoprotein N/Nx (3,28) also had an *in vitro* stimulatory effect on cell cultures from VHSV-immunized (11) and survivors of VHSV infection (10) trout, suggesting that it also induced an *in vivo* immunological response, as in rabies (12,19,20) and infectious hematopoietic necrosis virus (IHNV), another fish rhabdovirus (27).

Fish are primitive vertebrates with an adaptative immune system not yet clear to possess true, B cells (18,25), T cells (10), antigen-presenting cells (33–36), CTL restricted by histocompatibility (38), histocompatibility system (31), or IgM immunoglobulins (30). Because fish cytotoxic-like responses to viral infections are largely unknown, we began the search for possible fish (trout) viral (VHSV) CTL by studying a cytotoxic epitope of similar sequence identified in the mammalian (mouse)/vesiculovirus (VSV) model. Mammalian

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cytotoxic T lymphocytes (CTL) recognize and destroy virus-infected cells through the recognition of some viral epitopes 7–9 amino acids long presented in association with MHC class I molecules (37). Some of these epitopes have been well characterized in mammalian vesiculoviruses, one of the groups of rhabdoviruses (37). Therefore, this study focuses on the presence and the *in vivo* effects in trout of a peptide sequence present in the nucleoprotein of VHSV which is similar to a CTL epitope of VSV.

The CTL immunodominant peptide to mouse class I H-K^b (GYVYQGL) was identified on vesicular stomatitis virus (VSV-Indiana) by Van Bleek and Nathenson (37). Rhabdoviral sequences were obtained by using the PCGene package of programs (Intelligentics, Inc., California, USA) and the EMBL-25 data bank or the direct references (Table 1). Homologous sequences to the mouse immunodominant peptide were searched with the subprograms SCANSIM and PALIGN of the PCGene package by using an unitary matrix and a high gap penalty. Amphiphatic indexes, Rothbard/Taylor motifs, and IAd motifs were searched with the program TSITES (13,23).

The sequence of the mouse immunodominant VSV-Indiana nucleoprotein peptide of class I H-K^b-restricted cytotoxic recognition, ^{53}GYVYQGL (37), was used to perform a computer search among the available amino acid sequences of the nucleoproteins of other rhabdoviruses, VHSV (5), IHNV (16), rabies (PV strain), VSV (New Jersey, Indiana, San Juan, Chandipura) (1,14,22,37), and Sonchus yellow NET (SYNV) a plant rhabdovirus (40).

Highly analogous sequences were present in most of the vesiculoviruses (VSV strains) studied and partially analogous sequences were found in some of the other rhabdoviruses (Table 1). The complete heptapeptide sequence of the VSV-Indiana immunodominant epitope was found in the VSV-San Juan and the internal core of 5 amino acids was conserved in the VSV-New Jersey. The most related motif sequence XYVXQGX (X is any amino acid) and in a similar position in the nucleoprotein sequence (amino terminal) appeared only in VHSV. Both VHSV 07.71 (^{70}SYVLQGN) and VHSV Makah (^{70}SYVLQGS) nucleoprotein sequences contained this motif (Table 1). Other related but more distant sequences to GYVYQGL appeared in VSV-Chandipura (^{54}SHVYDGI) (22), IHNV-Cedar (^{156}LATSQGI) (15), Rabies-PV (^{423}RYVSVSS) (32), and SYNV-PV.263 (^{134}GYYYTQL) (40), but none has the sequence XYVXQGX. In contrast, a pentapeptide SXYSX (40) highly conserved in some rhabdoviruses (nucleoprotein amino acid residues, 338–377 in SYNV, 298–336 in rabies, and 281–297 in VSV-Indiana and VSV-New Jersey) was not found in the nucleoprotein sequence of the VHSV. Furthermore, no similar sequences to the heptapeptide were found in the glycoprotein G sequences of any of the rhabdoviruses mentioned above and no other similar pentapeptide core was found in the whole nucleoprotein N sequence of VSV-Indiana and VHSV 07.71.

TABLE 1. VESICULOVIRUS AND VHSV NUCLEOPROTEIN SEQUENCES SHOWING MAXIMAL HOMOLOGY TO THE MOUSE H-K^b CYTOTOXIC IMMUNODOMINANT HEPTAPEPTIDE ^{53}GYVYQGL FROM VSA-INDIANA[a]

Virus	*Strain (ref.)*	*Sequence*
VSV	Indiana (37)	^{53}GYVYQGL
VSV	San Juan (14)	^{53}GYVYQGL
VSV	New Jersey (1)	^{53}AYVYQGI
VHSV	07.71 (5)	^{70}SYVLQGN
VHSV	Makah (6)	^{70}SYVLQGS

[a]VSV, vesicular stomatitis virus; VHSV, viral hemorrhagic septicemia virus. The number above the left of the first amino acid (single letter code) is their amino terminal position in the nucleoprotein. Identical amino acids to the VSV-Indiana heptapeptide are underlined. The numbers in parentheses corresponds to the reference.

All the heptapeptides in the vesiculoviruses began in about the same relative positions (53 or 54) with respect to their nucleoprotein amino-terminal amino acid. The relative positions of the first amino acid of the partially conserved heptapeptide in the nucleoproteins of other rhabdoviruses were 70, 156, 134, and 423 for VHSV (07.71 and Makah), IHNV, SYNV, and rabies, respectively.

A first estimation of the possible immunogenicity in trout of the peptide of VHSV 07.71 was undertaken by using similar computer protocols (13,23) as employed for the prediction of 70–80% of mammalian T cell epitopes to scan the nucleprotein sequence. This analysis revealed a number of potential trout T cell-like epitopes especially toward their C termini (not shown) but none in the region of the VHSV heptapeptide sequence.

Bath immunization was used to assay for the VHSV peptide. Bath immunization is the most practical procedure to vaccinate fish (2,4,9) with subunit vaccines (8,16,27,39). Most probably, this method involves antigen uptake from the water throughout the gills to the gill macrophages and then to the lymphoid organs from the blood (8,10,11,26). Experiments were performed in groups of 34 fingerling trout (0.5–2 g of body weight per fish), held in 30-liter closed-system aquaria filled with dechlorinated water, at 10–14°C and provided with biological filters. VHSV isolates 689, 798, and 144 (2) were attenuated by 20 passes in epithelial papillosum carp (EPC) culture and used as supernatant from infected EPC cells. The partially conserved VHSV nucleoprotein peptide H_2N-^{70}SYVLQGN-COOH (conserved amino acids are underlined) was chemically synthesized and resin-cleaved (Clontech Lab, California, USA). The peptide was 86.5% pure as assessed by high-pressure liquid chromatography on an analytical Dynamax-300 AC4 column. One day before trout immunization, 5 mg of the peptide was dissolved in 5 ml of 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, aged glutaraldehyde added to a 1% final concentration, and the mixture kept at 4°C for 1 day. To obtain control immunizations, protein extracts were obtained from *Yersinia ruckeri*, a well known trout pathogen (8). After disruption of the bacteria with a French press, the extracts were centrifuged and the supernatants adjusted of 1 mg/ml and treated as above. To immunize fingerling trout by bath immunization with the peptide, the *Yersinia* extracts, or the attenuated VHSV (2), the amount of water in the aquaria was reduced to 1–2 liters and cooled to 8–10°C. Phytohemagglutinin (PHA-M, Flow Labs, Ayrshire, Scotland) was added to the aquaria simultaneously with the crosslinked peptide or the *Yersinia* extracts as an adjuvant for bath immunization, because it was the most potent *in vitro* immunostimulator (10,26). The 5 mg of the crosslinked peptide plus 2 mg of phytohemagglutinin (PHA), the 5 mg of crosslinked *Yersinia* extracts plus 2 mg of PHA or the 10^5 $TCID_{50}$ VHSV per ml were added per aquarium. The trout were held for 2 hr with strong aeration. Then the aquaria were filled with water and the flow through the filters was restored. One month after immunization the trout were challenged with 10^5 $TCID_{50}$ VHSV-07.71/ml (about 1 lethal dosage 50%, according to prior optimization experiments) in 2 liters of water for 2 hr at 9–10°C. Dead fish were removed from each tank, frozen at −40°C, and deaths recorded daily. All the dead fish were confirmed to contain VHSV nucleoprotein antigens by sandwich ELISA using monoclonal antibodies (28). Percent mortality was calculated by the formula, number of fish dead/number of initial fish × 100.

As Figure 1 shows, bath immunization of fingerling trout with the VHSV peptide, in the conditions used (crosslinking of the peptide with glutaraldehyde), not only did not produce protection against challenge but enhanced the mortality of the peptide-immunized trout with respect to nonimmunized trout controls. In contrast, parallel immunization of fingerling trout with attenuated VHSV reduced the mortality after the challenge and neither enhancement nor reduction was obtained by using crosslinked *Yersinia* extracts as another control.

This work is a preliminary attempt to define cytotoxic-like T cell epitopes on fish viruses. Because of the difficulties in analyzing such responses in fish when compared to studies in mammals, since the basic understanding of the fish immune system is much less developed and reagents are much harder to obtain, the study began with a well-characterized mouse immunodominant cytotoxic T lymphocyte (CTL) viral peptide.

The VSV heptapeptides homologous to the mouse cytotoxic immunodominant epitope of the Indiana strain (37) are highly conserved in several strains of vesiculoviruses (Table 1) and therefore they could be candidates for a conserved CTL epitope. Even though there is no reason to suppose that trout and mouse would present similar molecules to their immunological cytotoxic cells (since even different mice strains will present different viral sequences), the sequence motif XYVXQGX was present in the VHSV 07.71 (^{70}SYVLQGN)

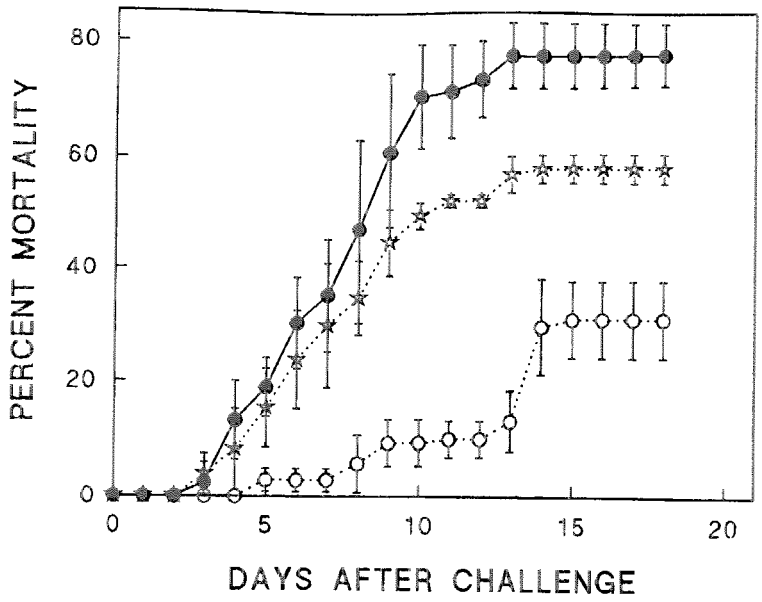

FIG. 1. Percent mortality of trout immunized with the VHSV 07.71 nucleoprotein crosslinked peptide ^{70}SYVLQGN and challenged with virulent VHSV 07.71. (*) Control nonimmunized fingerling trout; (●) crosslinked SYVLQGN immunized fingerling trout; (○) attenuated VHSV-immunized fingerling trout. The mortality profile of control fingerling trout immunized with crosslinked *Yersinia* extracts was very similar to the one of nonimmunized trout. Averages and standard deviations from three separate experiments using different fingerling trout populations (34 trout per experiment) are represented in the figure.

(5) and in the VHSV-Makah (^{70}SYVLQGS) (6) in contrast to its absence in other rhabdoviruses and the absence of an earlier reported sequence motif (SXYSX) common to many rhabdoviruses (32,40).

The *in vivo* enhancement of fish mortality after immunization with a short peptide like the VHSV heptapeptide described above had not been described before. Similar enhancement of mortality but with a different mechanism (antibody-dependent) had been reported *in vivo,* in natural situations or under laboratory experimental conditions, and for other viruses including rhabdoviruses as well as for VHSV (29). The present findings could be a first example of a cytotoxic-dependent *in vivo* enhancement of mortality in fish if that mechanism could be demonstrated. Other possible explanations for this enhancement could be an increase of VHSV infectivity in the absence of anti-VHSV neutralizing antibodies (24,29), a requirement for endogenously processed peptides (17,35,37), an autoimmune reaction due to cross-reactivity with some trout cells, immune tolerance because of incomplete immune maturation of the fingerling trout used (8,38), and/or immune tolerance because of oral immunization in mammals (however, although it is well known that in mammals oral immunizations can induce tolerance depending on the dose used, the mechanism for bath immunizations in fish is not oral but through the gills) (8). In addition other factors that might influence these kind of immunizations are time/dosage of exposure, amount of virus and the presence of defective interfering particles (7), booster immunizations, adjuvants, method of crosslinking, temperature, fish age, etc. (4,8,9,26,27). Further experiments would be needed to clarify the mechanisms involved in the observed enhancement effect, however, this trout mortality-enhancing effect could be of importance in defining vaccination strategies against VHSV in a genetically heterogeneous trout population and might explain at least some of the failures for this and/or other subunit vaccines in trout (8,29).

ACKNOWLEDGMENTS

Thanks are due to D. Frías for ELISA, cell preparation, and care of the aquarium. We appreciated the help of J.P. Coll in typing. This work was supported by Research Grants CT920036 from the AIR Program of the European Economic Comunity and AGF92-0059 from the Comision Interministerial de Ciencia y Tecnología (CICYT), Spain.

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