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Specific Regulation of the Chemokine Response to Viral Hemorrhagic Septicemia Virus at the Entry Site▽

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The fin bases constitute the main portal of rhabdovirus entry into rainbow trout (*Oncorhynchus mykiss*), and replication in this first site strongly conditions the outcome of the infection. In this context, we studied the chemokine response elicited in this area in response to viral hemorrhagic septicemia virus (VHSV), a rhabdovirus. Among all the rainbow trout chemokine genes studied, only the transcription levels of CK10 and CK12 were significantly upregulated in response to VHSV. As the virus had previously been shown to elicit a much stronger chemokine response in internal organs, we compared the effect of VHSV on the gills, another mucosal site which does not constitute the main site of viral entry or rhabdoviral replication. In this case, a significantly stronger chemokine response was triggered, with CK1, CK3, CK9, and CK11 being upregulated in response to VHSV and CK10 and CK12 being down-modulated by the virus. We then conducted further experiments to understand how these different chemokine responses of mucosal tissues could correlate with their capacity to support VHSV replication. No viral replication was detected in the gills, while at the fin bases, only the skin and the muscle were actively supporting viral replication. Within the skin, viral replication took place in the dermis, while viral replication was blocked within epidermal cells at some point before protein translation. The different susceptibilities of the different skin layers to VHSV correlated with the effect that VHSV has on their capacity to secrete chemotactic factors. Altogether, these results suggest a VHSV interference mechanism on the early chemokine response at its active replication sites within mucosal tissues, a possible key process that may facilitate viral entry.

Viral hemorrhagic septicemia virus (VHSV) belongs to the *Novirhabdovirus* genus within the *Rhabdoviridae* family and is the etiological agent of a lethal disease for many cultivated fish species worldwide, including rainbow trout (*Oncorhynchus mykiss*). In fish, the fin bases constitute one of the main portals of pathogen entry and pathogen multiplication prior to dissemination through the host, as has been demonstrated for many different pathogens (5, 11, 34–36). This is also true for VHSV and a very closely related salmonid pathogen, the infectious hematopoietic necrosis virus (IHNV) (17, 43, 44), since bioluminescence imaging of live infected rainbow trout revealed the fin bases and not the gills as the major portal of entry (17), prior to dissemination to hematopoietic tissues, where these viruses replicate most frequently (6, 48). Upon bath exposure, viral replication was already visible as early as 8 h postinfection in this area, whereas no replication was observed at this point in the gills. Moreover, when fish were exposed to a nonpathogenic recombinant IHNV, viral replication remained limited to the fin bases, suggesting that in this case the local immune response was sufficient to block further viral dissemination (17). For VHSV, viral replication in excised fin tissue has even been shown to correlate with mortality after waterborne infection (43, 44), highlighting again the importance of this early replication at fin bases in the outcome of the infection. However, whether the level of viral replication in skin tissues conditions that the amount of virus that arrives in the internal organs is too high for the internal defenses to eliminate, or if it is that the external fin tissues send the appropriate "danger" signals to the immune system enabling the systemic defenses to clear the virus in internal organs, remains unsolved.

Chemokines constitute one of the first secreted immune factors upon an encounter with a pathogen that not only orchestrate immune cell recruitment to the area of inflammation but also condition the immune response that is mounted as they regulate the immune functions of their target cells (14). Chemokines have been shown to be crucial for the elimination of many different viruses (2, 7, 9, 41), but, on the other hand, inappropriate persistence of chemokine expression in viral infections can drive tissue damage and inflammation (2, 9, 19). Although the mucosal tissues (epithelium and associated immune tissue) such as the skin or the gills provide a first line of defense against viral entry, early innate signaling molecules such as chemokines are crucial for protection against viral infections. Therefore, for a complete understanding of VHSV pathogenesis, it is of great importance to study the chemokine response to VHSV at these mucosal sites. Through the comparison of the chemokine response between the fin bases and the gills, in which the virus replicates very differently, we may deduce whether the chemokine response is a consequence of viral encounter or if viral replication influences the response.

In rainbow trout, 22 different chemokine genes have been identified to date even though for most of them functional

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studies to determine their immune roles have not yet been performed, and in some cases only their chemotactic capacities have been described (18, 29, 37). The extensive duplication events and the fact that chemokines (one of the eight most rapidly changing proteins as a response to different infectious experiences [42, 53]) evolve more quickly than other immune genes make difficult the establishment of true orthologues between fish and mammalian chemokines. Therefore, no clear inferences as to chemokine functions can be made based on their similarities to potential mammalian counterparts, and their roles have to be experimentally addressed. However, recent, very complete studies have completely changed the previous phylogenetic grouping of fish chemokines into groups or clades that better reflect the ascription of orthologues and homologues to their mammalian counterparts (22, 42). Phylogenetic analysis of teleost CXC chemokine sequences has identified six different CXC chemokine clades: CXCa, CXCb, CXCc, CXCd, CXCL12, and CXCL14 (reviewed in reference 22), but in rainbow trout representatives of only three clades have been identified so far: interleukin-8 ([IL-8] clade CXCa) (27), gamma interferon-inducible protein ([γIP] CXCb) (25), and CXCd1/CXCd2 (CXCd1/2) (54). With respect to CC chemokines, after the identification of CK1 (13), CK2 (31), and CK3 (EMBL accession number AJ315149), 15 new rainbow trout CC chemokine sequences were identified within expressed sequence tag (EST) databases (26), bringing the total to 18. Recently, seven large groups of fish CC chemokines were established through phylogenetic analysis: the CCL19/21/25 group, the CCL20 group, the CCL27/28 group, the CCL17/22 group, the macrophage inflammatory protein (MIP) group, the monocyte chemotactic protein (MCP) group, and a fish-specific group (42).

In this work, we analyzed the chemokine response to VHSV in the fin bases and gills by choosing representatives of each of these mentioned phylogenetic groups, and in the cases in which we observed an important effect of VHSV on their expression, we proceeded to study all the chemokines within that group. Our results revealed that while only two specific chemokines were upregulated in response to VHSV at the fin bases, a much wider effect was observed in the gills, where we detected no viral replication. Moreover, our studies revealed that the chemokines that play a major role in mucosal immunity are mainly those belonging to phylogenetic groups CCL19/21/25 and CCL27/28. As the skin was revealed as a major chemokine-producing tissue and a major viral replication site within the fin bases, we studied the capacity of different skin cell types to support viral replication in combination with studies concerning the effects that VHSV had on their capacity to produce chemotactic factors. Having observed that dermis cells support active replication but that viral translation is interrupted within epidermis cells, a correlation between these differences in their susceptibilities to VHSV with the effect that VHSV has on their capacities to secrete chemotactic factors might be established. Our results highlight the very specific chemokine response elicited by VHSV in the area of viral entry in which the skin plays a major role and suggest a viral interference effect on the chemokine response, a key mechanism to begin an effective local inflammation and correct triggering of the systemic immune response.

MATERIALS AND METHODS

Fish. For bath infection experiments, healthy specimens of rainbow trout (*Oncorhynchus mykiss*) were obtained from Centro de Acuicultura El Molino (Madrid, Spain), located in a VHSV-free zone. Fish were maintained at the Centro de Investigaciones en Sanidad Animal (CISA-INIA) laboratory at 14°C with a recirculating water system and 12/12-h light-dark photoperiod and fed daily with a commercial diet (Trow, Spain).

Prior to any experimental procedures, fish were acclimatized to laboratory conditions for 2 weeks, and during this period no clinical signs were ever observed. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

Virus preparation. VHSV (0771 strain) was propagated in the RTG-2 rainbow trout cell line. Cells were cultured at 18°C in minimal essential medium (MEM; Invitrogen, Carlsbad CA) supplemented with 10% fetal calf serum (FCS; Invitrogen), containing 100 units/ml penicillin and 100 μg/ml streptomycin. The virus was inoculated on RTG-2 cells grown in MEM with antibiotics and 2% FCS at 14°C. When cytopathic effect was extensive, supernatants were harvested and centrifuged to eliminate cell debris. Clarified supernatants were used for the experiments. All virus stocks were titrated in 96-well plates according to Reed and Müench (45).

VHSV bath infection. For the VHSV challenge, 30 rainbow trout of approximately 4 to 6 cm were transferred to 2 liters of a viral solution containing 5×10^5 50% tissue culture infective doses ($TCID_{50}$)/ml. After 1 h of viral adsorption with strong aeration at 14°C, the water volume was restored to 5 liters. A mock-infected group treated in the same way was included as a control.

At days 1, 3, and 6 postinfection, seven trout from each group were sacrificed by overexposure to MS-222. The area surrounding the base of the dorsal fin and the gills were removed for RNA extraction in the case of four or five fish and for immunohistochemistry in the case of the other three.

cDNA preparation. Total RNA was extracted using Trizol (Invitrogen) following the manufacturer's instructions. Tissues were first homogenized in 1 ml of Trizol in an ice bath; 200 μl of chloroform was added, and the suspension was then centrifuged at 12,000 × *g* for 15 min. The clear upper phase was aspirated and placed in a clean tube. A total of 500 μl of isopropanol was then added, and the samples were again centrifuged at 12,000 × *g* for 10 min. The RNA pellets were washed with 75% ethanol, dissolved in diethyl pyrocarbonate (DEPC)-treated water, and stored at −80°C.

RNAs were treated with DNase I to remove any genomic DNA traces that might interfere with the PCRs. One microgram of RNA was used to obtain cDNA from each sample using Superscript III reverse transcriptase (Invitrogen). In all cases, RNAs were incubated with 1 μl of oligo$(dT)_{12–18}$ (0.5 μg/ml) and 1 μl of 10 mM dinucleoside triphosphate (dNTP) mix for 5 min at 65°C. After the incubation, 4 μl of 5× first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCO_2$) 1 μl of 0.1 M dithiothreitol (DTT), and 1 μl of Superscript III reverse transcriptase were added, mixed, and incubated for 1 h at 50°C. The reaction was stopped by heating at 70°C for 15 min, and the resulting cDNA was diluted in a 1:10 proportion with water and stored at −20°C.

Evaluation of chemokine gene expression by real-time PCR. To evaluate the levels of transcription of the different chemokine genes studied, real-time PCR was performed with an Mx3005P quantitative PCR (QPCR) instrument (Stratagene) using SYBR green PCR Core Reagents (Applied Biosystems). Reaction mixtures containing 10 μl of 2× SYBR green Supermix, 5 μl of primers (0.6 mM each), and 5 μl of cDNA template were incubated for 10 min at 95°C, followed by 40 amplification cycles (30 s at 95°C and 1 min at 60°C) and a dissociation cycle (30 s at 95°C, 1 min 60°C, and 30 s at 95°C). For each mRNA, gene expression was corrected by elongation factor 1α (EF-1α) expression in each sample and expressed as $2^{-\Delta CT}$ (C_T is threshold cycle), where ΔC_T is determined by subtracting the EF-1α C_T value from the target C_T as previously described (10). The primers used were designed from sequences available in the GenBank using the Oligo Perfect software tool (Invitrogen) and are shown in Table 1. All amplifications were performed in duplicate to confirm the results. Negative controls with no template were always included in the reaction mixtures. As controls for effective viral infection in the fin bases, the levels of expression of the interferon (IFN)-induced Mx gene and the VHSV N gene were also evaluated by real-time PCR using primers previously described (10).

Light microscopy and immunocytochemistry. Fin tissue including the fin bases obtained from control and VHSV-infected fish at different times postinfection were fixed in Bouin's solution for 24 h, embedded in paraffin (Paraplast Plus; Sherwood Medical), and sectioned at 5 μm. After dewaxing and rehydration, some sections were stained with hematoxylin-eosin in order to determine the levels of infiltration or any other apparent damage or pathological changes. Other sections were subjected to an indirect immunocytochemical method to

TABLE 1. Chemokine genes analyzed in this study with accession numbers, primer sequences, and phylogenetic clade or group to which each of these chemokines is assigned

Gene	Accession no.	Primer sequence (5′→3′)[a]	Phylogenetic clade/group
EF1-α	AF498320	F: GATCCAGAAGGAGGTCACCA R: TTACGTTCGACCTTCCATCC	
IL-8	AJ279069	F: ATTGAGACGGAAAGCAGACG R: CTTGCTCAGAGTGGCAATGA	CXCa
γIP	AJ417078	F: TGGACTGGTGAACCGTGTTA R: TCTTGGCAAATGGAGCTTCT	CXCb
CXCd	DQ191448 (1) DQ191448 (2)	F: GCTCACACTGCTCTAAGGAAGAA R: GGAGAGAGTCTCAATGGAACGT	CXCd
CK1	AF093802	F: GATGGCTGAAAGGCTACACC R: TGGGATTTGTTCTCCTGACG	CCL20
CK3	AJ315149	F: AGATCACCGTTCCCATCATC R: GTGACTTTCTGGCCATCTCC	Fish CC group
CK5B	CA374135	F: TTTGCTGATCGTCAGATACCC R: GTGTCTGCTCCCCAGACTTC	MIP group
CK6	CA355962 (A) CA355812 (B)	F: TGAAAGGCCTACGAATCTGC R: GTTGTTGTTGGCTGGTTGTG	CCL17/22
CK7A	CA343117	F: CCGAGAATCCCTCTTCAACA R: TCATCGTCGTCTTGGCAGTA	MCP group
CK9	CA378686	F: GGCTCTTATGGGAACTGCTG R: CTGGGATTGGCACAAACAG	CCL19/21/25
CK10	CA361535	F: ATTGCCAAGATCCTCTTCTGTGTTC R: CCTGAGGCTGGTAACCTATGACAAC	CCL19/21/25
CK11	BX072681	F: CCTTTGAGCATACTAATGCGAGTGG R: GTCTGCACAATACTTCCTCCCATTG	CCL27/28
CK12	CA358073 (A) CA346383 (B)	F: GACATCGATGCCACTGTGTT R: GGAGATGGTTCGCTCCAGAC	CCL19/21/25
CCR7	CX721232 CU065128	F: TTCACTGATTACCCCACAGACAATA R: AAGCAGATGAGGGAGTAAAAGGTG	
CCR9	AJ003159.1	F: TCAATCCCTTCCTGTATGTGTTTGT R: GTCCGTGTCTGACATAACTGAGGAG	

[a] F, forward; R, reverse.

detect VHSV using 1P1D11, a monoclonal antibody (MAb) specific to the G protein of VHSV, obtained from N. Lorenzen at the Danish Institute for Food and Veterinary Research (Århus, Denmark) (32). The sections were first incubated for 30 min in phosphate-buffered saline (PBS; pH 7.2 to 7.4) containing 5% bovine serum albumin ([BSA] PBT). Then sections were incubated overnight at 4°C with the MAb at an optimal dilution of 1:100 in PBS with 1% BSA. After washing in PBT, the sections were exposed to anti-mouse IgG biotin-conjugated antibody (Sigma) diluted 1:100 for 1 h at room temperature. The samples were then washed in PBT and incubated for 1 h with avidin-biotin-alkaline phosphatase mouse IgG. The immunocytochemical reactions were then revealed by incubation with Fast-Red (Sigma) diluted in Tris-HCl buffer (pH 7.6) for 15 min at room temperature. The specificity of the reactions was determined by omitting the first antiserum and comparing the results obtained in control fins. Slides were examined with an Axiolab (Zeiss) light microscope.

Skin primary cultures. Complete skin cultures were established after round sections of skin (diameter, 1 cm) were removed with a scalpel. For each rainbow trout, four different sections were obtained, and each section was then placed in 24-well plates with 1 ml of Leibovitz medium (L-15; Invitrogen) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5% FCS.

In other cases, only epidermal cells were removed from round sections of 1-cm diameter by scratching the skin surface with a scalpel. Posterior histological examination of the area showed that only epidermal cells were removed through this technique.

To determine the susceptibility of each of these cultures to VHSV, cultures were infected with VHSV at a final concentration of 5×10^4 $TCID_{50}$/ml in culture medium with 2% FCS or mock infected with medium alone and incubated at 14°C for different time points, depending on the experiment performed.

Isolation of PBLs. Peripheral blood leukocytes (PBLs) were isolated from labeled rainbow trout from which fin explants or epidermal cultures had been established following the method previously described (16). Briefly, blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with L-15 medium supplemented with antibiotics, 10 units/ml heparin, and 2% FCS. The resulting cell suspension was placed onto 51% Percoll and centrifuged at $500 \times g$ for 30 min at 4°C. The interface cells were collected and washed twice at $500 \times g$ for 5 min in L-15 medium containing 0.1% FCS. The viable cell concentration was determined by Trypan blue exclusion. Cells were resuspended in L-15 medium with 2% FCS at a concentration of 1×10^6 cells/ml.

Chemotactic capacity of supernatants from primary skin cultures infected with VHSV. Complete skin or epidermal cell cultures were infected with VHSV at a final concentration of 5×10^5 $TCID_{50}$/ml in culture medium with 2% FCS or mock infected with medium alone. After 3 days of incubation at 14°C, culture supernatants were collected, and their capacity to induce the migration of PBLs from the same individual rainbow trout was tested. The same day that supernatants were collected, PBLs were extracted from each trout, and the chemotaxis assay was later performed.

The chemotaxis assays were performed in chemotaxis chambers in 24-well plates (Costar-Corning Life Sciences). A total of 600 μl of 1:2 dilutions of the different supernatants in culture medium was placed in the wells. Controls with medium alone and medium and VHSV were also included. After introducing the chemotaxis chambers into each of the wells, 100 μl of the PBL cell suspensions was loaded in the upper part of the chamber. The upper and lower chambers were separated by a 3-μm-pore-sized polycarbonate filter. After 2 h of incubation at 20°C, the number of cells that had migrated to the bottom of the wells was quantified by flow cytometry (FACSCalibur; Becton Dickinson). Cell number was determined at a constant flow time (1 min) of the medium in the lower chamber. The migrating cells were analyzed based on forward and side light scatter parameters. All experiments were performed in duplicate.

Western blot analysis of viral proteins. Cell lysates were prepared from either fin cultures or epidermal cell cultures exposed to VHSV as described above. Electrophoresis of cell lysates and Western blotting were performed as described previously (12, 49) using the IP5B11 monoclonal antibody recognizing the N protein of VHSV provided by N. Lorenzen at the Danish Institute for Food and Veterinary Research (Århus, Denmark).

TABLE 2. Levels of transcription of a selection of rainbow trout CXC and CC chemokines in the fin bases in response to VHSV bath infection

Chemokine	Mean relative transcription[a]					
	Day 1		Day 3		Day 6	
	Control	VHSV	Control	VHSV	Control	VHSV
IL-8	0.0152 ± 0.02	0.0022 ± 0.00	0.0274 ± 0.05	0.0060 ± 0.00	0.0359 ± 0.02	0.0396 ± 0.05
γIP	0.0272 ± 0.01	0.0284 ± 0.02	0.0699 ± 0.13	1.1948 ± 0.73	0.0130 ± 0.01	0.0548 ± 0.07
CXCd	0.2902 ± 0.30	0.0279 ± 0.01	0.0225 ± 0.01	0.0431 ± 0.07	0.0350 ± 0.04	0.0064 ± 0.00
CK1	ND	ND	ND	ND	ND	ND
CK3	0.6826 ± 0.76	0.2642 ± 0.18	0.3118 ± 0.44	2.7193 ± 2.64	1.1951 ± 1.16	1.2815 ± 0.60
CK5B	0.0245 ± 0.01	0.0108 ± 0.00	0.0182 ± 0.02	0.0243 ± 0.02	0.0146 ± 0.01	0.0057 ± 0.00
CK6	0.3394 ± 0.41	0.0443 ± 0.04	0.2033 ± 0.38	0.0305 ± 0.02	0.0309 ± 0.02	0.2558 ± 0.47
CK7A	ND	ND	ND	ND	ND	ND
CK9	235.0912 ± 198.07	58.8618 ± 68.73	54.0372 ± 77.60	38.4374 ± 36.95	52.7418 ± 46.44	10.7460 ± 13.26
CK10	0.0309 ± 0.02	0.0168 ± 0.01	0.0146 ± 0.01	**2.0167 ± 1.10**	0.0158 ± 0.00	**0.9253 ± 0.45**
CK11	72.8666 ± 123.19	4.0194 ± 3.08	36.9005 ± 68.64	7.1217 ± 7.58	7.8178 ± 6.53	1.0905 ± 1.25
CK12	1.1877 ± 0.91	1.2991 ± 0.93	0.9388 ± 1.04	**33.4860 ± 30.00**	3.6679 ± 2.54	1.0162 ± 0.66

[a] At days 1, 3, and 6 postinjection, four trout from both the infected and the mock-infected group were sacrificed, RNA was extracted, and the levels of expression of the different chemokines were studied through real-time PCR in triplicate. Data are shown as the mean chemokine gene expression relative to the expression of endogenous control EF-1α ± standard deviation. Values in bold constitute relative expression levels significantly higher than those of the respective controls ($P < 0.05$). ND, not detected.

RESULTS

Chemokine gene expression at the fin bases in response to VHSV. In order to elucidate the chemokine role at the fin bases, the main portal of VHSV entry, we studied the levels of expression of a wide selection of rainbow trout chemokine genes by real-time PCR following VHSV bath infection. Table 2 shows the mean levels of transcription of all the chemokine genes studied at the fin bases in response to the VHSV infection at days 1, 3, and 6 postinfection. We first determined the level of expression of one representative rainbow trout chemokine gene ascribed to either one of the CXC clades or CC chemokine groups previously described. As this first screening revealed an important effect of VHSV on CK12 regulation of transcription, we also included the other rainbow trout chemokines belonging to the CCL19/21/25 group, CK9 and CK10. Out of the 12 genes studies, VHSV was capable of significantly upregulating the levels of expression of only CK10 and CK12 at day 3 postinfection and of only CK10 at day 6 postinfection, revealing a very specific response, as VHSV had been proved to significantly upregulate the transcription of many of the other chemokine genes in other organs such as spleen or head kidney (38). No significant downregulation was observed.

One noticeable result was the level of constitutive transcription that we observed in fin bases for CK9 and CK11. The mean constitutive expression levels for these two chemokines varied throughout the sampling time points from 52 to 235 times the level of expression of the EF-1α housekeeping gene for CK9 and from 7 to 72 times for CK11, suggesting an important role of these chemokines in this fin base area. In response to VHSV, the levels of expression of these highly expressed chemokines decreased moderately, but differences were not significant.

Chemokine gene expression at the gills in response to VHSV. Since the chemokine induction pattern in response to the virus at the fin bases was much more specific than what we had previously observed in immune organs (38), we studied in parallel the chemokine response to another mucosal tissue, the gills. Previous studies had revealed that although some viral particles could be found in the gills upon bath infection, this is not the main site of viral entry for fish rhabdoviruses (17), and viral replication can be detected in only a few specific cells (4). In this case, the chemokine response to the virus was very different from that observed in the fin bases (Table 3). At day 1 postinfection, VHSV provoked significant upregulation of the levels of transcription of CK1, CK3, CK9, and CK11. At day 3, the only upregulation that is maintained is that of CK3, whereas at this point we found unexpected downregulation of CK10 and CK12 transcription in response to the virus, which was maintained for CK12 at day 6. These results show that the response to a viral infection at mucosal surfaces is highly specific and most probably is dependent on both the cell composition of the mucosal tissue and the degree of viral susceptibility of the different cell types present.

Viral gene and Mx transcription in response to VHSV in the fin bases and gills. To further understand the distinct chemokine response to VHSV of these two mucosal tissues, we also evaluated the levels of transcription of the N viral gene as well as the transcription of the IFN-induced Mx gene since the levels of expression of Mx in fish have been shown to correlate well with an efficient immune response to viral replication (33).

Figure 1 shows Mx transcription in response to VHSV at the fin bases (Fig. 1A) and gills (Fig. 1B). While the levels of Mx were significantly induced in the fin bases in response to VHSV at day 3 postinfection, the levels of Mx transcription in the gills were not significantly modulated by the viral encounter. These results are in correlation with those obtained for the transcription of the N VHSV gene since although a significant viral transcription was detected at both days 1 and 3 postinfection at the fin bases (Fig. 2), no viral transcription was ever detected in our gill samples.

VHSV replication at the fin bases. To further understand the relation between chemokine production and viral replication and confirm in which specific tissues the virus was replicating at the fin bases, we next performed immunohistochemical studies

TABLE 3. Levels of transcription of a selection of rainbow trout CXC and CC chemokines in gills in response to VHSV bath infection

Chemokine	Mean relative transcription[a]					
	Day 1		Day 3		Day 6	
	Control	VHSV	Control	VHSV	Control	VHSV
IL-8	0.0005 ± 0.00	ND	0.0004 ± 0.00	0.0002 ± 0.00	0.0046 ± 0.00	0.0041 ± 0.01
γIP	0.0006 ± 0.00	0.0006 ± 0.00	0.0004 ± 0.00	ND	1.2317 ± 1.66	1.3387 ± 1.49
CXCd	0.0629 ± 0.09	0.0049 ± 0.01	0.1149 ± 0.15	0.04 ± 0.03	0.0275 ± 0.06	1.1964 ± 1.31
CK1	0.0036 ± 0.00	**0.0536 ± 0.03**	ND	ND	ND	ND
CK3	0.0341 ± 0.03	**5.7203 ± 5.25**	0.2413 ± 0.31	**7.380 ± 4.79**	0.0041 ± 0.01	0.0039 ± 0.01
CK5B	ND	ND	0.0004 ± 0.00	0.0002 ± 0.00	0.0680 ± 0.06	0.0309 ± 0.03
CK6	0.4987 ± 1.00	0.0137 ± 0.01	0.05108 ± 0.07	0.0925 ± 0.12	0.0986 ± 0.18	0.1102 ± 0.19
CK7A	ND	ND	ND	ND	ND	ND
CK9	0.0611 ± 0.03	**27.4551 ± 18.66**	0.1843 ± 0.22	0.2556 ± 0.25	0.7172 ± 1.09	3.8930 ± 0.02
CK10	0.0117 ± 0.01	0.0120 ± 0.02	1.6597 ± 1.97	ND	0.5842 ± 1.09	0.01601 ± 0.02
CK11	0.0117 ± 0.01	**21.9521 ± 18.15**	0.5777 ± 0.90	281.5384 ± 304.76	1.0340 ± 1.26	1.5975 ± 2.23
CK12	0.2628 ± 0.25	0.1809 ± 0.17	1.766 ± 1.20	0.1714 ± 0.09	279.6089 ± 168.53	12.7088 ± 8.22

[a] At days 1, 3, and 6 postinjection five trout from both the infected and the mock-infected group were sacrificed, RNA was extracted, and the levels of expression of the different chemokines were studied through real-time PCR in triplicate. Data are shown as the mean chemokine gene expression relative to the expression of endogenous control EF-1α ± standard deviation. Values in bold constitute relative expression levels significantly higher than those of the respective controls, whereas values underlined constitute expression levels significantly lower than those of the respective controls ($P < 0.05$). ND, not detected.

to detect VHSV G gene expression in this area (Fig. 3). The fin base section in which we studied the chemokine response is represented in Fig. 3a, showing the presence of the different skin layers, muscle, and cartilage that may support viral replication differently upon viral entry. Among these different tissues, we detected the G viral protein in the dermis and muscle but never in the epidermal layer of the skin (Fig. 3b to f).

VHSV actively replicates only in dermis cells and not in skin epidermis. To verify the results obtained through immunohistochemical studies of complete fin bases in which we had seen different susceptibilities to VHSV in the different skin layers, we conducted *in vitro* experiments comparing the response of the complete dissected skin to epidermal cell cultures in which only this outermost layer was present, as verified by histology. When studying viral transcription through real-time PCR analysis of N gene expression, we detected active transcription in both explants and epidermal primary cultures (Fig. 4A), indicating that the virus was capable of entering both endodermal and epidermal cells, thus transcribing its viral genome. Lower levels of transcription were observed in epidermal cells than in dermal cells, but the differences were not significant. No significant differences in the levels of Mx induction in response to

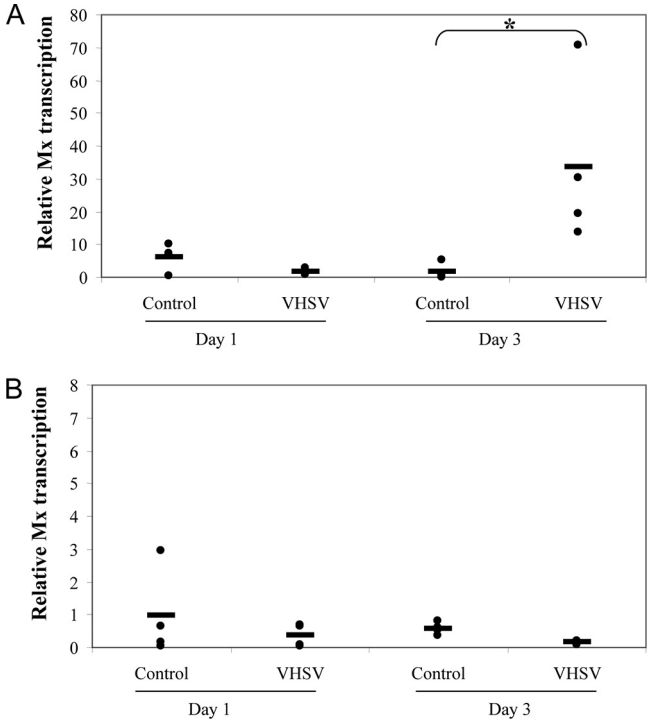

FIG. 1. Levels of Mx gene transcription in response to VHSV bath infection in fin bases (A) and gills (B). Samples were collected from both infected and mock-infected controls after 1 or 3 days of VHSV infection; RNA was obtained, and the levels of transcription of the Mx gene were determined by real-time PCR. Individual data (circles) were analyzed in triplicate and are shown as the mean gene expression (black bars) relative to the expression of endogenous control EF-1α. *, relative mean expression significantly higher than the relative mean expression in the respective control ($P < 0.05$).

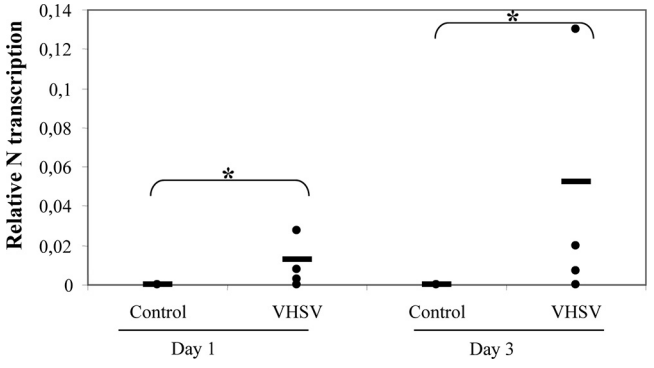

FIG. 2. Levels of VHSV N gene transcription in the fin bases in response to VHSV. Fin bases were collected from both infected and mock-infected controls after 1 or 3 days of VHSV infection; RNA was obtained, and the levels of transcription of the N viral gene were determined by real-time PCR. Individual data (circles) were analyzed in triplicate and are shown as the mean gene expression (black bar) relative to the expression of endogenous control EF-1α. *, relative mean expression significantly higher than the relative mean expression in the respective control ($P < 0.05$).

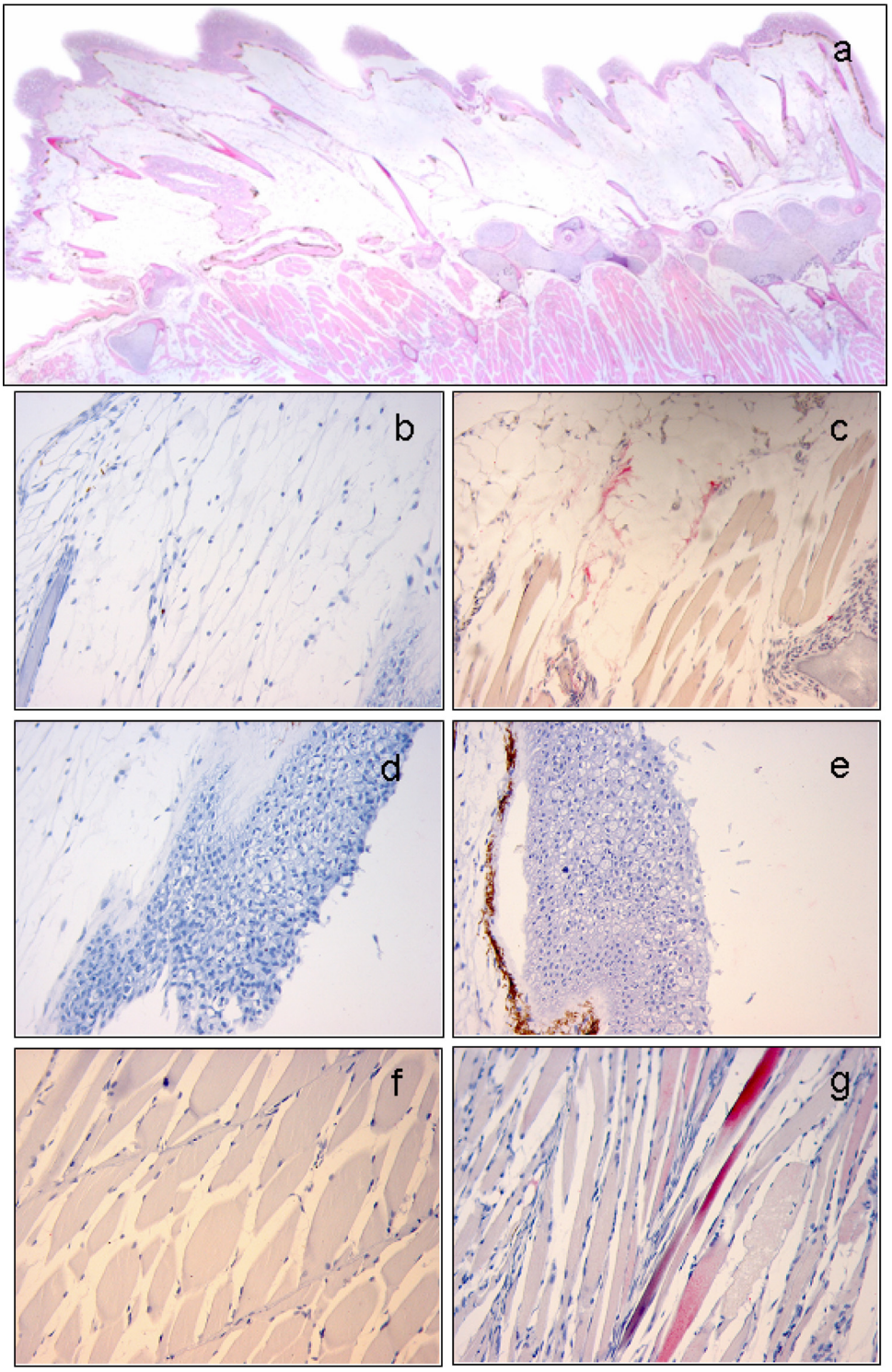

FIG. 3. Immunohistochemical staining of fin bases infected with VHSV. Although the immunohistochemical staining was performed with both control and infected fish obtained at days 1, 3, and 6 postinfection, no significant differences in the level or distribution of staining were observed in infected fish at different days postinfection. Therefore, samples taken at day 1 postinfection are shown.(a) Control fin base area stained with hematoxylin-eosin corresponding to the area sampled in chemokine expression studies (magnification, ×10). (b, d, and f) Images from control fish showing dermis, epidermis, and muscle, respectively, in which no staining was observed (magnification, ×20). (c, e, and g) Images from infected fish showing dermis, epidermis, and muscle, respectively (magnification, ×20). Specific staining for VHSV in red was always observed in muscle and dermis but not in epidermis.

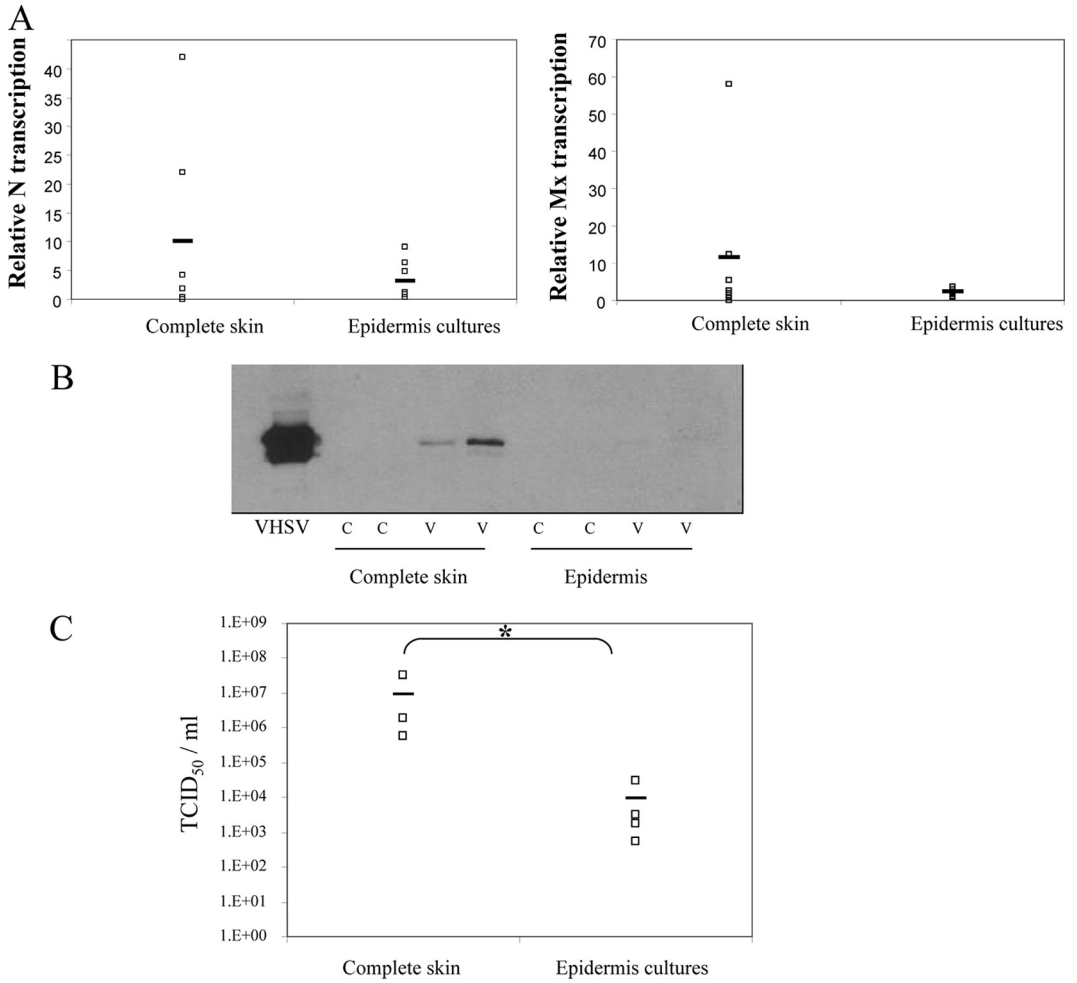

FIG. 4. Cultures were established either from complete skin in which both epidermal cells and dermal cells were present or exclusively from epidermal cells. These two types of skin cultures were infected *in vitro* with VHSV or mock infected to determine their susceptibility to VHSV. (A) Levels of transcription of the viral N gene and Mx gene transcription determined through real-time PCR in complete skin or epidermis cultures after 3 days of incubation with the virus *in vitro*. Data are shown as gene expression relative to the expression of endogenous control EF-1α obtained in individual cultures or as stimulation indexes in the case of Mx, obtained by dividing the levels of Mx expression observed in response to VHSV to those observed in the respective mock-infected culture. (B) Detection of N viral protein through Western blotting in fin explants or epidermis cell cultures infected with VHSV (V) or mock infected (C) *in vitro* at day 3 postinfection. (C) Viral titer obtained in four independent cultures after 7 days of infection with VHSV at a final concentration of 5×10^4 $TCID_{50}$/ml.

VHSV were observed between epidermis and complete skin. However, in accordance with what we had previously observed through immunohistochemistry, viral N protein expression was strongly detected in fin explants in which dermis tissue was also present but was only weakly detected in epidermis cultures (Fig. 4B). Finally, the titration of viral yields in these cultures further confirmed these results (Fig. 4C) since the viral titers were significantly higher after 7 days of infection in complete skin cultures than in epidermis cultures, in which the viral titer obtained was even lower than the viral input. Therefore, it seems that VHSV is capable of entering both the dermis and the epidermis, but this outermost layer is capable of interrupting the viral cycle to abort the infection at some point before the translation of the viral genes.

Epidermal cells are the main contributors to chemokine production. As a step toward the understanding of the contribution of each skin layer to the chemokine response observed at the fin bases, we compared the chemokine production capacity of the different skin layers through the primary skin cultures established. Among the CXC chemokines studied, the skin transcribed only IL-8 at similar relative levels in epidermal cells and in complete skin, indicating that both dermis and epidermis contribute to IL-8 mRNA expression (Fig. 5). Among CC chemokines, primarily the chemokines previously catalogued within the CCL19/21/25 and CCL27/28 groups, that is, CK9, CK10, CK11, and CK12, were expressed in the different skin layers. In the case of CK9, the epidermis does not seem to be a major source for its transcription. Apart from chemokines in these phylogenetic groups, epidermal cells strongly expressed CK6, and both types of skin cultures expressed CK3.

VHSV infection affects the chemotactic capacity of dermal and epidermal skin cells differently. Since VHSV replicates differently in dermal and epidermal cells, we next wanted to

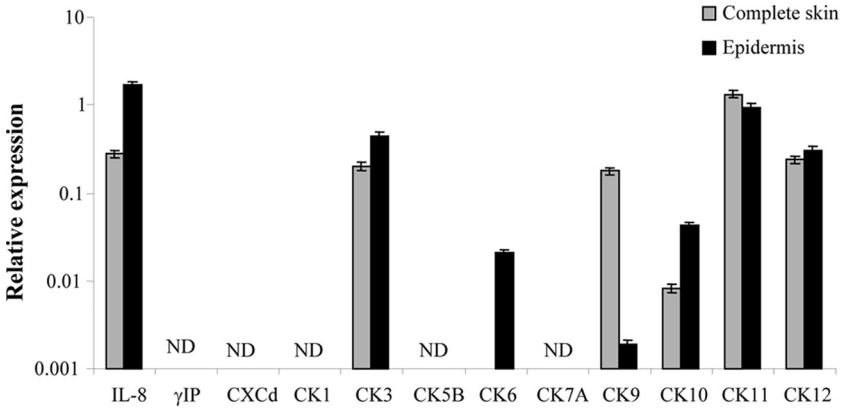

FIG. 5. Levels of transcription of the different chemokine genes in the different skin cultures established. RNA was extracted from either complete skin cultures or epidermis cell cultures from which supernatants were collected, and the levels of transcription of these chemokines were studied through real-time PCR. Data are shown as mean chemokine gene expression ± standard deviation relative to the expression of endogenous control EF-1α of four independent cultures. ND, not detected.

determine the effect that VHSV had on their capacity to produce chemotactic factors and then compare these responses to the effects observed in the original samples in which the chemokine transcription levels were determined. For this, we obtained supernatants from skin sections or epidermal primary cultures either uninfected or infected with VHSV and compared their abilities to attract autologous PBLs.

Supernatants derived either from fin explants or from epidermal cell cultures strongly attracted PBLs (Fig. 6). However, when these cultures were infected with VHSV prior to the supernatant generation, the effects differed in both types of cultures. When fin explants were infected with VHSV, the chemotactic capacity of the supernatants significantly decreased, whereas when epidermal cell cultures were infected with VHSV, the chemotactic capacity of the supernatants significantly increased. Clear ascription of these viral effects to a specific chemokine was not possible as there may have been an overall effect in which many of these skin chemokines cooperate. What seems clear is that epidermal and dermal cells produce very different secretions of chemotactic factors in response to VHSV.

DISCUSSION

Complete knowledge of the early immune mechanisms triggered at the site of viral entry into the host provides us with important information for the understanding of viral pathogenesis. It has been recently demonstrated that rhabdovirus enters the fish through the fin bases and, moreover, that early replication in this first site strongly conditions the outcome of the infection (17). While a virulent IHNV replicates in this area as a first step to distributing itself through the organism, a low-virulence IHNV remains confined to this area, highlighting the importance of the early local immune mechanisms for controlling rhabdoviral infections.

In this context and given the central role of chemokines in antiviral defense, we have determined which rainbow trout chemokines are modulated by a viral encounter in this fin base

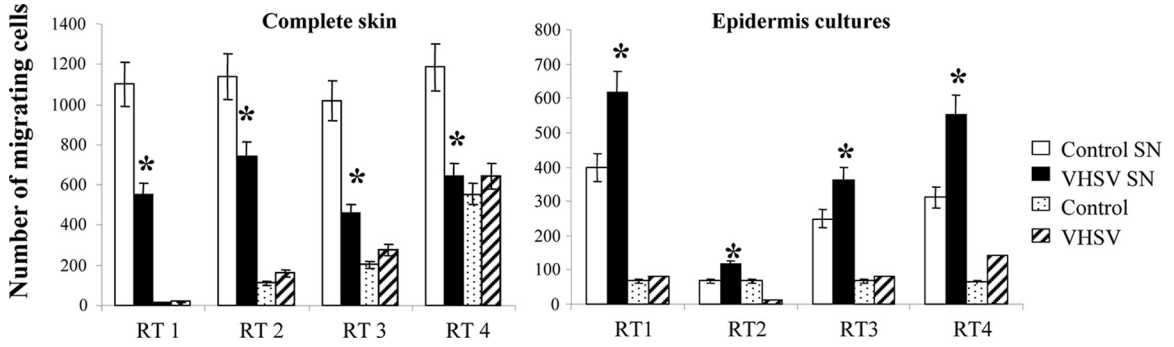

FIG. 6. Effect of VHSV on the capacity of the different skin cultures to secret chemotactic factors. Cultures were established either from complete skin in which both epidermal cells and dermal cells were present or exclusively from epidermal cells. These two types of skin cultures were infected *in vitro* with VHSV or mock infected and incubated for 3 days at 14°C. At this point, supernatants (SN) from VHSV-infected cultures and from mock-infected cultures were collected to determine the capacity of these supernatants to induce migration of autologous PBLs by comparing the migration to the migration observed toward medium alone (Control) or medium with VHSV. The chemotaxis assay was performed as described in Materials and Methods, and fluorescence-activated cell sorting analysis was used to enumerate the number of migrating cells. Experiments were performed in triplicate, and data are shown as the mean number (± standard deviation) of migrating cells obtained in individual rainbow trout (RT). *, migration levels toward supernatants from infected cultures significantly different from migration levels obtained with supernatants from mock-infected cultures ($P < 0.05$).

area in comparison to the viral effect in another mucosal tissue, the gills, in which the virus replicates poorly. Our results revealed that the local chemokine response is much stronger in a low-replication tissue such as the gills than in one in which the virus efficiently replicates such as the fin bases. More in-depth studies of this fin base area demonstrated that epidermal cells and dermal cells support VHSV replication to different levels and thus produce chemotactic factors at different levels in response to the virus, pointing again to the idea that viral replication interferes with the chemokine response. This viral interference may be an important pathogenicity factor that may explain why the virus enters the body through the fin base in which the epidermal layer is thinner and even interrupted, highlighting as well the importance of the epidermis in the fish antiviral defense.

Although IL-8 is strongly expressed constitutively in the skin, when studying the effect of VHSV on CXC chemokines of mucosal tissues, we included all the genes that have been characterized in rainbow trout to date and found no significant effect of the virus on their levels of transcription. This suggests that these chemokines, which are known to act preferentially on neutrophils and some lymphocyte subsets (8, 40), do not have a preferential role in mucosal antiviral immunity. CC chemokines, on the other hand, act primarily on monocytes instead of neutrophils, as well as on other specific lymphocyte subsets (50). In this case, we selected a group of chemokine genes belonging to different phylogenetic groups that had been proven to be strongly regulated in response to VHSV in lymphoid organs such as spleen and head kidney (38). In fact, VHSV strongly upregulates γIP, CXCd, CK3, CK5B, CK6, and CK12 in spleen and γIP, CXCd, CK3, and CK12 in head kidney. In our current study, only CK10 and CK12 were significantly upregulated in response to VHSV infection in the fin bases, whereas CK1, CK3, CK9, and CK11 were strongly upregulated in the gills.

The great differences that were observed in the chemokine response to VHSV in fin bases and gills do not seem to be only a consequence of different chemokines being secreted by different cell types as the constitutive chemokine profiles for both mucosal tissues are very similar; thus, it seems that the key difference is whether active viral replication is taking place or not. Having seen in our experiments that the infected fish demonstrated strong symptomatology from viral infection and began to die as early as 6 days postinfection (data not shown), we further studied viral replication in these locations. Viral replication was confirmed by analysis of viral gene transcription and immunohistochemistry in the fin bases, and an efficient IFN response was mounted, as determined through the study of the IFN-induced Mx gene. In contrast, no viral N protein expression was detected in the gills, despite the fact that other authors had described a low-moderate VHSV replication level in the gills focused in the cells lining the vessels of the primary gill arch (4). Therefore, it seems that efficient viral replication is not needed for the induction of an effective chemokine response, but, on the other hand, the infected tissues have a suppressed reaction.

Furthermore, through the development of fin explants in which both epidermal and dermal cells are present and epidermis cell cultures in which no dermis cells were present in combination with immunohistochemistry studies, we could conclude that both epidermal and dermal cells supported viral transcription although the levels of transcription were slightly lower in epidermal cells. However, viral N protein expression was observed only in cultures in which dermal cells were present, indicating that epidermal cells were able to block the viral cycle at some point before viral protein expression, in accordance with what was observed by immunohistochemistry. This blockage of viral protein expression was also consistent with viral titration of primary skin cultures as the virus produced a significantly higher viral yield in complete skin cultures than in epidermis cell cultures in which the viral yield did not increase throughout the infection period. Similarly, RTS11 rainbow trout monocyte-macrophages have also been shown to block VHSV replication at some point of the viral cycle before the translation of viral proteins (49). Consequently, the effect that the virus had on the capacity of these cells to produce chemotactic factors also differed between dermal and epidermal cells since, while VHSV provokes an upregulation of the chemotactic factors produced by the epidermis, it provokes downregulation of the chemotactic factors produced by epidermal and dermal cells together. It has been difficult to point to a chemokine as responsible for the different viral effects, as it may be an overall effect of different chemokines. What seems clear, however, is that in cells that can control the viral infection, an induced chemokine response is observed, whereas in cells in which there is active viral replication, this defense mechanism is impaired. It may be possible that this limitation is a consequence of a general shutoff mechanism induced by VHSV upon translation, as widely demonstrated for rhabdovirus (23), but although this may explain the reduction of the chemotactic activity, it would not explain the absence of chemokine transcription upregulation as the shutoff does not affect mRNA synthesis (24). Some viruses such as poliovirus can block secretion of proteins in infected cells, thus blocking chemokine release, but this again would not explain the direct effect of gene transcription.

On the other hand, it is well known that many viruses have developed strategies to either exploit or avoid chemokine networks and thus replicate more efficiently (28). For large DNA viruses, the most common strategy is the encoding of chemokine homologs, chemokine receptor decoy homologs, or soluble chemokine binding proteins (1, 28, 47), but some RNA viruses have also developed strategies to directly interfere with chemokine synthesis. For example, the hepatitis C virus complex of nonstructural proteins 3 and 4A (NS3/4A) downregulates the transcription of CCL5, IL-8, and γIP through the inhibition of the retinoic acid-inducible gene I (RIG-I) pathway (46). Moreover, many viruses interfere with the NF-κB pathway (21), which is known to be directly responsible for the transcription of many chemokine genes (30, 52).

Finally, in a context in which the exact immune function is unknown for most rainbow trout chemokines, our results point to an important role in mucosal immunity of chemokines CK9, CK10, CK11, and CK12, ascribed to phylogenetic groups CCL19/21/25 and CCL27/28 by Peatman and Liu (42). All of these chemokines were either modulated by the virus at mucosal sites or were produced in very high constitutive levels. Interestingly, major roles in mucosal immune responses have been demonstrated for mammalian chemokines belonging to these two groups, CCL19/21/25 and CCL27/28 (3, 15, 20, 39,

51); therefore, although much more work should be done to determine if the rainbow trout chemokines are the true orthologues of the mammalian chemokines, it seems that some functional equivalence is observed.

In conclusion, we have demonstrated that a very restricted chemokine response to VHSV is observed in the area of primary replication, the fin bases, where the virus actively replicates in the dermis and muscle cells, while a much stronger chemokine response is observed in the gills. Within the fin bases, epidermal cells are capable of blocking viral replication before viral translation while the virus replicates in the dermis, in which the virus is capable of limiting the production of chemotactic factors. More work should be done to determine the exact mechanism through which the virus is capable of limiting the chemokine response upon its active replication.

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