Vertical and horizontal transmission of tilapia larvae encephalitis virus: The bad and the ugly

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A B S T R A C T

Impairment of innate immunity in tilapia larvae after vertical and horizontal infection with the newly characterized tilapia larvae encephalitis virus (TLEV) was accessed by evaluation of cell-mediated reactive oxygen species (ROS) production in affected fish with the use of horseradish peroxidase-amplified luminol-dependent chemiluminescence assay. The priming in-vivo infection with TLEV resulted in downregulation of ROS response in both vertically- and horizontally-infected fish; this suppression was further exacerbated by specific in-vitro booster infection with the same virus. Application of Ca ionophore and phorbol myristate acetate as alternative nonspecific boosters enabled restoration of ROS release in vertically-infected but not in horizontally-infected larvae. The results indicate severe TLEV-imposed phagocyte dysfunction in affected larvae. The difference in restoration potential of ROS production after vertical and horizontal virus transmission is interpreted in the frame of principal distinctions between the two modes.

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Introduction

The mode of pathogen transmission determines its survival and persistence in the host. Depending on the environmental conditions, priority is given to vertical or horizontal transmission, and the most effective combination of these two major routes is evolved. Accordingly, development of adequate preventive measures of disease control must be based on correct evaluation of the survival mechanism employed by a given pathogen in a particular environment. Nowadays, assessment of this issue comes to acquire more and more importance (Stewart et al., 2005; Van den Bosch et al., 2010). In this work, we attempted to evaluate a damaging potential of vertical and horizontal virus transmission through ROS response of the affected host.

Generation of ROS is an intrinsic self-protective feature of living organisms. The cells involved in ROS production are phagocytic leukocytes whose primary function in immune defense of organism against unfavorable environmental factors (infection, stress and pollution) is phagocytosis, and generation of ROS (usually referred to as respiratory or oxidative burst) is one of the most frequently accessed responses. The respiratory burst of “professional” phagocytes (granulocytes, monocytes and macrophages) is accompanied by spontaneous or enzymatically mediated production of various ROS, which are powerful microbicidal agents (Gilbert and Colton, 1999; Edwards, 2005).

Modulation of ROS response to pathological challenge varies greatly; the complexity and versatility of this process can be exemplified by controversies and inconsistencies in the data published on influenza (Masini et al., 1984; Arora and Henrichon, 1994) and HIV (Muñoz et al., 1999; Salmen et al., 2007).

In fish, similar to other vertebrates, phagocytosis is one of the major defensive mechanisms (Avtalion and Shahrabani, 1975; MacArthur and Fletcher, 1985). Like in mammals, fish phagocytes are also capable of generating ROS, both in a healthy state (Kadomura and Edwards, 2000). The mechanism of respiratory burst regulation in phagocytes seems to be as complex as in mammals (Novoa et al., 1996), affected by multiple factors (Stave et al., 1984), diverse in different fish species (Nikoskelainen et al., 2006), and, depending on the virus, may result in stimulation, indifference and suppression of ROS production (Novoa et al., 1996; Tafalla et al., 1998; Mohankumar and Ramasamy, 2006).

We have recently described a whirling syndrome-associated disease of tilapia larvae that induced high mortality rates (Shlapobersky et al., 2010). The causal agent of this disease was found in larvae brain tissue.

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and we referred to the disease as viral encephalitis of tilapia larvae. By virtue of morphological, biophysical and phylogenetic analyses, the causal agent was attributed to Herpesviridae family and designated tilapia larvae encephalitis virus (TLEV). The virus was capable of both vertical and horizontal transmission. In this work, we studied severity of impairment of the innate cellular immunity of tilapia larvae induced by vertical and horizontal TLEV transmission by monitoring a phagocyte-mediated ROS response of the infected fish.

Results

Experimental design and structure of the groups to be tested are presented in Fig. 1 and Table 1, respectively.

ROS response to TLEV

Fig. 2 summarizes the delta values of ROS response in experimental groups (the difference between ROS concentrations in test subjects and respective controls) and shows the sign of the deviation of ROS values in test subjects from those in controls. Compared to the uninfected controls (1548 ± 200 cpm/g), the BCL values in minced tissues of larvae infected in-vivo with TLEV, were found to be suppressed twofold, both in vertically-infected (740 ± 89 cpm/g) and horizontally-infected (681 ± 75 cpm/g) subgroups, in both subgroups (1 VER and 1 HOR, respectively) suppression of BCL response was significant (p < 0.01).

Similarly, in-vitro addition of TLEV to cell suspensions of the above in-vivo infected two subgroups resulted in further inhibition of ROS response. Compared to the control group (392 ± 59 cpm/g), StCL values measured in vertically- and horizontally-infected subgroups equaled 111 ± 40 (2 VER) and 126 ± 28 cpm/g (2 HOR), respectively; inhibition in both groups was significant (p < 0.02 and p < 0.01, respectively). Thus both vertical and horizontal routes of TLEV infection, both in priming and booster, resulted in a distinct decrease of CL counts in affected larvae compared to respective controls.

ROS response to nonspecific boosters

Fig. 2 demonstrates a quite different pattern of the StCL response that was observed when the cells of the two diseased subgroups (1 VER and 1 HOR) were stimulated nonspecifically. Depending on the

<table>
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<th>Table 1 Experimental groups’ design.</th>
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Pointed are basic and stimulus constituents of respective controls and test specimens (samples) within the experimental groups. Naïve, uninfected TLEV-negative healthy asymptomatic red tilapia larvae. TM, virus transmission mode, vertical (VER) for blue tilapia larvae and horizontal (HOR) for red tilapia larvae. Accordingly, all groups were composed of VER- and HOR-infected subgroups. Cohabitation (HOR virus transmission) in group 1 was performed as described elsewhere (Shlapobersky et al., 2010). Samples 2-5 were basically composed of group 1 larvae infected in-vivo with TLEV by respective TM, 20 subjects in each subgroup. Cal and PMA, two non-specific stimuli of ROS production, Ca ionophore and phorbol myristate acetate, respectively.

Fig. 1. Schematic representation of the experimental design. Blue tilapia larvae were maternally infected with TLEV (vertical virus transmission). Shortly after specific clinical manifestation of the disease (dark skin pigmentation and whirling syndrome), there was onset of mortality, and mortality rate reached 90–95%. The survivors did not display the above symptoms and, like their mothers, turned to be asymptomatic TLEV-positive carriers. Red tilapia larvae were originally healthy and TLEV-negative: part of them was used as naïve controls, and another part was infected with TLEV by cohabitation with infected blue larvae (horizontal virus transmission). Noteworthy, red larvae did not reveal dark skin pigmentation prior to whirling syndrome that typically appeared in blue larvae; instead, they turned to be slightly pale, which also enabled their distinction from healthy asymptomatic controls. Thus, horizontally infected red larvae were collected in the period between pale pigmentation (preceded to whirling) and the distinct time point of whirling manifestation and onset of mortality (known from our previous work). High mortality rate was recorded in both tilapia subspecies, the survivors turned to be TLEV-carriers. Detailed in Shlapobersky et al., 2010.
mode of virus transmission, the response differed. In vertically-infected cells (3 VER), addition of Cal resulted in restoration of ROS response and its further threefold augmentation beyond the relevant control level (565 ± 100 cpm/g vs. 180 ± 55 cpm/g, respectively, \( p < 0.02 \)). In contrast, elevation of the StCL response in horizontally-infected cells (3 HOR, 271 ± 46 cpm/g) was insignificant (\( p > 0.05 \)).

Likewise, ROS production was restored and amplified beyond the control level, albeit less effectively, by applying PMA, which resulted in significant (\( p < 0.01 \)) stimulation of the StCL response in vertically-infected cells (4 VER, 2.569 ± 334 cpm/g vs. 1.049 ± 152 cpm/g); again, slight rise of the StCL response in horizontally-infected cells (4 HOR, 1.286 ± 288 cpm/g) was insignificant (\( p > 0.05 \)).

To test whether a nonspecific booster may be effective in the immune suppression induced by in-vitro TLEV priming and further exacerbated by in-vitro TLEV booster (subgroups 2 VER and 2 HOR), Cal was added to minced tissues of larvae from these two subgroups. Exposure to Cal resulted in augmentation of the StCL response; again, compared to the control level (350 ± 53 cpm/g), the amplification of ROS response was significant in vertically-infected cells (5 VER, 587 ± 66 cpm/g, \( p < 0.01 \)) and insignificant in horizontally-infected cells (5 HOR, 271 ± 46 cpm/g, \( p > 0.05 \)).

Vertical vs. horizontal virus transmission

Table 2 summarizes relative ROS values in test samples vs. relative ROS values in respective controls. All the data indicate a negative impact of TLEV on ROS production in groups 1 and 2 (downregulation of ROS response in both VER and HOR subgroups). Interestingly, the cumulative specific virus-induced suppression of ROS release in these two groups was equal in vertical and horizontal transmission (0.76 relative units). Based on this result, we might have expected a similar effect in both transmissions when applying nonspecific stimuli (Cal and PMA) in attempt to restore ROS production. However, depending on the virus transmission mode, the results strikingly differed. Application of nonspecific stimulatory boosters was successful to overcome the effect of specific suppression imposed by maternally transferred virus (vertical transmission) but proved to be inefficient in specific suppression imposed by horizontal transmission.

Discussion

Given a high mortality rate of tilapia larvae caused by the newly described virus (TLEV), a major objective in this work was evaluating an impairment of innate cellular immunity in affected subjects and comparing severity of this damage in larvae infected by two different routes, vertical and horizontal virus transmission. To this end, we accessed a cellular immune response of fish by monitoring the phagocyte-mediated ROS response.

ROS response to TLEV

In both modes of virus transmission, ROS release proved to be suppressed. Recently we have suggested a distinct negative correlation between ROS production and severity of pathological states (Sinyakov et al., 2007, 2010). In this concept, within a reasonable range of ROS response and outside a deleterious effect of the oxidative burst (ROS overproduction), the sign of ROS deviation from its basal level (i.e. increase or decrease in ROS production) acquired a critical significance: stimulation of ROS is indicative of an active resistant potency of ROS-producing cells in protective immune response whereas suppression of ROS is indicative of severe impairment or exhausted potential of these cells to resist pathology. In this meaning, we may interpret a prompt downregulation of ROS release as a direct indication of severe TLEV-imposed phagocyte dysfunction in affected fish mediated through virus-specific cellular receptors.

Thus we substantiate our assumption that TLEV is an extremely dangerous herpes encephalitis virus that disables fish phagocytes and promotes rapid and fatal development of brain pathology. Indeed, ROS production in fishes during the larval stage is concentrated in the head area (Kadomura et al., 2007), and ROS-producing cells might be one of the primary TLEV targets. Decreased generation of ROS can lead to impaired immune defense (Storz, 2007) and result in severe diseases (Segal, 1996). Myeloperoxidase deficiency in fish also makes immature immune system of larvae particularly vulnerable to external pathogens (Dubansky et al., 1998; Lanza, 1998).

ROS response to nonspecific boosters

To access severity of TLEV-mediated phagocyte dysfunction with regard to ROS production, we added nonspecific stimulators of ROS (PMA and Cal) to virus-infected cells. Both of these compounds are involved in activation of protein kinase C and Ca channels (Suena

### Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ratio</th>
<th>SI</th>
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<tbody>
<tr>
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</tr>
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<td>VER2:ctrl2</td>
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<td>VER3:ctrl3</td>
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<td>VER5:ctrl5</td>
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<tr>
<td>HOR1:ctrl1</td>
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<tr>
<td>HOR2:ctrl2</td>
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<tr>
<td>HOR3:ctrl3</td>
<td>1.51</td>
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</tr>
<tr>
<td>HOR4:ctrl4</td>
<td>1.23</td>
<td></td>
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<tr>
<td>HOR5:ctrl5</td>
<td>0.69</td>
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Pointed are relative values of ROS production calculated as ratios of chemiluminescence in relevant controls and test specimens (samples) within the experimental groups structured as indicated in Table 1. SI, stimulation index of ROS production calculated as ROS value after stimulus treatment divided by ROS value prior to stimulus treatment.
Both compounds, albeit with different efficacy, were able to trigger ROS release in maternally infected subjects (vertical virus transmission) but were incapable of surmounting a specifically induced downregulation of ROS response in cohabitation infected larvae (horizontal virus transmission).

**Vertical vs. horizontal virus transmission: which one is worse?**

The major finding that emerged from the work with nonspecific stimuli was the fact that the restoration potential of ROS production was consistently higher in vertically-infected fish, while none of horizontally-infected groups developed ROS response beyond the control level. To explain this phenomenon, we will refer to the basic features that distinguish the two modes of virus transmission.

Vertical transmission is accompanied by ‘natural’ or ‘intrinsic’ immunological tolerance and induction of specific immune suppression of the protective networks of progeny towards maternally-derived components, which are recognized as self, including possibly presenting pathogen(s) (Burnet, 1969; McCullagh, 1996; von Siebenthal et al., 2009). In this situation, particularly in freshwater and seawater without environmental pressure from competing pathogens, the optimal survival strategy of a novel emerging virus would be a mild infectivity along with conferring a healthy carrier status to a susceptible host. Under the most favorable conditions of host tolerance and immune suppression, the virus takes full control over the host and survives in a latent state. The protective immunity network is “switched-off,” neutralized and disabled but it is not destroyed since it did not oppose to the virus. Thus it retains a definite and significant restoration potential of ROS production (a resistance potential) after being activated by “switching-on” triggers.

We have suggested earlier that, in order to survive in the original natural environment, the newly emerged virus might apply the tactics of a balanced co-existence with its new host. In this case, TLEV may reside in tilapia in a dormant state as a latent neurotropic infection, a typical feature of alphaherpesviruses (Jones, 1998; Mettenleiter, 2003; Mettenleiter et al., 2008). Critical mutation(s) that trigger conversion of TLEV from a dormant state to a terminating lethal virus might occur when carrier fish are transferred to aquaculture. In the frame of interrelation between pathogen virulence and host population structure (Boots et al., 2004; Nair, 2005), abundant supply of vulnerable host (in particular, intensively cultured farmed larvae and fry with immature immunity) might become an easy target for the virus that acquired a drastically increased virulence.

In contrast, horizontal (or waterborne) transmission proceeds as an uncompromised fighting of the virus with the immune system of a healthy host, which recognizes the virus as a non-self threat and activates protective networks to oppose this threat. In horizontal transmission by cohabitation, the virus attack may be substantially supported by an ally. In aquaculture systems, along with unavoidable handling stress, one of the major unfavorable environmental impacts is confinement due to high densities of rearing fishes (Portz et al., 2006). Confinement stress greatly contributes to weakening of the immune system of fish and makes it more vulnerable to pathogen invasion (Avtalion, 1981; Wise et al., 1993; Cubero and Molinero, 1997). Importantly, along with other systems affected, confinement stress was evidenced to suppress the phagocyte-mediated ROS production (Vazzana et al., 2002). Facing combined impact of the virus and confinement stress, immature immune system of larvae is usually incapable of successful resistance.

The final result of horizontal transmission—the virus control over the host—might be indistinguishable from that of vertical transmission (cumulative 0.76 relative units, Table 2), but the status of the impaired host immune system differs principally: it is irreversibly destroyed or badly damaged in the former case, and it is disabled, neutralized or mildly damaged in the latter case. Accordingly, the restoration potential of protective immunity in vertically-induced infection is superior to that in horizontally-transferred disease, which may reasonably explain our observations.

**Concluding remarks**

In our recent work, we have primarily characterized a newly emerging herpes-like encephalitis virus responsible for high mortality rate of tilapia larvae (TLEV). We have also described vertical and horizontal modes of TLEV transmission. In the present work, to gain further insight into the mechanisms involved in TLEV-induced pathology, we have found that one of the principal innate cellular immune responses of tilapia larvae, ROS-producing activity, had been strongly compromised, and the phagocyte-mediated ROS production had been efficiently suppressed. The level of downregulation of ROS response was essentially the same in both vertical and horizontal virus transmission. However, vertically-infected larvae possessed a distinct potential for restoration of ROS production whereas the impairment of innate immunity after horizontal transmission appeared irreversible. In addition to our previous remark that the disease may be easily overlooked in the routine practice of larvae harvest, these results further emphasize a serious potential threat of TLEV for tilapia aquaculture.

**Methods**

**Virus**

Virus was isolated from the naturally infected tilapia larvae which exhibited the whirling symptoms of a generalized infection. The virus isolation and purification procedures were essentially the same as described elsewhere (Shlapobersky et al., 2010).

**Fish**

Fig. 1 outlines a primary experimental design with regard to the two subspecies of tilapia larvae used in this work and the two principal modes of TLEV transmission applied to these subspecies. Vertical transmission of the disease was achieved in the subjects of locally developed inbred gynogenetic line of blue tilapia (Oreochromis aureus) larvae (aged 20–25 days and weighing 40–60 mg), which were descendants of TLEV-carrier mothers. Their counterparts from the originally healthy asymptomatic red tilapia larvae of the same age and weight were taken as naïve uninfected controls and were allowed to contact with maternally infected subjects of blue tilapia larvae by cohabitation to carry out horizontal transmission of the disease. Blue and red tilapia larvae TLEV-infected by vertical and horizontal virus transmission were taken for ROS analysis shortly before the whirling syndrome manifestation.

Fish cell suspensions were prepared as described elsewhere (Belotsky et al., 1990). Briefly, larvae of each group were weighed and homogenized by scissors in plastic cuvettes containing 0.2 ml 0.15 M phosphate buffered saline (PBS, pH 7.3), supplemented with 5 mM glucose, and 0.2 ml PBS was added again thereafter. Alternative methods of homogenization including pressing of tissues through the mesh resulted in cell damage and thus rendered the cells incapable of adequate responding. Minced non-fractionated homogenate of whole-body cells contained all major types of cells known to be involved in ROS production such as blood and kidney phagocytes (neutrophils and macrophages). This approach enabled interpretation of the results as a total integral ROS response of larvae to the stimuli applied.

Importantly, fish leukocytes are extremely sensitive to routine treatments usually applied to mammals’ cells, i.e. centrifugation, hypotonic shock (to remove erythrocytes), and even intensive shaking in a tube (while preparing a specimen for ROS measurements). All these manipulations might induce an immediate high spontaneous respiratory burst, which interferes with monitoring a
true ROS response to any stimulus applied. To avoid artifact results, maximal care was taken while working with homogenates, stirring of suspensions was carried out with minimally possible physical impact.

**ROS measurements**

Chemiluminescence (CL) assay is an adequate and the most popular method of choice for studying free radical reactions in cells and tissues; CL intensity is directly proportional to a steady-state concentration of the radicals involved, which is a distinct advantageous feature of this technique (Vladimirov and Proskurina, 2009). Estimation of the phagocytic activity of cells with regard to ROS production is one of the most important and widely used applications of the assay. Luminol-dependent CL technique has been applied to follow-up the formation of ROS in homogenate specimens of tilapia larvae cells subjected to virus infection in-vivo and in-vitro (specific stimulus) as well as exposed to in-vitro treatment with Ca ionophore (Cal) and phorbol myristate acetate (PMA) (nonspecific stimuli). However, the cellular CL response after application of both specific and nonspecific stimuli proved to be beyond the sensitivity of the assay and could not be detected. This failure, which can be attributed to the intrinsic myeloperoxidase deficiency in fish (Iida and Wakabayashi, 1995; Dubansky et al., 1998), prompted us to change the assay design and to use horse radish peroxidase (HRP) as an exogenous peroxidase source (Dahlgren and Stendhal, 1983) to artificially amplify otherwise undetectable ROS production in minced larval tissues. Following this modification, we were able to carry out CL measurements. To standardize the results, the output CL measured in counts per minute (cpm) was divided by homogenate weight (g) and thus reflected the ROS concentration in the same number of cells per 1 g (cpm/g).

The exogenous HRP-amplified luminol-dependent CL assay was employed throughout with the use of Biocounter M1550L (Lumac, Holland) for monitoring CL changes. To ensure for intracellularly generated hydrogen peroxide to be detected on the outside, the CL reaction was allowed to proceed in the presence of azide (Dahlgren and Stendhal, 1983) to prevent for intracellularly generated hydrogen peroxide to be detected on the outside, the CL reaction was allowed to proceed in the presence of azide (Dahlgren and Stendhal, 1983) to artificially amplify otherwise undetectable ROS production in minced larval tissues. Following this modification, we were able to carry out CL measurements. To standardize the results, the output CL measured in counts per minute (cpm) was divided by homogenate weight (g) and thus reflected the ROS concentration in the same number of cells per 1 g (cpm/g).

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**Statistics**

Five independent measurements were made within every one of the experimental groups. The results (M ± SE) were expressed as peak values of CL in counts per minute per gram of minced homogenate (cpm/g). The unpaired two-tailed t-test was applied for evaluation of differences between the groups.

**References**


