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RESEARCH ARTICLE

Impact of cyprinid herpesvirus-3, which causes interstitial nephritis and gill necrosis, on the activity of carp (*Cyprinus carpio* L.) macrophages and lymphocytes

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Abstract. The aim of the study was to determine the impact of CyHV-3 on the development of interstitial nephritis and gill necrosis in carp, *Cyprinus carpio* L., and on the metabolic activity of pronephric macrophages analyzed with respiratory burst activity (RBA) stimulated by PMA and the proliferative response of pronephric lymphocytes stimulated by mitogens ConA and LPS. CyHV-3 isolate was used in the *in vitro* experimental study and quantified with plaque assay using koi fin cell (KFC) culture incubated at 24°C for 96 h. The macrophage or lymphocyte suspensions were deposited in 96-well culture plates and incubated with CyHV-3 (1×10^6 pfu ml^{-1} RPMI-1640) at 16, 18, 22, and 2°C. The results indicated that CyHV-3 decreased the level of macrophage respiratory burst activity in comparison with the control at 16, 18, and 22°C, but increased it at 28°C. A similar pattern was observed with lymphocyte proliferation stimulated by ConA and LPS. The results showed that CyHV-3 decreased the proliferative response of pronephric lymphocytes stimulated by ConA and LPS in comparison to the control at 16, 18, and 22°C, while it only increased the proliferative response of lymphocytes stimulated by ConA at 28°C. This preliminary *in vitro* study demonstrated the strong inhibitory influence of CyHV-3 on macrophage activity and the proliferative response of lymphocytes. This inhibitory effect is determined by temperature.

Keywords: carp, CyHV-3, macrophage activity, lymphocyte proliferation

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Introduction

A decade ago, considerable losses noted in carp, *Cyprinus carpio* L., culture were attributed to a new syndrome caused by a virus of the family Herpesviridae and described as koi herpesvirus (KHV) (Gray et al. 2002, Haenen et al. 2004). Later studies indicated that KHV differed insignificantly from the virus that caused mass carp kills in Israel and Europe, and according to the clinical pathology it caused, it was identified as carp interstitial nephritis and gill necrosis virus, or CNGV (Pikarsky et al. 2004). Currently, this virus is referred to as cyprinid herpesvirus-3 (CyHV-3) (Waltzek et al. 2005). Differences in the structures of KHV and CyHV-3 are not significant: both viruses have twenty-sided cores approximately 100-110 nm in diameter, and both are enveloped viruses with a threaded coating on the core structure similar to that of herpes virus DNA. The difference between them is in the size of the double molecule of DNA, which is larger in CyHV-3 than in KHV (Pikarsky et al. 2004, Waltzek et al. 2005). However, there is little data in the available literature regarding the pathogenesis of the virus or on its impact on immunocompetent cells and nonspecific cellular and humoral defense mechanisms. Characteristic features of CyHV-3 include that it is highly pathogenic, and the disease caused by it is

septicemic. Exudative fluid collects in the body cavities of infected fish, and their internal organs become enlarged with numerous necrotic lesions. These pathological changes in carp and koi carp occur in kidney hematopoietic tissues, hepatocytes, the spleen, and throughout the digestive tract. Necrosis of skin and gill cells is particularly significant as it causes mass fish kills. Infected cells exhibit characteristic degenerative pathology including nuclear disintegration and vacuolization and reduced cytoplasmic surface tension (Pokorova et al. 2005, Siwicki et al. 2006, Novotny et al. 2010). The aim of the study was to determine the impact of CyHV-3 on the macrophage metabolic activity and proliferative response of lymphocytes of the carp head kidney.

Materials and methods

The study was performed on 10 carp individuals with body weights of 180-200 g, which were obtained from the Experimental Fisheries Department, Inland Fisheries Institute in Żabieniec. The fish were held in a closed recirculating system at a water temperature of 20±1°C, and the water physicochemical parameters were monitored continually. The fish were anesthetized with Propiscin (IFI Żabieniec) before the head kidney and spleen were excised for in vitro tests. After preparation, the head kidneys and spleens were submerged in RPMI-1640 medium (Sigma). The cells were isolated using the method described by Siwicki et al. (2008). Lymphocytes and macrophages were isolated from the head kidney after gradient centrifugation in a Histopaque-1077 (Sigma). The cells were stained with a 0.1% trypan blue solution, and then their viability and concentration were determined microscopically.

CyHV-3 isolated from carp exhibiting clinical disease symptoms and cultured on koi fin cells (KFC) was used in the study. The virus was identified with PCR using primers to amplify CNGV/CyHV-3 thymidine kinase (Siwicki et al. 2006).

The metabolic activity of the macrophages isolated from the kidneys was determined based on their respiratory burst activity (RBA) after stimulation with phorbol myristate acetate (PMA; Sigma) using the method described by Siwicki et al. (2008). Cell suspensions (100 µl volume) from each of the ten fish specimens at a concentration 1×10^6 ml^{-1} RPMI-1640 were placed in a 96-well culture plates (Nunclon) and incubated with a 20 µl solution of CyHV-3 virus (1×10^6 pfu ml^{-1} RPMI-1640) for 2 h at temperatures of 16, 18, 22, and 28°C. The control was cell suspensions from each fish that were not exposed to the virus. The cells were then rinsed in RPMI-1640 and were subjected to nitroblue tetrazolium (NBT Sigma, 2 mg ml^{-1}) with the addition of PMA (Sigma, 50 µg ml^{-1}) for 30 m at analogous temperatures. After incubation, the plates were rinsed with 70% ethyl alcohol and 120 µl 2 M KOH and 140 µl DMSO (Sigma) were added, and optical densities (OD) of the control and test samples were read at a wavelength of 620 nm on a spectrophotometer (MRX 3 Dynatech).

The lymphocyte proliferation (LP) response of cells isolated from carp kidney and stimulated with concanavalin A (ConA, Sigma) and lipopolysaccharide (LPS, Sigma) was determined with spectrophotometry after incubation with CyHV-3 (Siwicki et al. 1999). ConA (64 µg ml^{-1}) or LPS (160 µg ml^{-1}) in volumes of 100 µl were added to suspensions of lymphocytes (100 l volume) from each fish (10 individuals) at concentrations of 5×10^6 ml^{-1} RPMI-1640 with the addition of 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% Hepes buffer (Sigma), antibiotics (penicillin/streptomycin: 100 U/100 µg ml^{-1}), and 10% fetal calf serum (FCS) and then incubated at temperatures of 16, 18, 22, and 28°C. After a two-hour incubation the lymphocyte culture was infected with CyHV-3 (20 µl, 1×10^6 pfu ml^{-1} RPMI-1640). The control was cultured lymphocytes that were not infected with the CyHV-3 virus. Following a 72-h incubation at different temperatures, a 20 µl solution of MTT (3[4,5-dimethylthiazole-2-yl]2,5-diphenyltetrazoliu m bromide, Sigma) at a concentration of 5 mg ml^{-1} was added for a period of 4 h. Then, the fluid was drained from the culture, 100 µl isopropanol (Sigma) was added, and after a 10 min incubation the optical

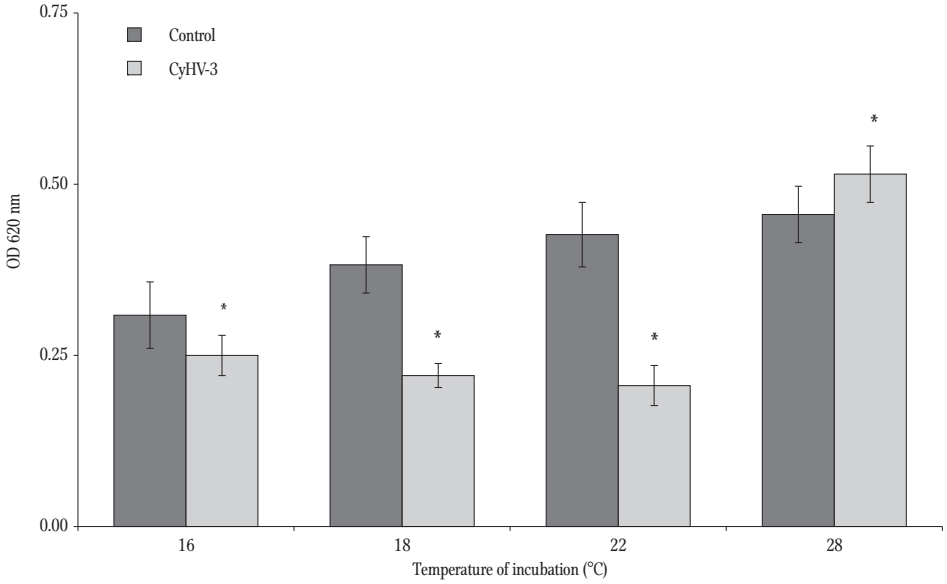

Figure 1. Impact of the CyHV-3 virus on respiratory burst activity of macrophages isolated from carp kidneys and incubated at temperatures of 16, 18, 22, and 28°C (mean values ± standard deviation; n=10; * statistically significant at P<0.05).

density (OD) was read at a wavelength of 620 nm with a spectrophotometric microreader (MRX 3 Dynatech).

The results were analyzed statistically to determine means and standard deviation (SD) and multivariate analysis of variance (ANOVA) was applied. The significance of differences in comparison to the control group were determined at a level of $P < 0.05$. Statistica for Windows 7.1 (Stat-Soft Inc. 2004) was used.

Results

The results of the study indicated that the CyHV-3 virus strongly suppresses the metabolic activity of macrophages isolated from carp kidneys depending on temperature. The highest statistically significant ($P < 0.05$) lowering of macrophage RBA was noted at the temperatures of 18 and 22°C (Fig. 1), but this was also statistically significant ($P < 0.05$) in comparison to the controls (cells not exposed to CyHV-3) at a temperature of 16°C. However, statistically significantly ($P < 0.05$) increased macrophage RBA was noted in comparison to that of the control at a temperature of 28°C.

The analysis of the results of the study of the impact of the CyHV-3 virus on the proliferative response of lymphocytes stimulated with ConA and LPS and then incubated at different temperatures indicated unequivocally that the virus has a strong suppressive impact. At the incubation temperatures of 16, 18, and 22°C, the proliferative response of lymphocytes stimulated with both ConA and LPS was lowered statistically significantly. The greatest suppressive impact was noted following stimulation with ConA at temperatures of 18 and 22°C (Fig. 2) and following stimulation with LPS at a temperature of 22°C (Fig. 3). However, at the temperature of 28°C a statistically significant ($P < 0.05$) increase in the proliferative response of lymphocytes stimulated with ConA was noted, but no such increase was noted with regard to lymphocytes stimulated with LPS.

Discussion

One of the characteristic aspects of fish herpesviruses, and particularly of the KHV and CyHV-3 viruses, is their high pathogenicity, and the diseases caused by them are of a septicemic character. Exudative fluid collects in the body cavities of

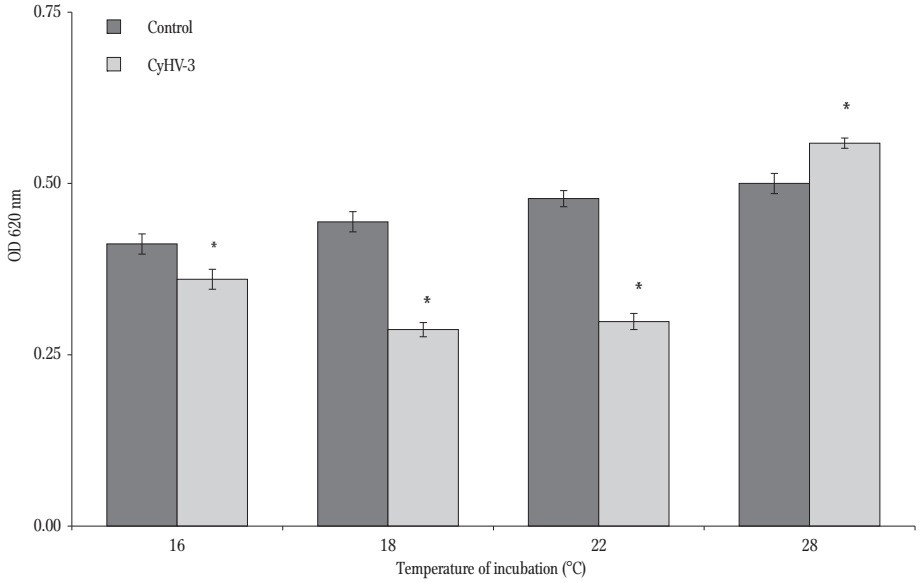

Figure 2. Impact of the CyHV-3 virus on the proliferative response of lymphocytes isolated from carp kidneys and stimulated with ConA and incubated at temperatures of 16, 18, 22, and 28°C (mean values ± standard deviation; n=10; * statistically significant at P<0.05).

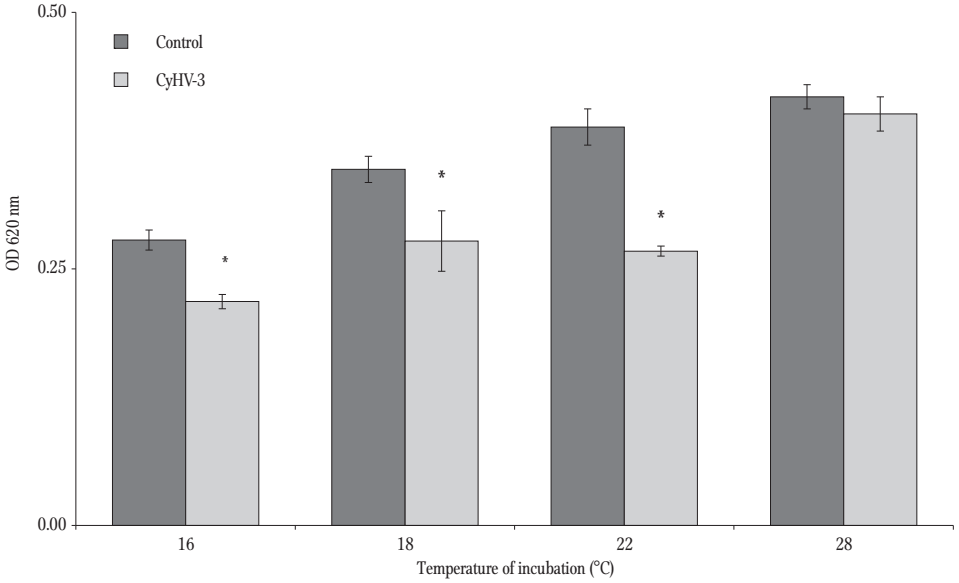

Figure 3. Impact of the CyHV-3 virus on the proliferative response of lymphocytes isolated from carp kidneys and stimulated with LPS and incubated at temperatures of 16, 18, 22, and 28°C (mean values ± standard deviation; n=10; * statistically significant at P<0.05).

infected fish, and their internal organs become enlarged with numerous necrotic lesions. These symptoms in carp are most frequently observed in the kidney hematopoietic tissues, hepatocytes, and the spleen. Necrosis of skin and gill cells is particularly significant as it causes mass fish kills. Infected cells exhibit characteristic degenerative pathology including nuclear disintegration and vacuolization and reduced cytoplasmic surface tension (Novotny et al. 2010). The CyHV-3 virus is also frequently associated with secondary bacterial (*Aeromonas*, *Pseudomonas*, *Flavobacterium*) or fungal (*Saprolegnia*, *Achlya*) infections, which suggests the strong suppression of nonspecific defense mechanisms that predispose fish to such infections (Siwicki et al. 2004).

The impact of the CyHV-3 virus on macrophages and T and B lymphocytes is strictly dependent on temperature. At temperatures of 16, 18, and 22°C, the tested virus had a strong suppressive impact, but at the temperature of 28°C an increase in the activity of macrophages and T and B lymphocytes was noted, which could suggest that the virulence of the virus is lowered. It is also possible that macrophages and lymphocytes are more resistant to the virus at higher temperatures. Other studies and clinical observations have indicated that the optimal temperature for the development of carp interstitial nephritis and gill necrosis is above 20°C (22-26°C), while the virus can develop at temperatures of 18-25°C (Ronen et al. 2003, Haenen et al. 2004). However, at temperatures exceeding 30°C there is a sudden inhibition of the development of infection. The latency period between viral exposure and the occurrence of symptoms is strictly dependent on temperature and ranges from 10-12 days (Ronen et al. 2003, Pikarsky et al. 2004), and the lower the temperature is, the longer the disease takes to develop (Gilad et al. 2003, Pokorova et al. 2005).

The results obtained suggest unequivocally that the CyHV-3 herpesvirus inhibits the activity of macrophages and lymphocytes that are responsible for the proper functioning of nonspecific defense mechanisms and determine the specific immune response in fish. Macrophages, which have immediate cytotoxicity against infected cells, play a key role in antiviral immunity regardless of the presence of specific antibodies or complementary components. Macrophages inhibit viral replication in cells by excreting arginase, which interferes with viral arginine utilization. T lymphocytes also play a key role in antiviral immunity thanks to their ability to kill cells infected with virus. The results presented here of this initial study indicate that the CyHV-3 virus has a strong immunotropic impact especially in its ability to inhibit the activity of macrophages and lymphocytes in carp. This supplies the impetus for further study of the morphology and pathogenicity of this new herpesvirus that causes mass carp kills in pond culture the world over.

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Author contributions. A.K.S., and E.M.Z. designed and performed the experiment; A.K.S., and B.K. analyzed the data; A.K.S., and K.K. wrote the paper.

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