Respiratory burst in neutrophilic granulocytes of carps (*Cyprinus carpio*): cytometric studies

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ABSTRACT: Present studies aimed at flow cytometric analysis of respiratory burst in neutrophilic granulocytes in carps, at various stages of their ontogeny. Cytometric evaluation of the repiratory burst in PMN cells of carps demonstrated augmented values of the mean fluorescence channel of PMA-stimulated PMN cells, as compared to the value of the fluorescence channel noted for PMN cells which were not activated or did not respond to activation in response to the applied stimulator. Intensity of the fluorescence, compared to the mean fluorescence channel of the cells which were not stimulated by PMA, was most pronounced in 11- to 21-month old carps, average in the youngest carps aging 3 to 9 months, and the lowest in the eldest, 23- to 29-month old carps. In cases of analysis of the mean fluorescence channel in the selected fraction of PMA-stimulated PMN cells, as compared to the mean fluorescence channel of granulocytes which did not respond by the stimulation reaction, the most intense fluorescence was noted to develop in the eldest, i.e. 23- to 29-month old fish. In the remaining carps, on the other hand, the difference in the mean fluorescence channel was significantly lower. In parallel, the highest fraction of PMA-stimulated granulocytes was found in 11- to 21-month old carps, a lower fraction in the youngest carps aging 3 to 9 months and the lowest fraction of such granulocytes in the eldest carps, 23 to 29 months of age.

Keywords: carp; PMN cells; respiratory burst; cytometry

Flow cytometry opens the potential for monitoring metabolic activity of immune system cells. It was also used in studies on immune system cells in the fish even if surface markers specific for cells of the animals still await a more thorough definition. Till now, the method has found application in genetic, pathogenetic studies, in studies on immunopathological processes (Rodriguez *et al.*, 1991, 1993, 1995; Perez *et al.*, 1994; Chilmończyk *et al.*, 1995; Chilmończyk and Monge, 1999) as well as in evaluation of immune cell functions and some parameters of cell-mediated immunity (Ellsaesser *et al.*, 1985; Evans *et al.*, 1987; Bly *et al.*, 1990; Secombes and Fletcher, 1992; DeLuca *et al.*, 1993; Morgan *et al.*, 1993; Lamas and Ellis, 1994; Voccia *et al.*, 1994; Chilmończyk *et al.*, 1995, 1997; Pettersen *et al.*, 1995; Slierendrecht *et al.*, 1995; Hamdani *et al.*, 1998; Siegl *et al.*, 1998; Chilmończyk and Monge, 1999) Present studies aimed at flow cytometric analysis of respiratory burst in neutrophilic granulocytes in carps, at various stages of their ontogeny.

MATERIAL AND METHODS

Fifty-four carps were examined, aging 3 to 29 months, 30 to 1 000 g in body weight, which were studied in three experimental groups: group I of 20 carps, aging 3 to 9 months, group II of 20 carps, aging 11 to 21 months, and group III of 14 carps, aging 23 to 29 months. Condition and health status of the fish were examined using techniques generally accepted in the veterinary-medical diagnosis. Blood of carps was studied. Blood samples were taken from tail vein to tubes containing heparin (50 IU per ml blood), in field conditions, immediately after taking the fish out of the pond. Laboratory tests were performed 60 to 80 min after blood sampling, i.e. immediately after delivery of the blood samples to the laboratory.

Cytometric studies on oxygen metabolism in neutrophilic granulocytes (PMN cells) were performed by the technique of Hasui *et al.* (1989), adapted to fishes. Diluted samples of blood (100 µl) were supplemented

each with 15 µl 0.3 mM DCFH-DA (2´7´-dichlorofluorescein, Acros, USA) and, then, following 30 min incubation at room temperature (21°C) with 2 µl PMA (phorbol 12-myristate 13-acetate, Sigma, USA, 1 mg per ml 95% ethanol) in order to stimulate hydrogen peroxide production by neutrophilic granulocytes. After another incubation of 20 min at room temperature, 300 µl Ortho-mune Lysing Reagent (Ortho Diagnostics System, Germany) was added to each sample. So prepared samples were left for 20 min and, then, fluorescence of 2,7-dichlorofluorescein (DCF) was measured within the green light band (515 to 548 nm) using Cytoron Absolute flow cytometer, employing logarythmic signal amplification. Intensity of the fluorescence was measured estimating values of the so called mean fluorescence channel, calculated using the Immuno Count 2 software. Intensity of the photoelectric signals, converted to numerical values, permitted to define the extent of labelling phagocytic cells with the fluorochrome. Results of the studies are summed up in Tables 1 and 2.

RESULTS AND DISCUSSION

Cytometric analysis of respiratory burst in neutrophilic granulocytes of carps demonstrated increased values of the mean fluorescence channel in PMA-stimulated neutrophilic granulocytes, as compared to the mean fluorescence channel obtained for neutrophilic granulocytes which were not activated or did not respond to activation with the applied stimulator. Intensity of neutrophilic granulocyte fluorescence, compared to the mean fluorescence channel of cells which were not stimulated by PMA, was most pronounced in carps aging 11 to 21 months, average in the youngest fish, aging 3 to 9 months, and the lowest in the eldest, 23- to 29-month old carps (Table 1). A distinct pattern was observed upon analysis of the mean fluorescence channel in a separated fraction of PMA-stimulated, neutrophilic granulocytes, as compared to the mean fluorescence channel in granulocytes which did not respond by activation reaction. In this case, the most pronounced fluorescence developed in the eldest carps, aging 23 to 29 months. On the other hand, in the remaining carps aging either 3 to 9 months or 11 to 21 months the difference in the mean fluorescence channel was significantly less pronounced (Table 2). The highest proportion of PMA-stimulated PMN granulocytes was noted in 11- to 21-month old fish, lower in 3- to 9-month old carps and the lowest in 23- to 29-month old carps (Table 2).

Results of Hamdani *et al.* (1998) indicate that respiratory burst activity is manifested both by cells of non-fractionated leukocyte population and by neutrophilic granulocytes isolated with the use of monoclonal antibodies, E3D9 (Pettersen *et al.*, 1995). However, the property has been found to characterise first of all the $E3D9^+$ cells, neutrophilic granulocytes (Secombes and Fletcher, 1992; Lamas and Ellis, 1994; Hamdani *et al.*, 1998) even if the process has also been demonstrated in macrophages (Secombes and Fletcher, 1992; Lamas and Ellis, 1994). Thus, the observations made in our own studies resemble those made by other authors (Secombes and Fletcher, 1992; Lamas and Ellis, 1994; Pettersen *et al.*, 1995; Hamdani *et al.*, 1998). It pertains in particular the observations on the significant role of oxygen metabolism in defensive mechanisms of neutrophilic granulocytes. Our studies have shown in addition that numerical values of the mean fluorescence channel, determined using PMA stimulator, represent a good objective indicator of the cell metabolic and bactericidal activity of neutrophilic granulocytes and may play the role of an index of phagocytic capacity of the cells. However, it should be added that results of our studies have been obtained in a population distinguished only on grounds of FSC/SSC characteristics and not identified by respective monoclonal

Table 1. Mean fluorescence channel of PMN cells in blood of healthy carps with or without supplementation of PMA

Group of carps	Mean fluorescence channel for population of PMN cells		Difference* (range)
	DCFH	DCFH+PMA	
I (n = 20)	81.1 ± 6.3	131.3 ± 10.4	47.2–57.3
II (n = 20)	84.6 ± 7.1	152.7 ± 12.3	76.1–78.9
III (n = 20)	91.7 ± 8.7	108.9 ± 8.9	21.3–23.8

* difference in the value of mean fluorescence channel between samples with and without stimulator

Table 2. Mean fluorescence channel of PMA-stimulated PMN cells in blood of healthy carps

Group of carp	Mean fluorescence channel for population of PMN cells		Difference** (range)
	DCFH+PMA	% stimulated PMN cells*	
I (n = 20)	184.5 ± 11.2	68.3 ± 5.4	87.9–90.3
II (n = 20)	179.6 ± 14.6	88.7 ± 8.1	89.3–95.2
III (n = 14)	174.2 ± 12.5	45.4 ± 3.7	116.4–118.3

* percentage of PMN cells which positively responded to stimulator supplementation

** difference in results of the "mean fluorescence channel" between PMN cells responding (stimulated) to non-responding to the stimulator

antibodies (Secombes and Fletcher, 1992; Lamas and Ellis, 1994; Pettersen *et al.*, 1995; Hamdani *et al.*, 1998). The respiratory burst activity has pertained the cell fraction characterised by high values of FSC and SSC. It should also be stressed that oxygen metabolism of neutrophilic granulocytes, measured by flow cytometry, has not correlated with ontogenetic development of the carps and, thus, the data obtained till now with the conventional techniques, used earlier for evaluation of the phenomenon and in immunological diagnosis in fish (Stosik, 1995), have not been confirmed.

SUMMARY

Cytometric analysis of oxygen metabolism in neutrophilic granulocytes demonstrated increased values of the mean fluorescence channel in PMA-stimulated cells as compared to the result obtained for neutrophilic granulocytes, which were not activated or did not respond to stimulation by the activation. The obtained data did not correlate with ontogenetic development of studied carps.

REFERENCES

Bly J., Miller N.W., Clem L.W. (1990): A monoclonal antibody specific for neutrophils in normal and stressed channel catfish. Dev. Comp. Immunol., *14*, 211–221.

Chilmończyk S., Monge D. (1999): Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens. Fish Shelfish Immunol., *9*, 319.

Chilmończyk S., Voccia I., Monge D. (1995): Pathogenesis of viral haemorrhagic septicemia virus: cellular aspects. Vet. Res., *26*, 501–511.

Chilmończyk S., Voccia I., Tarazona J.V., Monge D. (1997): Flow cytometric analysis of fish leucocyte populations exposed to pollutants and pathogens: modulatory effects induced by experimental procedures. In: Zelikoff J.T. (ed.): Ecotoxicology: Responses, Biomarkers and Risk Assessment. SOS Publications, Fair Haven. 171–184.

DeLuca D., Wilson M., Warr G. (1983): Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol., *13*, 546–551.

Ellsaesser C.F., Miller N.W., Cuchens M.A., Lobb C.J., Clem L.W. (1985): Analysis of channel catfish peripheral blood leucocytes by bright-field microscopy and flow cytometry. Trans. Am. Fish. Soc., *114*, 279–285.

Evans D.L., Smith E.E. Jr., Brown F.E. (1987): Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*). Dev. Comp. Immunol., *11*, 95–104.

Hamdani S.H., McMillan D.N., Pettersen E.F., Wergeland H., Endresen C., Ellis A.E., Secombes C.J. (1998): Isolation of rainbow trout neutrophils with an anti-granulocyte mo-noclonal antibody. Vet. Immunol. Immunopathol., *63*, 369–380.

Hasui M., Harabayashi Y., Hattori K., Kobayashi Y. (1989): Simultaneous measurement by flow cytometry of phagocytosis and hydrogen peroxide production of neutrophils in whole blood. J. Immunol. Meth., *117*, 53–58.

Lamas J., Ellis A.E. (1994): Atlantic salmon (*Salmo salar*) neutrophil responses to *Aeromonas salmonicida*. Fish Shell-fish Immunol., *4*, 201–219.

Morgan J.A.W., Pottinger T.G., Rippon P. (1993): Evaluation of flow cytometry as a method for quantification of circulating blood cell population in salmonid fish. J. Fish Biol., *42*, 131–141.

Perez S.I., Rodriguez S., Vilas P. (1994):. Flow cytometry in fish virology. In: Stolen J.S., Fletcher T.C., Rowley A.F., Zelikoff J.T., Kaattari S.L., Smith S.A. (eds.): Techniques in Fish Immunology. SOS Publications, Fair Haven. 161–173.

Pettersen E.F., Fyllingen I., Kavlie A., Maaseide N.P., Glette J., Endresen C., Wergeland H.I. (1995): Monoclonal

antibodies reactive with serum IgM and leukocytes from Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol., *5*, 567–584.

Rodriguez S., Vilas P.M., Palacios M., Perez S.I. (1991): Detection of infectious pancreatic necrosis in a carrier population of rainbow trout, *Oncorhynchus mykiss*, by flow cytometry. J. Fish Dis., *14*, 545–553.

Rodriguez S., Perez S.I., Vilas P. (1993): Flow cytometric analysis of infectious pancreatic necrosis virus attachment to fish sperm. Dis. Aquat. Org., *15*, 153–156.

Rodriguez S., Vilas P.M., Gutierrez C., Perez S.I. (1995): *In vitro* quantitative kinetic study of infectious pancreatic necrosis viral antigen by flow cytometry. Fish Pathol., *30*, 1–5.

Secombes C.J., Fletcher T.C. (1992): The role of phagocytes in the protective mechanisms of fish. Ann. Rev. Fish Dis., *2*, 53–71.

Siegl E., Nebe B., Blunk H., Rychly J. (1998): Detection of mitogen induced stimulation of leukocytes from the rainbow trout (*Oncorhynchus mykiss*) by flow cytometric analysisof intracellular calcium. Comp. Biochem. Physiol., *119*, 915–923.

Slierendrecht W.J., Lorenzen N., Glamann J., Koch C., Rombout J.H.W.M. (1995): Immunocytochemical analysis of a monoclonal antibody specific rainbow trout (*Oncorhyn-chus mykiss*) granulocytes and thrombocytes. Vet. Immunol. Immunopathol., *46*, 349–360.

Stosik M. (1995): Selected immunological and haematological parameters in cultured healthy and sick carps (*Cyprinus carpio* L.) in the course of their ontogeny (in Polish). [Thesis of habilitated doctor.] Scientific Editorial Office, University of Szczecin, Szczecin.

Voccia I., Krzystyniak K., Flipo D., Fournier M.(1994): *In vitro* mercury-related cytotoxicity and functional impairment of the immune cells of rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicol., *29*, 37–48.

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