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Responses of Circulating Fish Phagocytes to Paper Mill Effluent Exposure

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Identification of pollutant-sensitive biomarkers for the monitoring of aquatic pollution is a current thrust in the ecotoxicology research (Ahokos et al. 1976; Atchison et al. 1987; McCarthy and Shugart 1990). This approach has met with the successful development of early warning assays such as that of inhibition of (δ-aminolevulenic acid dehydratase (ALAD) by lead exposure in aquatic and terrestrial organisms (Hodson et al. 1977; Anderson 1988). Immune system biomarkers though do not fall in the specific category, they have demonstrated their utility in the pollution monitoring programs (Bekesi et al. 1979; Lee and Chang 1985). Pollutant-induced immune function alterations reflect the toxic potential of the pollutant towards the immune system on one hand and the health status of the affected organism on the other. A slight misappropriate functioning of the immune system would predispose the host towards the disease causing events including microbial pathogenicity. We have undertaken investigations to evaluate fish immune system under the environmental chemical induced stress. In the present paper, we report the investigations on the freely circulating phagocytes (macrophages) of an air-breathing catfish, *Heteropneustes fossilis* Bloch. The circulatory cells of fish hemopoaetic system, in particular those behaving like phagocytes provide defense against invading agents (Zeeman and Brandley 1981; Manning 1994). These cells are also potential targets of environmental toxicants encountered by the fish. We investigated short-term and long-term responses of circulating phagocytes of fish peritoneal cavity and those found adhered to gills towards exposure to paper mill effluent, which is a complex mixture of organic and inorganic pollutants (Hamm et al. 1986; Suntio et al. 1988; van den Heuvel et al. 1995).

MATERIALS AND METHODS

Fish stock of *Heteropneustes fossilis* (Bloch) of both sexes were obtained from natural resources and were kept in glass aquarium measuring 12 in. X 12 in. X 24 in. Fish were acclimatized for fifteen days before use in chlorine free tap water and were hand fed on fresh autoclaved goat liver meat. Healthy fishes were divided into four large groups. Three groups of

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fishes were exposed to different concentrations (0.5, 1.0, 2.0% v/v) of paper mill effluent collected from the neighboring paper mill installations. Fourth group served as control without effluent exposure. The effluent and water change was made every other day. Fish were sacrificed at the intervals of 15, 30, 60 and 90 days.

In order to study the activation pattern of phagocytes of fish, normal fish were challenged with 3% thioglycollate medium (E. Merck, Bombay) injected intraperitoneally. On the third day phagocytes were collected from the treated and control fish (injected with normal saline). For collection of phagocytes, fish were anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chem. Co., USA) and the caudal vein was severed to avoid the contamination of red blood cells in cell preparations. An abdominal incision was made and peritoneal cavity and gill were thoroughly rinsed with cold Hank's balanced salt solution (HBSS pH-7.2) in separate petri dishes, kept in incubator for one hour for adherence. Supernatant from these dishes was decanted and cells were detached with the help of a cell scraper in fresh cold HBSS. Detached cells were centrifuged in siliconized tubes at 2500 rpm for 10 min (X 3) and the final cell pellet resuspended in 1 mL of medium RPMI-1640. Cells were counted by using a hemocytometer. The cell viability was determined using trypan blue dye exclusion method (Raisuddin et al. 1993). Differential counts were performed to assess the population of macrophages in the cell suspension.

For evaluation of *in vitro* cytotoxic potential of paper mill effluent, cells were exposed to different concentrations (5, 10, 20, 50, 100% v/v) of paper mill effluent. Cells obtained from both the sources of normal fishes were suspended in RPMI-1640 (pH 7.2) containing 5% fetal calf serum (FCS) and a concentration of $6x10^6$ cells/ml was obtained. An aliquot of 1 mL of above suspension was mixed with 1 mL of paper mill effluent in phosphate buffer saline (PBS, pH 7.2) to achieve the desired concentrations. They were incubated at 37 °C in a metabolic shaking water bath for 4 hr. Cell viability in each set of tubes was determined using trypan blue exclusion method.

Phagocytic activity of phagocytes was evaluated using the suspension assay as described by Fujiki and Yano (1997) with some modification. Briefly, 0.lmL aliquot of $l0xl0^6$ cells/ml density in RPMI-1640 medium was mixed with 0.lml of medium containing 20% FCS and $100x10^6$ cells/ml of heat treated (at 100 °C for 1 hr) yeast cells. The mixture was incubated at 35 °C for one hour with occasional shaking. After incubation, 50µL volume of this mixture was smeared on glass slide, air-dried and stained with Wright-Giemsa stain. The slides were observed under a light microscope (Olympus BX40) using oil immersion. At least 500 cells were counted. Phagocytic activity was expressed as phagocytic index and phagocytic capacity. Results of each study were statistically analyzed using student's 't' test. The level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

It was observed that a significant number of cells isolated from peritoneum and those adhered to gills behave like phagocytes. Challenge with thioglycollate medium induced a significant ($p < 0.001$) increase in the cell numbers obtained from gill and peritoneum (Table 1). Such observation is identical to that observed in rodents (Eduardo et al. 1995). A substantial number of cells were adhered to petri-plates that were assumed phagocytes because of their inherent adhering property. *In vitro* cytotoxicity study showed that cells isolated from both the sites, i.e. peritoneum and gill respond in a dose-dependent manner (Figure 1). Paper mill effluent was toxic to cells at all the concentration levels.

Table 1. Population of inactivated and activated phagocytes isolated from peritoneum and gills of normal fish.

Peritoneal exudate cells					
Inactivated cells			**Activated cells**		
Total number of cells ($x10^6$)	Adhered phagocytes ($x10^6$)	%age of adhered phagocytes	Total number of cells ($x10^6$)	Adhered phagocytes ($x10^6$)	%age of adhered phagocytes
16.88±1.040	5.28±0.268	31.27	22.56* ±0.300	8.86±0.169	39.27
Gill adhered cells					
23.88±0.570	7.28±0.259	30.48	28.49* ±0.228	10.88±0.286	38.18

*=$p<0.001$

As regards phagocytic response, it was observed that there was an initial stimulation of phagocytes as demonstrated by significantly increased phagocytic index and phagocytic capacity ($p < 0.001$). This stimulation was more pronounced at the concentration levels of 0.5 and 1% (Figure 2, 3). Long-term exposure, however, induced a suppressive effect, as there was significant ($p < 0.001$) reduction of phagocytic index as well as phagocytic capacity after an exposure of 90 days (Figure 2, 3). An almost identical pattern of response was observed for the phagocytes isolated from both the sites. It may be assumed that they have same lineage. Our electron microscopy studies also confirm this assumption as cells from both the sites show similar morphological characteristics (presented in other communication). It, however, would be inappropriate, if a parallel is drawn between gill adhered phagocytes of fish and mammalian alveolar macrophages. Due to circulatory nature of fish hemopoaetic system the

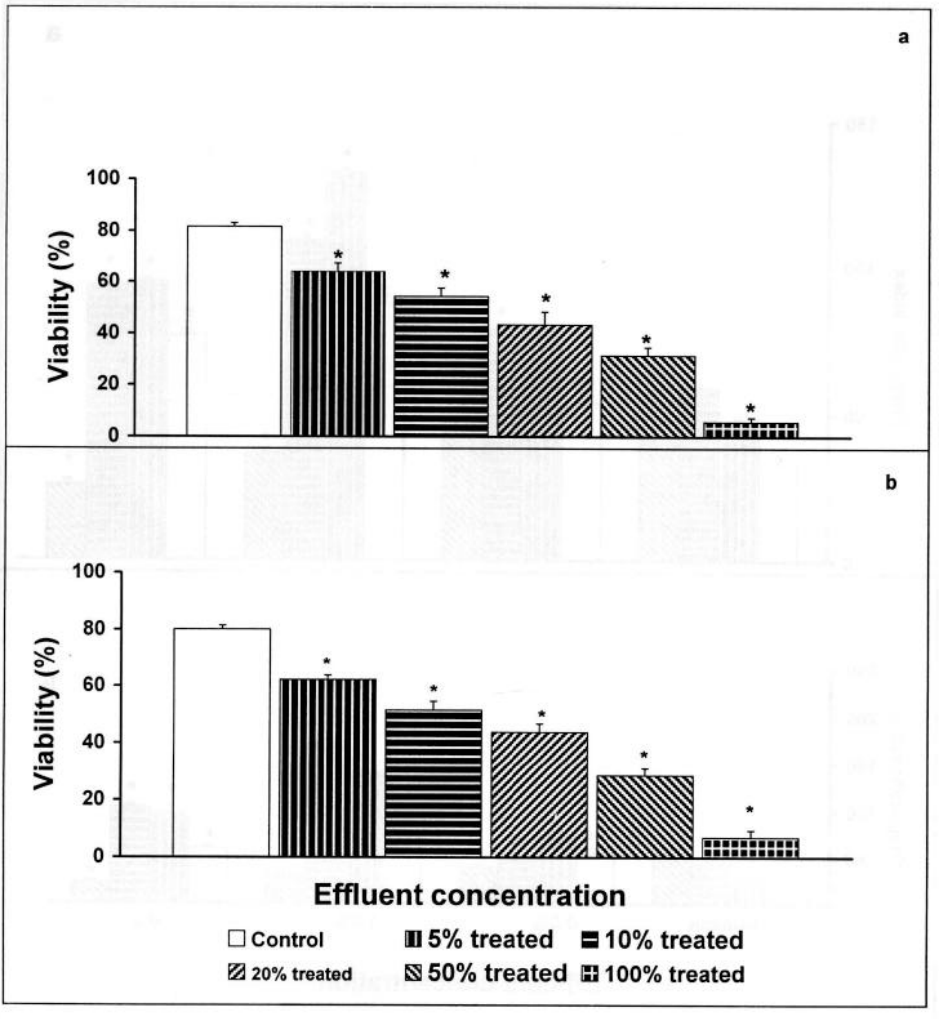

Figure 1. *In vitro* cytotoxicity of paper mill effluent in phagocytes isolated from **a)** peritoneum and **b)** gill of normal fish. Cells were incubated for 4 hr at 37 °C. The cell viability was determined using trypan blue dye exclusion method. * = $p < 0.001$ when compared with control group (n=6).

phagocytes tend to reach various tissues sites more in numbers at the site of stimulus. Since fish gills contiguously receive, water full of antigenic and particulate material, presence of a large number of phagocytes at this tissue seems plausible (Flano et al. 1997).

Paper and pulp mill effluent contains complex mixture of dissolved lignin and cellulose degradation products, various other wood extracts and chlorinated organic compounds. It is characterized by high concentration of heavy metals, total suspended solids, high pH, increased chemical oxygen

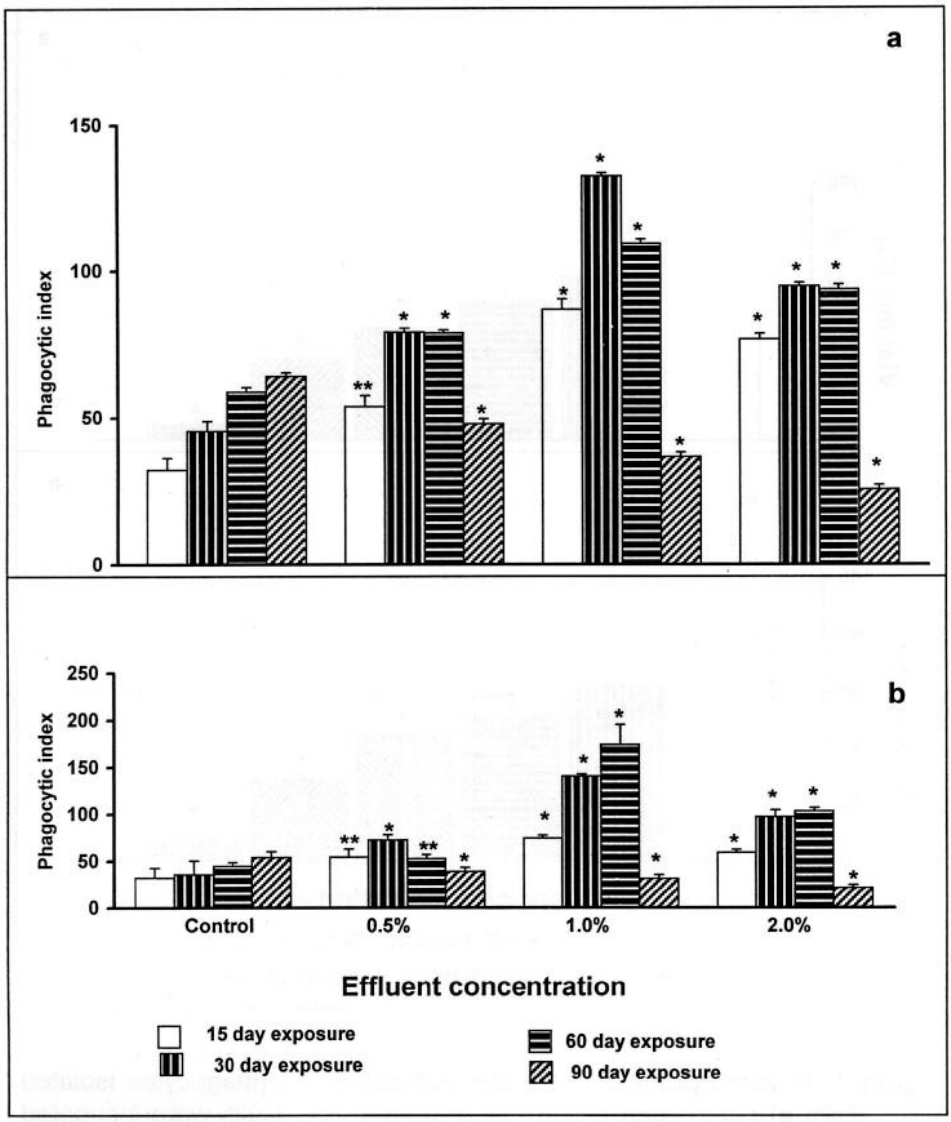

Figure 2. Effect of paper mill effluent on phagocytic index of fish phagocytes isolated from **a)** peritoneum **b)** gill. Phagocytic index was calculated using the formula A x B where A= percentage of phagocytes engulfing atleast two yeast cells; B= average number of yeast cells engulfed by phagocytosis positive cells. *=p<0.001, ** = p<0.01 when compared with control group (n=5).

demand and low dissolved oxygen that apparently increases the biological oxygen demand. Heavy metals include mercury, cadmium, lead, chromium, copper and zinc (Hamm et al. 1986; Suntio et al. 1988). Some of these constituents such as heavy metals have toxic potential while others including lignin and cellulosic material may temporarily stimulate the

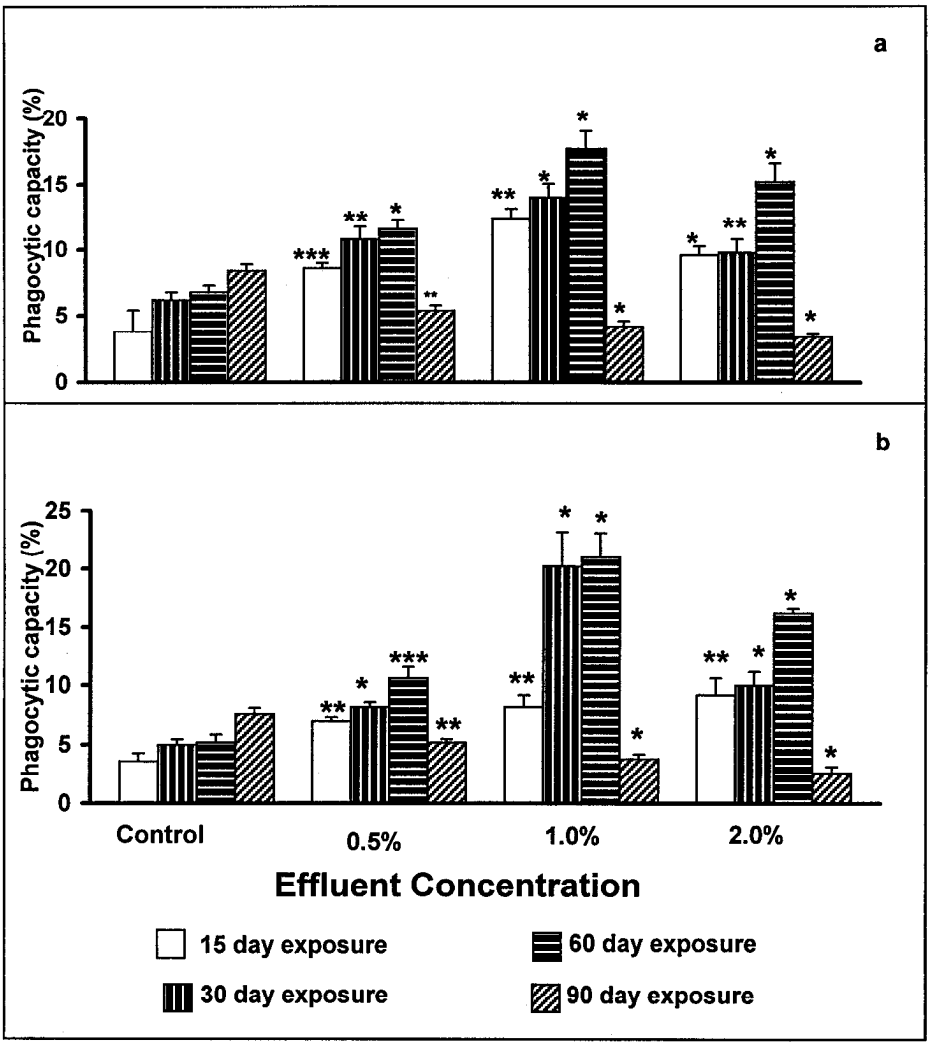

Figure 3. Effect of paper mill effluent on phagocytic capacity of fish phagocytes isolated from **a)** peritoneum and **b)** gill. Phagocytic capacity expressed as mean percentage of cells engulfing 2 four heat-treated yeast cells. * = $p < 0.001$, ** = $p < 0.01$, *** = 0.02 when compared with control group (n=5).

immune cells mainly because of their characteristic chemical composition. Toxic potentials of several of these constituents have been reported by Wong et al. (1992) and Voccia et al. (1994). The initial stimulation of phagocytes may be attributed to the exposure to cellulosic and lignocellulosic components of paper mill effluent which because of their molecular size may act as elicitor of phagocytes (Blazer 1991). Long term exposure resulting in bioaccumulation of toxic heavy metals seems to

overwhelm the activated state of phagocytes (Zelikoff 1993). Fish phagocytes (macrophages) are involved in nonspecific and specific immune reactions, such as phagocytic killing of microorganisms and antigen presentation (Ellis 1977; Zelikoff et al. 1991). These vital functions can be altered by numerous environmental, nutritional and physiological factors (Fletcher 1986). Long term exposure to paper mill effluent will definitely augment the disease development. It is concluded that a biological response to an environmental pollutant largely depends on the type of pollutant in question. The level and duration of exposure also play important role and a holistic approach has to be adopted while designating a particular biological response as biomarker of pollution.

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