Development of an *in vitro* Environmental Monitoring System by Using Immune Cells

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Phagocytic abilities between casein-induced Sprague-Dawley (SD) rat macrophage and kidney macrophage of fish were compared. First of all, latex bead phagocytosis abilities of macrophages from rat and fish by the same water sample were compared. *In vitro* exposure of the water samples to the induced SD rat macrophages for 4 hr at concentrations ranging from 10 × to 80 × of the water sample reduced the latex bead phagocytosis of the macrophages with concentration dependency. This result was similar with that of the head kidney macrophages from *Cyprinus carpio* and *Carassius auratus*. The most important advantage of using rat macrophage is its availability, because it is very hard to catch resident fishes in highly polluted rivers, and it is almost impossible to determine the extent of biological crisis in the river. These critical difficulties of previous monitoring systems could be solved by the application of casein-induced SD rat macrophages. With the above result, rat macrophage was used to investigate the acute toxicity of polluted water samples and compare pollution levels of the Kumho, Kum and Mankyung Rivers and Miho Creek in the southern part of Korea. Finally, it was confirmed that the *in vitro* monitoring system for exposure of SD rat macrophage to polluted water samples was available for environmental monitoring purposes.

Key words —— environmental monitoring, fish, rat, macrophage, phagocytosis

INTRODUCTION

Development near rivers is responsible for increasing water pollution. The water leads to an impairment of the balance between aquatic life and its habitat.[1)] Petroleum-derived hydrocarbons, insecticides and herbicides are known as mutagens. The occurrence of these and other pollutants in fish can cause acute effects, and the high concentration of the toxicants can lead to injury or death in a short period of time.[2)] These facts were enough to pose a scientific question about eligible environmental monitoring systems.

Macrophages have been regarded as an important part of the cellular immune system of fish, and function to protect the host by phagocytizing foreign material including disease-causing agents.[3)] Therefore, changes in their phagocytic abilities have been of interest to ascertain the effect of environmental pollutants on the normal function of macrophages.[4)]

Cyprinus carpio and *Carassius auratus* are fish species that have been known as common fauna in most stream waters in Korea. However, they are not easy to catch insufficient quantity for environmental monitoring purposes because of their decreasing population with increasing environmental pollution levels. On the other hand, Sprague-Dawley (SD) rats are have been commonly used for many experiments in most labs.

This study has focused on similarities of phagocytic aspects between macrophages from fishes (*C. carpio* and *C. auratus*) and SD rats through *in vitro* exposure of immune cells to polluted water, and on developing a monitoring system to evaluate and compare the acute toxicity of polluted water samples from various stream sites and spots on macrophages from SD rats. The water samples were obtained from four main water streams running through big cities located in the southern part of South Korea: the Kumho, Kum and Mankyung Rivers and Miho Creek running through Taegu, Taejun, Chunju and Chungju City, respectively.

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MATERIALS AND METHODS

Water Sample Pre-Treatment —— River water samples were collected from upstream downstream points of the Kumho, Kum and Mankyung Rivers and from Miho Creek. Each collected river water sample (1 l) was acidified with concentrated sulfuric acid to pH 3. Active components were adsorbed for 24 hr by stirring with 0.5 g/l of Amberlite XAD-4 resin (Fluka), based on the static adsorption mode of the combined solid-phase extraction method. Pollutants adsorbed to the resin were eluted with a mixture of dichloromethane (DCM) and ethyl acetate (9 : 1) in a soxhlet apparatus, then dried under reduced pressure.[5] Residues were dissolved in 100% ethyl alcohol (1 ml) and stored at –20°C.

Macrophage Separation from SD Rat and Fish ——

SD Rat Macrophage Separation: Six grams of Casein were dissolved in 100 ml of distilled water. The prepared 6% casein solution was introduced into the rats by peritoneal injection at the rate of 15 ml/400 g. The SD rats used in this study were 9 weeks old and 250 g. 10 days after the injection, macrophages (10^6 cells/ml) were separated. The cell suspensions were centrifuged at 312 × *g* for 6 min at 4°C. The cell pellet was resuspended in Hanks' balanced salt solution. Total cell counts were obtained using a hemacytometer technique.

Fish Macrophage Separation: Fish used in this study were *C. carpio* and *C. auratus*. The National Institute of Environmental Research in Seoul, Korea supplied them, and their weights were between 30 and 80 g and between 50 and 200 g, respectively.

The separated head kidney of the fishes was homogenized with woven nylon mesh, and macrophages obtained from the organ were collected using the continuous gradient percoll separation method;[6] 9 volumes of percoll with 1 volume of sterile 1.5 M NaCl were mixed to make stock isotonic percoll (SIP); an initial density of 1.065 g/ml was obtained by mixing 4 ml of the head kidney cell suspension in pH 7.6 heparin/L-15 with 4.2 ml of SIP. To make a self-generated continuous gradient, the prepared sample solution was centrifuged for 20 min, 20000 × *g*, at 5°C. Macrophages were obtained from the third band with a density distribution of 1.069–1.075 g/ml. The collected cell suspended solution was washed three times by centrifugation for 10 min at 300 × *g* and at 4°C with 10 ml of incomplete L-15. The washed cell suspension was counted in a hemacytometer, after a 1 : 2 dilution with a 0.4% solution of Trypan Blue in HBSS, to determine cell viability (routinely ≥ 90%).

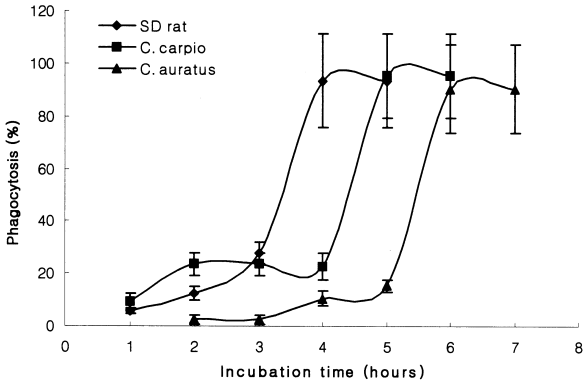

Fig. 1. Comparison of Latex Bead Saturation Time between SD Rat, *C. Carpio* and *C. Auratus* Macrophage Phagocytic Activies

Macrophage Phagocytic Activity Test —— The separated macrophages (10^6 cells/ml in 0.96 ml) were treated with the pretreated water samples (40 μl), then mixed well with latex beads (2 μl). The treated macrophage samples were incubated for 4, 5 and 6 hr: the incubation time was set differently by the origin of macrophage: 4-hr incubation for SD rat macrophage at 37°C, 5-hr for *C. carpi*o at 14°C, and 6-hr for *C. auratus* at 14°C.

The number of macrophages showing phagocytosis and total macrophages were counted to measure the ratio.

RESULTS AND DISCUSSION

Latex bead saturation time of macrophages from fish and rats was measured to set the incubation time prior to the toxicity tests of water samples, effects on the macrophages.

The macrophages obtained from fish and rats were mixed with latex beads and incubated at 14°C and 37°C, respectively, with different incubation time schedules (Fig. 1). 93.4% of SD rat macrophages engulfed latex beads at 4 hr of incubation; *C. auratus* and *C. carpio* macrophages engulfed 95.2 and 90.3% at 5 and 6 hr of incubation, respectively (Fig. 1).

The Phagocytosis patterns of macrophages from *C. carpio* and SD rat by serial treatment using a standard material, cadmium cloride, were investigated.

The macrophages obtained from the *C. carpio* and rat mixed with latex beads were treated with 0.004, 0.04, 0.4 and 4 mM of $CdCl_2$, then incubated

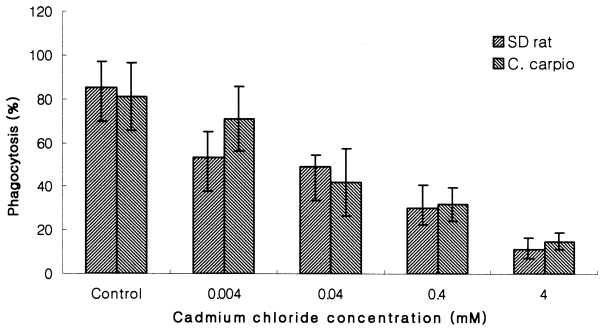

Fig. 2. Comparison of Latex Bead Phagocytosis Patterns by Cadmium Chloride Serial Treatment between SD Rat and *C. Carpio* Macrophage

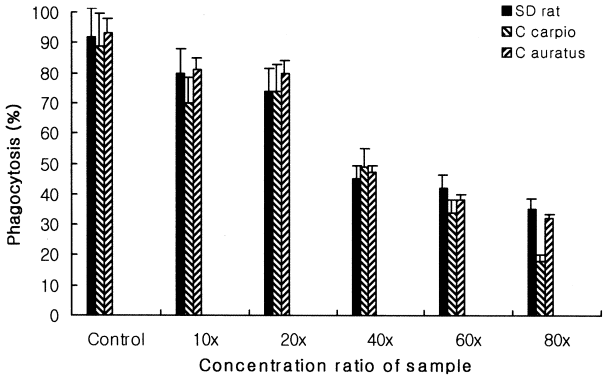

Fig. 3. Comparison of Phagocytic Ability Reduction between Macrophages from *C. carpio*, *C. auratus* and SD Rat by Concentration Ratio Changes from a Kumho River up Stream Water Sample

at 14°C for 5 hr and 37°C for 4 hr, respectively. Approximately 81 and 85% of *C. carpio* and SD rat macrophages engulfed latex beads as a control; 67% and 54% at 0.004 mM of $CdCl_2$; 43% and 48% at 0.04 mM; 31% and 30% at 0.4 mM; 14% and 12% at 4 mM (Fig. 2).

Figure 3 shows the effects of polluted water on macrophages. Phagocytosis of the macrophages treated with Kumho River upstream water sample clearly decreased in a concentration dependent manner. At the 40-fold concentration of the sample, the reduction of phagocytic activities was significantly changed ($p < 0.05$) from the control: phagocytosis of rat, *C. carpio* and *C. auratus* were $45 \pm 5\%$, $49 \pm 3\%$ and $47 \pm 5\%$, respectively.

From the preliminary results, optimal conditions for applying SD rat macrophages to monitoring indirect biological effects were determined: 4 hr of incubation time (Fig. 1) and a similarity in pattern change of phagocytic activity (Fig. 2), as well as at least a 40-fold concentration of water samples

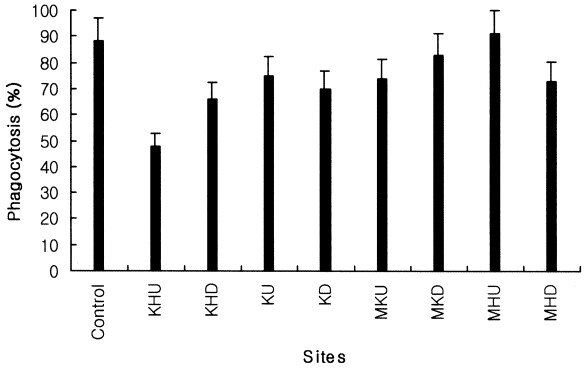

Fig. 4. Comparision of Site Water Pollution Levels by Macrophage Activity Reduction

*KH, K, MK and MH stand for Kumho River, Kum River, Mankyung River and Miho Creek, respectively. *U and D mean up and downstream.

(Fig. 3). Using these parameters, water pollution levels of the Kumho, Kum and Mankyung River and Miho Creek running through Taegu, Taejun, Chunju and Chungju City, respectively, were evaluated. 40-fold concentrated water samples from the streams were used to treat the casein induced SD rat macrophage.

Kumho River water samples significantly reduced phagocytic activity: $47 \pm 5\%$ and $65 \pm 3\%$ by the up and down stream water samples, respectively. The up and down-stream values differed significantly ($p < 0.05$) from the control (Fig. 4). This result provided supporting evidence for the necessity of environmental monitoring of the river.

Five spots from up and down-stream in the Kumho River were selected to screen its overall pollution levels. The range of SD rat phagocytosis reductions by 40-fold concentrated water samples from the spots were between $32 \pm 4\%$ and $63 \pm 3\%$: all the values from the spots showed a significant ($p < 0.05$) decrease in phagocytic activity of SD rat macrophages (Fig. 5).

Casein-induced SD rat macrophages were used for comparing pollution levels from up and downstream in the Kumho River, Kum River, Mankung River and Miho Creek, all located in southern portion of Korean peninsula; 40× concentrated up and downstream water samples from the rivers were introduced to the immune cell. Figure 4 shows that samples from the Kumho River were highly polluted compared with the rest of river water samples. The results from this study were in good correlation with previously reported COD and heavy metal pollution levels, that is, the COD and metal pollution levels were worst in the Kumho River (data not shown).

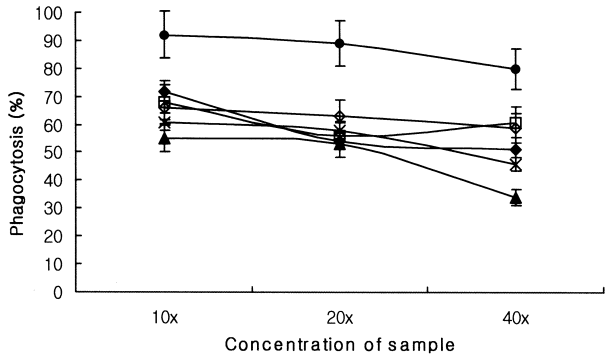

Fig. 5. SD Rat Macrophage Phagocytic Activity Reduction by Various Concentration Ratio of Kumho River Main Stream Spot Samples

*KH1, KH2, KH3, KH4 and KH5 mean spots of Kumho river from up to down stream.

This is because industrial regions are located along with Kumho River. Especially, the dye industry and other factories are concentrated on sampling site KH3. This site showed the highest pollution level among Kumho river sites (Fig. 5). This result is also consistent with previously reported COD and metal pollution levels (data not shown). From the results and data analyses above, it could be said that the declining macrophage phagocytic activity was related to the level of pollution at specific sites, and immune cell activity change is therefore one available monitoring method.

So far, macrophages from resident fish living in polluted water resources have been used to uncover the severity of water pollution levels, according to many studies.[4,7] Unfortunately, it is very hard to catch resident fish in highly polluted rivers, and thus it is almost impossible to determine the biological crisis in these rivers. These critical difficulties of previous monitoring systems could be solved by the application of casein induced SD rat macrophages, or at least, this study suggested a solution for the difficulties in environmental studies from both a scientific and economical point of view.

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