INTRODUCTION

Fish living in polluted environments are exposed to many chemicals. Especially, there is growing concern about the possible harmful effects by the exposure to xenobiotic compounds that are capable of disrupting the endocrine system. Effects on the reproductive systems of animals by these endocrine-disrupting chemicals has been emphasized. The neuroendocrine and immune systems are recognized to be intimately linked with and involved in bidirectional communication, which performs a homeostatic function. Actually, immunological cells such as macrophages or lymphocytes have estrogenic receptors, and their immunologic functions can be modulated by estrogen. Many estrogenic chemicals are accumulated in the aquatic environment and affect aquatic animals such as fish, crustacean and shellfish. The effects by endocrine-disrupting chemicals, such as polychlorinated biphenyl (PCB), tetrachlorodibenzo-\(p\)-dioxine (TCDD) or tributyltin (TBT), on the immune system have been studied previously in aquatic mammals and fish.

Bisphenol A is a major component of epoxy resins. Epoxy resins are used in protective coatings on food containers and in paints and adhesives. Nonylphenol is a degraded polyethoxylate, which is a major group of non-ionic surfactants commonly used as constituents in detergents, paints and plastics. The major route of entry of bisphenol A and nonylphenol into the environment is via wastewaters discharged by industries. Bisphenol A and nonylphenol have been detected in seawater, river and sewage effluents in Japan at concentrations of micrograms per liter (maximum levels observed were 0.71 \(\mu\)g/L for bisphenol and 4.6 \(\mu\)g/L for nonylphenol). It is well known that bisphenol A and nonylphenol have estrogenic activity; however, any other effects of these chemicals on aquatic organisms have not yet been reported. In the present study, we investigated the effect of bisphenol A and nonylphenol on carp phagocytic cells.

MATERIALS AND METHODS

Fish

A total of 100 common carp \(Cyprinus carpio\) (mean weight = 120 g) was obtained from Sunaso Fisheries Farm in Miyazaki, Japan. Fish were maintained in
outdoor tanks with running fresh water at a temperature of 16°C for 2 weeks, and fed commercial diets (Maruha Co. Ltd, Tokyo, Japan) twice daily.

Chemicals

Bisphenol A and nonylphenol (p-n-nonylphenol standard) were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). These chemicals were dissolved in a small amount of dimethylsulfoxide (DMSO; Wako Pure Chemical Industries) and added to distilled water (DW). 17β-Estradiol (water-soluble grade), which was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan), was used for the control experiments.

Macrophage isolation

The head kidney phagocytic cells of carp were isolated according to the modified method described by Braun-Nesje et al.7 The cells were removed from the fish and pushed through a nylon mesh and suspended in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) containing 1% streptomycin/penicillin (S/P; Sigma-Aldrich), 0.2% heparin (Sigma-Aldrich) and 10% carp serum obtained from sexually immature fish (CS). The cell suspension was placed on 34/51% Percoll gradient and centrifuged at 400 ¥ g for 40 min at 4°C. The macrophage-enriched cells from the 34/51% Percoll interface were separated and centrifuged at 500 ¥ g for 5 min and then washed three times with RPMI-1640 medium.

Using six individual fish, the direct effects of bisphenol A and nonylphenol on fish phagocytic cells were analyzed by superoxide anion assay (nitroblue tetrazolium; NBT), nitric oxide (NO) production and phagocytosis. As the positive control, the same concentration of 17β-estradiol was used in the study.

Preparation of leukocyte monolayer on 96-well microculture plates and their chemical treatments

Nitroblue tetrazolium and NO production of fish phagocytic cells in 96-well microculture plates were examined. The viable cells were adjusted to 10⁶ cells/mL in Hanks’ balanced salt solution (HBSS; Nissui Pharmaceutical Co. Ltd), and 100 µL of this suspension was added to each well (Corning Co., Corning, NY, USA). After incubating for 2 h at 20°C, the unattached cells were washed off with HBSS. Viable phagocytic cells, including neutrophils (=20%) and macrophages (=80%), were counted by the trypan blue exclusion method, and the cell number in the monolayer was determined to be approximately 2.5 ¥ 10⁵ cells/mL. Monolayers were fed 0.99 mL of RPMI-1640 supplemented with 10% CS, 1% S/P, and 0.01 mL of either bisphenol A or nonylphenol solution (final concentration of each chemical was 0.1 nM, 1 nM, and 10 nM, respectively) and maintained for 10 h at 20°C.

Detection of superoxide anions in phagocytic cells

The production of superoxide anions in phagocytic cells was determined by the reduction of NBT as described by Sakai et al.8 The cells were washed twice with HBSS, and 100 µL of NBT solution (1 mg/mL in RPMI-1640 medium), to which 1 µg/mL of phorbol myristate acetate (PMA; Sigma-Aldrich) had been added, was added to each well and incubated for 60 min at 20°C. After the removal of the medium from the cells, NBT reduction was halted by the addition of methanol. The formazan in each well was dissolved in 120 µL of 2 M KOH and 140 µL of DMSO, and the optical density was measured with a multiscan spectrophotometer (Amerham Pharmacia Co., Uppsala, Sweden) at 620 nm, using NBT solution containing PMA without cells as the blank. Triplicate wells were used for each variable that was analyzed.

Phagocytosis assay

Phagocytosis was examined according to the method described by Yoshida et al.9 The cell number was adjusted to 10⁷ cells/mL in a RPMI-1640 medium containing 10% of CS. The cells were allowed to adhere to a coverslip for 1 h, and loose cells were then removed by washing with the RPMI-1640 medium. The cells were fed with 4.95 mL of the RPMI-1640 supplemented with 10% CS, 1% S/P, and 0.05 mL of either bisphenol A or nonylphenol solution (final concentration of each of these chemicals were 0.1 nM, 1 nM or 10 nM, respectively), and maintained for 10 h at 20°C. After being washed twice with HBSS, latex particles (0.85 µm, 10⁹ particles/mL), which were obtained from Polysciences Inc. (Warrington, PA, USA), were suspended in the RPMI-1640 medium, and 10% of CS was then added and a coverslip applied, followed by incubation for 2 h at 20°C. The cells on the cover slip were fixed with methyl alcohol and stained with Giemsa. The number of adhered cells was approximately 5 ¥ 10⁵ cells per coverslip and the number of phagocytic cells per 300 cells
was counted microscopically. The percentage of phagocytic activity (PA) was then calculated.

**Nitric oxide production**

The NO production of fish phagocytic cells was measured using a modified assay described previously by Green et al.\(^\text{10}\) After treatment with either bisphenol A or nonylphenol for 10 h, the cells were washed by centrifugation at 400\(\times g\) for 10 min, and then stimulated by lipopolysaccharides (LPS) at a concentration of 20\(\mu \text{g/mL}\). After incubation for 96 h, the cell-free supernatants were assayed for nitrite using Griess’ reaction. Aliquots of 50\(\mu \text{L}\) of the supernatant were incubated with 100\(\mu \text{L}\) of 1% sulfanilamide and 100\(\mu \text{L}\) of 0.1% \(N\)-1-naphthylethylenediaminedihydrochloride (Sigma-Aldrich) in 2.5% \(\text{H}_3\text{PO}_4\) at room temperature for 5 min. Optical density was determined using a microplate reader at 570 nm, and nitrite concentrations were quantified by including them with \(\text{NaNO}_2\) (Sigma-Aldrich) standards.

The NBT, phagocytosis, and NO experiments were repeated twice and similar results were obtained.

**Statistics**

Data are expressed as the mean±SEM and were analyzed using Student’s \(t\)-test and one-way ANOVA, followed by the Student–Newman Keuls test \(P<0.05\), which was considered statistically significant.

**RESULTS**

**Production of superoxide anions**

The production of superoxide anions by carp phagocytic cells treated either with bisphenol A or nonylphenol increased significantly in comparison to the control \(P<0.05\) (Fig. 1a,b). The highest level of stimulation was recorded samples treated either with 0.1 nM of bisphenol A or 10 nM of nonylphenol \(P<0.05\). In contrast, \(17\beta\)-estradiol did not stimulate the production of superoxide anions in carp kidney phagocytic cells (Fig. 1c).

**Phagocytosis**

The phagocytic activity of carp kidney cells was significantly decreased by 0.1 nM bisphenol A \(P<0.05\), but not by 1 nM and 10 nM (Fig. 2a). No significant decrease was observed in the phagocytic activity of carp kidney cells treated with nonylphenol (Fig. 2b). Conversely, phagocytic activity was significantly suppressed by 10 nM of \(17\beta\)-estradiol (Fig. 2c).
shown in Fig. 3. The NO production of carp phagocytic cells was affected by treatment with either bisphenol A or nonylphenol. 17\(\beta\)-Estradiol also did not affect the production of NO in carp leukocytes.

**DISCUSSION**

Bisphenol A is known to inhibit macrophage functions in humans,\(^{11-13}\) Segura et al. have reported that bisphenol A can inhibit macrophage adhesion at a concentration of 10 pM.\(^{11}\) Furthermore, bisphenol glycidylmethacrylate, which is used as a dentin bonding agent, can suppress the mitochondrial activity of macrophages.\(^{12}\) Recently, Inadera et al. have reported that bisphenol A could inhibit monocyte chemoattractant protein-1 gene expression.\(^{13}\) Conversely, nonylphenol is widely used as an ingredient in detergents, paints and herbicides. The estrogenic effects of nonylphenol have been reported in mammals and fish, but its effect on the immune system has not yet been studied.

The present *in vitro* studies demonstrated that bisphenol A and nonylphenol affect the immune modularity of carp kidney leukocytes. Kidney cells incubated with bisphenol A increased their production of superoxide anions, but this stimulation was seen only at a concentration of 0.1 nM. Nonylphenol also caused an increase in the production of superoxide anions in carp kidney phagocytic cells at concentrations ranging from 0.1 nM to 10 nM. A maximum response was shown at a concentration of 10 nM. In contrast, 17\(\beta\)-estradiol did not stimulate superoxide anion production in kidney phagocytic cells at the various concentrations tested. Wang and Belosevic have also reported similar results.\(^{14}\) In mammalian studies, the release of superoxides by rat peritoneal macrophages was not affected by 17\(\beta\)-estradiol at concentrations between \(10^{-10}\) M and \(10^{-9}\) M, but stimulation occurred at concentrations above or below that range.

Phagocytosis is a process whereby cells internalize, kill and digest invading microorganisms. This ability can usually be determined by the intake of foreign particles, such as bacteria, blood cells or latex. In the present study, carp kidney cells incubated with bisphenol A suppressed phagocytosis, but this was not seen in those incubated with nonylphenol. Conversely, a concentration of 10 nM of 17\(\beta\)-estradiol also suppressed the phagocytic activities of carp kidney leukocytes significantly, which is in agreement with Wang and Belosevic, who have also reported that 17\(\beta\)-estradiol inhibits phagocytosis.\(^{14}\)

Nitric oxide is one of the molecules responsible for bactericidal action by neutrophils in mammals.
neither bisphenol A nor nonylphenol had any effect on NO production in carp kidney leukocytes. 17β-Estradiol also did not affect the production of NO. Wang and Belosevic have reported that goldfish macrophage cell lines treated with 17β-estradiol did not affect the production of NO. However, in mammals, Chao et al., have reported that 17β-estradiol significantly inhibited the release of nitrites by macrophages at most of the concentrations used, except for 10⁻¹⁰ M, at which the release of nitrites remained unaffected.

In the present study, bisphenol A and nonylphenol affected the function of carp phagocytic cells, suggesting that these chemicals act not only as environmental estrogens but also as immune modulators. However, the effects of bisphenol A and nonylphenol on carp macrophages are different from that by 17β-estradiol. There have been several reports on the immune-modulating effects in fish by endocrine-disrupting chemicals such as PCB and TBT. Polychlorinated biphenyl can suppress antibody production and macrophage activity in fish, while TBT also reduces macrophage activity in toadfish. However, it is still not known whether suppression by these chemicals induces estrogen receptors on immune-competent cells. Thus, the mechanism of immune modulation by carp macrophages by bisphenol A and nonylphenol should be investigated further.

The results of the present study demonstrate that bisphenol A and nonylphenol affect the macrophage activity in carp. In Japan, bisphenol A and nonylphenol have been detected in rivers and seawater, and fish living in polluted areas have been exposed to these compounds. It is likely that the immune system of fish is disrupted by exposure to water contaminated by bisphenol A and nonylphenol, and leads to increased susceptibility to disease. Thus, the effect of bisphenol A and nonylphenol on the immune systems of fish should be studied extensively, particularly the effects of these pollutants on the production of antibodies or complement activity.

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REFERENCES


