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Effect of Oral Immunization with Liposome-Entrapped Bacterial Antigen on Protection Against Experimental *Aeromonas Hydrophila*

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Abstract Liposome-entrapped atypical *Aeromonas hydrophila* antigen was prepared to investigate the potential protective efficacy for *A. hydrophila* infection. Carp (*Cyprinus carpio*) were immunized orally with liposome-entrapped *A. hydrophila* antigen. After immunization, significantly more antigen-specific antibodies were detected in serum, intestinal mucus and bile than non-immunized control group. The immunized carp were then challenged by immersion with 1×10^6 cfu/ml of *A. hyrdophila* for 60 min. Of the eight non-immunized carp, three carp died (62.5% survival), whereas five out of six (83.5%) of the immunized survived. Furthermore, development of skin ulcers was significantly inhibited in carp immunized with liposomes containing *A. hydrophila* antigen. These results suggest that liposomes containing *A. hydrophila* antigen have a potential for induction of protective immune responses against atypical *A. hydrophila* infection and also suggest the possibility of developing a vaccine that may ultimately be used for prevention of fish diseases.

Key words: *Aeromonas hydrorphila*, liposome, carp, ELISA

In many countries, intensive fish farming has become a key industry in recent decades. With the increasing scale of aquaculture, fish are reared at high population density. Even when environmental conditions are favorable and the fish are healthy, mass mortality will occur if infectious agents are introduced into the farm, causing great financial losses. Previously, treatment of diseases has focused on chemicals and antibiotics. Treatment of affected fish with antibiotics is effective, but gives rise to problems such as accumulated resistance in the bacteria, which renders the antibiotic useless.

Many vaccines and different methods of mass vaccination have been developed against bacterial and viral diseases in cultured fish. In general, three different methods are employed for administration of antigens: immersion, injection, and oral vaccination. The effectiveness of a vaccine depends largely on its mode of administration (Vinitnantharat et al., 1999). Since the vaccines should be administered to a large number of fish irrespective of size at any time in the culture cycle and in every type of culture system, oral vaccination via feed is well suited (Quentel and Vigneulle, 1997). It is the least stressful method to deliver antigens, is time and labor saving, and avoids stress caused by manipulation of the fish. To induce sufficient protective immune responses, antigens should reach the lymphoid tissue of the hindgut without destruction during passage through the gastrointestinal tract (Rombout et al., 1985; Davidson et al., 1993; Jenkins et al., 1994). Antigens are therefore incorporated in or adhered to the feed, or are covered with artificial constructs (Wong et al., 1992; Campbell et al., 1993; Joosten et al., 1995; Joosten et al., 1997; Azad et al., 2000).

Liposomes are multilayer vesicles composed of amphipathic phospholipids. They are non-toxic, biodegradable and only weakly immunogenic. They have been used to deliver a wide variety of biologically active substances to specific tissues and cells, and have also been used as immunological adjuvants (Gregoriadis, 1990). In particular, liposomes entrapping antigens are protected from low pH and enzymatic attack until they reach the target sites, so that the possibility of liposomes as carriers and adjuvants for developing oral vaccines has attracted considerable interest (Alving, 1991). Previous studies have demonstrated that

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liposomes consisting of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS) and cholesterol (Chol) (1 : 1 : 2, molar ratio) are stable in acidic solution, bile, and pancreatin solution (Han et al., 1997), and would serve as an effective oral delivery vehicle for inducing mucosal immune responses (Watarai et al.,1998).

Aeromonas hydrophila is a ubiquitous fish pathogen that causes the disease furunculosis. In some of the fish species, particularly the non-salmonids, the disease manifests itself differently from the classical furunculosis, and the casual agent is regarded as 'atypical' *A. hydrophila* (Austin, et al., 1999). A disease, which produces ulcers on the body surface or fins, has become prevalent in colored carp cultivation in Korea, and atypical *A. hydrophila* was isolated from ulcerative lesions of affected fish (Matoyama, 1999). Vaccination is thought to be effective for prevention against atypical *A hydrophila infection*. However, no effective vaccine has been developed until now. Recently, it was demonstrated that oral administration of liposome-entrapped antigens effectively induced both systemic and local intestinal immune responses in carp, *Cyprinus carpio* (*C. carpio*) (Irie et al., 2003). In the present study, therefore, liposomes containing *A. hydrophila* antigens were orally delivered to carp and immune responses were investigated. Furthermore, the ability of immunization to protect against *A. hydrophila* infection after challenge with the bacteria was evaluated.

MATERIALS AND METHODS

Materials

DPPC, DPPS, and Chol were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Bovine serum albumin (BSA) and horseradish peroxidase-conjugated anti-rabbit IgG were from Sigma and Jackson Immuno Research (West Grove, PA, USA), respectively.

Bacteria

Atypical *A. hyrdophila* was isolated from infected *C. carpio*. Based on comparative biochemical tests, the isolated bacterium was identified as *A. hydrophila*. *A. hydrophila* was cultured in heart infusion broth (BD Bioxdiences, USA) at 20°C. To determine LD50 concentration of the pathogen, the bacterium was cultured in the laboratory in LB broth at 37°C for 24 h, and then the cells were separated by centrifugation at 8,000 × g and injected into carp (size 25 to 30 g) intraperitoneally at room temperature by following the method of Saeed and Plumb (1986).

Preparation of bacterial antigen

A. hydrophila suspended in saline was treated with ultrasound for 20 min at 10 sec intervals on ice by Handy Sonic (model UR-20P power control 10, Tomy Seiko Co., Ltd, Tokyo, Japan), and then centrifuged at 14,000 × g for 20 min at 4°C. The supernatant was used as antigens.

Animals

Common carp were provided by a commercial carp breeding farm, Kunsan, Korea. They were reared in 160 L plastic aquaria that were filled with dechlorinated tap water (water temperature 23°C) and aerated. Fish weighing about 350 g were used for the experiments.

Preparation of liposomes containing *A. hydrophila* antigens

Liposomes entrapping *A. hydrophila* were prepared by the following procedure. DPPC (0.5 μmol), DPPS (0.5 μmol) and Chol (1 μmol), each dissolved in organic solvent, were mixed in a conical flask. The lipids were dried on a rotary evaporator, and were left to stand for 30 min in a high vacuum in a desicator. After the addition of 2 ml of sonicated *A. hydrophila* antigen solution (5 mg/ml) and incubation at room temperature for 90 min, the lipid film was dispersed by vigorous vortexing. Unencapsulated *A.hydrophila* was removed by centrifuging at 14,000 × g for 20 min at 4°C in 150 mM phosphate-buffered saline (PBS, pH 7.2). The resulting pellet of liposomes was suspended in saline and used for the immunization.

The amount of *A. hydrophila* trapped in liposomes was determined by the following method. Isopropyl alcohol (90 μl) was added to a 10 μl suspension of liposome-entrapped *A. hydrophila* and vortexed. The protein concentration of the resulting solutions was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), with bovine plasma gamma globulin as a standard.

Immunization of carp

Carp (n = 6 or 4) were immunized orally with 500 μl of each solution of liposome-entrapped *A. hydrophila* or *A. hydrophila* alone (200 μg/ml saline) by using a catheter. Immunization was repeated 3 times at 2-week intervals. Sera, bile and mucus of intestinal tract were collected 2 weeks after the last immunization. They were used for antibody assay. Furthermore, the other carp (n = 4) were used as non-immunized control. Sera, bile and intestinal mucus from non-immunized control carp were also used for antibody assay.

Preparation of intestinal mucus samples

Intestinal mucus was prepared as follows. Intestinal tract was cut with scissors to open the intestinal lumen. Then, intestinal mucus was collected from the intestine by gentle scraping with spatula and transferred to a tube. After the addition of 500 μl of PBS, the tube was vortexed for 30 sec, and centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was collected and assayed for antibody activity.

Antibody assay

Specific antibodies against *A. hydrophila* were determined by enzyme-linked immunosorbent assay (ELISA). Formalin-killed *A. hydrophila* (20 μg/ml) diluted with PBS was dispensed at 50 μl/well into 96-well microtiter plate (Corning, USA). The plates were left overnight at 4°C, and were washed 5 times with PBS containing 0.1% Tween 20 (washing solution). Next, 100 μl of PBS containing 0.5% BSA (blocking solution) was added to each well. Following incubation for 60 min at 25°C, the wells were washed with the washing solution, and 50 μl of serial two-fold dilution of test serum, intestinal mucus or bile, diluted with blocking solution, was then added to each well. The plates were incubated for 90 min at 25°C, washed with the washing solution, and 50 μl of anti-carp IgM rabbit IgG (1 : 100 dilution in PBS) was added. After incubation for 90 min at 25°C, the wells were washed with the washing solution and 50 μl of horseradish peroxidase-conjugated anti-rabbit IgG (1 : 8000 dilution in PBS) was added. The plates were then incubated for 90 min at 25°C and washed. Next, 100 μl of substrate solution (0.4 mg/ml o-phenylenediamine and 0.2 μl/ml of 30 % H_2O_2 in citrate buffer) was added and left to react for 10 min at 25°C. The enzyme reaction was stopped by adding 100 μl of 2 N H_2SO_4, and the absorbance at 492 nm was measured with a microplate reader (Model 450; Bio-Rad Lab). Antibody titers were presented as the reciprocal of endpoint dilution exhibiting A_{492} more than 3 times that of background.

Challenge with *A. hydrophila*

Carp (n = 6) were immunized orally with liposome-entrapped *A. hydrophila* (200 μl/ml saline). Immunization was repeated 3 times at 2-week intervals. Two weeks after the last immunization, immunized carp and non-immunized carp (n = 8) were immersed in *A. hydrophila* suspension (1×10^6 cfu/ml) for 60 min. Carp were observed daily for 30 days for clinical signs of infection and survival (%) of carp was calculated. Protective effects were evaluated on the basis of the prolongation of survival and inhibition of ulcer formation on the skin.

Statistical analysis

The Student's *t*-test was used to evaluate the results.

Results

Serum antibody responses in carp orally immunized with liposome-entrapped *A. hydrophila* or *A. hydrophila* alone

Serum antibody titers were measured in carp immunized orally with 100 μg of *A. hydrophila* entrapped in liposomes. Control fish were administered with the same dose of *A. hydrophila* alone. Antibody titers increased significantly 14 days after the primary immunization when fish were immunized with *A. hydrophila*-liposome as shown in Fig. 1. Fourteen days after the third immunization (42 days after the primary immunization), the highest antibody responses were observed. By contrast, little increase of antibody against *A. hydrophila* antigen was detected in the non-immunized control carp (data not shown). These results indicated that oral immunization with liposome-associated *A. hydrophila* antigens could induce significant antibody responses against the bacterial antigen in carp.

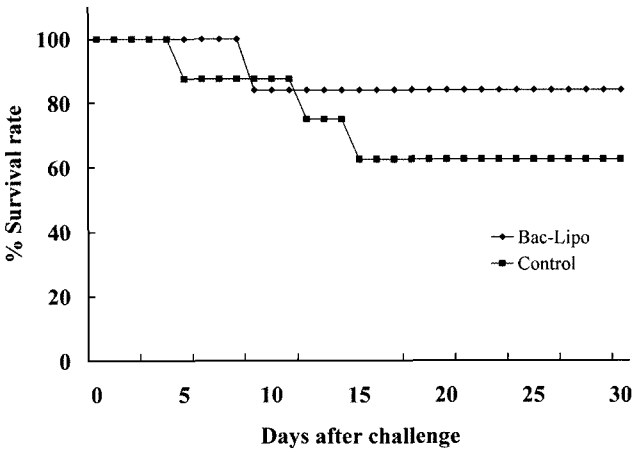

Fig. 1. Serum antibody responses in carp immunized with *A. hydrophila* entrapped in liposomes. Two groups of fish were immunized orally with *A. hydrophila* antigen entrapped in liposomes (n = 6) or *A. hydrophila* antigen alone (n = 4). Antibody titers were measured 2 weeks after the primary, secondary or third immunizations. (*) $p < 0.01$ vs *A. hydrophila* antigen alone. Data are expressed as mean values and standard errors of the mean from six or four fish.

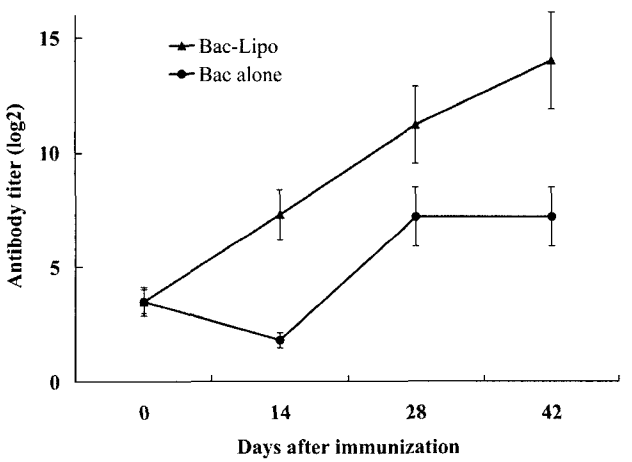

Fig. 2. Antibody production in the intestinal mucosa (A) and bile (B). Four individual fish were orally immunized with *A. hydrophila* antigen-containing liposomes 3 times, and antibody titers were measured 2 weeks after the third immunization. Data are expressed as mean values and standard errors of the mean from four fish. (*) $p < 0.01$.

Antibody production in intestinal mucosa and bile

To study the presence of antigen-specific antibodies in

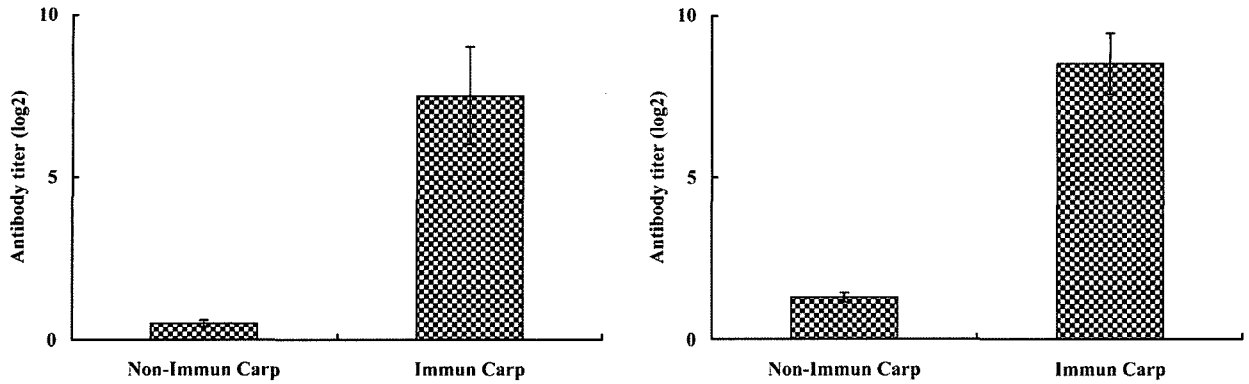

Fig. 3. Survival of carp after *A. hydrophila* challenge. Six fish were immunized orally with *A. hydrophila* antigen-containing liposomes 3 times. Non-immunized eight fish were used as control. Two groups of fish were challenged with *A. hydrophila* (1×10^6 cfu/ml) for 60 min.

intestinal mucosa and bile, carp were orally administered with *A. hydrophila*-liposomes 3 times. A clear increase of anti- *A. hydrophila* antibody titers was observed after the immunization (Fig. 2). On the other hand, antibody responses against *A. hydrophila* in non-immunized carp were very low (Fig. 2). The titers in the intestinal mucosa and bile of immunized carp were significantly higher ($p < 0.01$) than those of non-immunized control (Fig. 2).

Effect of immunization with *A. hydrophila* antigen-containing liposomes on challenge with *A. hydrophila*

Since antigen-specific antibody responses were generated in carp by oral immunization with liposomes containing *A. hydrophila* antigen, we next studied protective effect of *A. hydrophila* antigen-containing liposomes against challenge with *A. hydrophila.* Two weeks after the final immunization, the carp were challenged with *A. hydrophila.* Of the eight non-immunized carp in the control, after challenge with *A. hydrophila,* three carp died within 12 days post-infection whilst five carp (62.5%) survived for 30 days (Fig. 3). However, skin ulcers were observed in the surviving carp and were large and severe on the final day (day 30) of the experiment (Table 1). In the fish immunized with liposomes containing *A. hydrophila*, although one of six carp died on day 9 post-infection (Fig. 3), it showed a 4-day delay in the onset of death compared to non-immunized carp (Fig. 3). The rest of carp (83.3%) survived for 30 days (Fig. 3). Moreover, the development of skin ulcers was significantly inhibited in these carp (Table 1). These results indicated that immunization of carp with *A. hydrophila* antigens was effective for preventing *A. hydrophila* infection.

Discussion

The mucosal surface of the fish intestine is a major site of attack by many infectious agents. Therefore, to protect fish from enteropathogenic agents, induction of the mucosal immunity, and especially the intestinal immune response, is very important. Development of oral vaccines is crucial since the oral route of immunization can induce not only mucosal but also systemic immunity responses (Quentel and Vigneulle, 1997). However, the problem arises from antigen degradation by gastric acidity and proteolytic enzymes in the intestinal lumen, and extremely large doses are required to achieve satisfactory immune responses. It has been reported that liposomes are an effective carrier of antigen to be processed by antigenpresenting cells, such as macrophages (Gluck, 1995), and also that they are effective antigen-delivery vehicle for the induction of immune response (Han et al., 1997; Watarai et al., 1998). Previously, it was demonstrated that oral administration of liposomes containing BSA effectively induced immune responses in carp (Irie et al., 2003). Thus, in this study liposomes were used as carrier for induction of immune responses against *A. hydrophila* in carp.

The oral administration of liposomes containing *A. hydrophila* antigen was more effective for the induction of antigen-specific serum antibody responses than administration of *A. hydrophila* alone (Fig. 1). In addition, the presence of the antigen-specific antibodies in the intestinal mucosa and bile was also confirmed (Fig. 2). The result obtained here was probably due to the effective delivery of antigen entrapped in liposomes to antigen presenting cells and adjuvant activity of liposomes (Lawman et al., 1981; Garcon and Six, 1991; Gluck et al., 1995).

In this study, the efficacy of the immunization with *A. hydrophila* antigen-containing liposomes against challenge with the bacteria was evaluated. Good protection against *A. hydrophila* infection was induced in carp by immunization with liposome-associated bacterial antigen. The survival rate of carp after the challenge was 83.3% when they were immunized with *A. hydrophila* antigen-containing liposomes, whereas non-immunized carp showed 62.5% survival (Fig. 3). Furthermore, development of skin ulcers was significantly inhibited in carp immunized with liposomes containing *A. hydrophila* antigen (Table 1). These results indicate that

immunization with liposomes containing *A. hydrophila* antigen can induce protective immunity to *A. hydrophila*.

In this study, oral immunization with liposome-associated *A. hydrophila* antigen induced antigen-specific antibody responses and provided effective protection against *A. hydrophila* infection (Fig. 3 and Table 1). In fish, three main categories of mechanisms of protective immunity against infection have been reported: humoral antibody response, cell-mediated immune response and non-specific defense mechanisms (Ellis, 1997). Many reports strongly suggest that cell-mediated immunity is important in protection against furunculosis. It has also been demonstrated that immunization with liposomes could induce cell-mediated immunity (Lawman1 et al., 1981; Sugimoto et al., 1995; Ninomiya et al., 2002; Okada et al., 2003). Thus, it is likely that induction of cell-mediated immune responses would result in the protection against *A. hydrophila* infection in carp. However, further studies are required to clarify the mechanisms of induction of cell-mediated immunity in carp by immunization with liposome-associated antigens. Furthermore, it is well known that antibody isotypes, such as IgM, can effectively induce complement activation (Tizard, 2000). Thus, it is also possible that induction of antigen-specific serum IgM antibody after immunization with liposome-associated *A. hydrophila* antigen would lead to death of bacteria or facilitate phagocytosis by phagocytic cells through complement activation. Indeed, the complement activity is considered as an important host defense factor against *A. salmonicida* infection in Atlantic salmon (*Salmo salar*) (Marsden et al, 1996). However, additional studies are required to determine how administration of liposomes containing the bacterial antigen provides protection against *A. hydrophila* infection.

On the basis of this study, immune response against *A. hydrophila* induced by oral immunization with liposomes contaning *A. hydrophila* antigen can effectively protect against infection, therefore liposomes would be suitable carriers for development of novel vaccines against this bacteria. Furthermore, microbial antigen-containing liposomal vaccine might help in controlling of infectious diseases among fish population.

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