Comparative Study on Bacterial Outer Membrane Protein (BOMP) and Bacterial Outer Membrane Gene (BOMPG) Vaccination to Goldfish *Carassius Auratus* against *Aeromonas Hydrophila*

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Abstract

Aeromonas hydrophila is one of the serious bacterial pathogen in fresh water aquaculture industry. Recently, some attention has been given to bacterial outer membrane protein (BOMP) as potentially important vaccine components. Ornamental gold fish *Carassius auratus* immunized with the bacterial outer membrane protein encoding gene clone, BOMPG-pTZ57R/T that was named as BOMPG and the bacterial outer membrane proteins (BOMP) which were developed from a virulent strain of *A. hydrophila* and used as a vaccine candidate. In order to study the comparative effects of the vaccines, BOMPG and BOMP were delivered to the experimental fishes (16.4 ± 1 g mean weight) by intra peritoneal injection once in every 15 days interval. At the end of the experiment, the immunized and control fishes were challenged with virulent strain of *A. hydrophila* and assessed the immunological parameters. *C. auratus* succumbed to death 100% at five days when no vaccination was given whereas the vaccinated groups survived significantly ($F=34.64$; $P \leq 0.001$) of 70 and 80% respectively in BOMPG and BOMP after 10 days of challenge. Surprisingly the vaccinated groups help to decrease the *Aeromonas sp.* load in blood and muscle of treated fishes and improve the serum biochemical parameters significantly ($P \leq 0.001$) than the control group. The haematological and immunological parameters were also improve significantly ($P \leq 0.001$) in the BOMP vaccinated fishes due to the immune enhancement by the vaccines. Among the two different BOMP deliveries, the BOMP highly influenced to improve the immune system against *A. hydrophila* challenge than BOMPG. It may be the reason the BOMPG vaccine may easily degraded in vivo conditions.

Keywords: *Aeromonas hydrophila*; *Carassius auratus*; Outer membrane protein; Vaccine

Background

The ornamental fish trade in aquaculture sector has significant economic importance due to the high value of its exports. In 2007 alone, the value of export of ornamental fish across the world, as estimated by Food and Agriculture Organization of the United Nation (FAO), was reported to be 703 million dollars [1]. The art of rearing ornamental fish started around 800 B.C. in China with the gold fish *Carassius auratus*, which is in high demand and is still throughout the world even today [2]. Nowadays, ornamental fish aquaculture is facing lots of problems such as disease outbreaks, high mortality resulting in loss of economical output. *Aeromonas hydrophila* is an important fish pathogen in aquaculture systems and the diseases caused by this bacterium leading to severe damages and economic losses [3].

In this situation, the conventional treatment protocols against aquatic bacterial pathogens are rather difficult, non-effective, costly and causing environmental problems. Even though antibiotics and synthetic drugs give positive effects against pathogenic control, they cannot be advised due to their residual effects, resistant strain developments, bio-magnifications and suppression of immunity in host organisms [4]. Vaccination is the most efficacious and valuable tool in the prevention of infectious diseases, provided that they are administered prophylactically in anticipation of pathogen exposure.

Fish vaccination in the aquaculture industry has been considered to be very important in reducing economic losses caused by disease [5].

Recently BOMP is considered as highly immunogenic vaccine candidate and are located at host-bacterial interface and are important for host immune responses and as targets for drug therapy. BOMPs play a significant role in virulence as they comprise the outermost surface in contact with host cells and immune defence factors. Recombinant BOMPs have been tested as possible vaccine antigens for *A. hydrophila*. Fang et al. [3] showed significant protection against two isolates of *A. hydrophila* challenged in blue gourami, *Trichogaster trichopterus* immunised with a recombinant BOMP of 43 kDa. Recombinant BOMP (37 kDa) of *A. hydrophila* was also shown to be immunogenic in rohu carp [6].

Nucleic acid immunisation consists of inoculating a target animal with purified DNA or RNA containing a gene (s) under the control of an exogenous eukaryotic promoter, so that protective antigens are expressed within the host [7]. Nucleic acid vaccines potentially have a number of advantages over traditional attenuated, killed or subunit vaccines. They are cheaper and easier to produce than recombinant protein vaccines, have fewer adverse side effects and induce both cellular and humoral immune system [8]. The present study is to focus on the comparative effect of *A. hydrophila* BOMPG and BOMP used to immunize the ornamental gold fish, *C. auratus* against *A. hydrophila* challenge.

Materials and Methods

Source of BOMP clone (BOMPG)

A. hydrophila BOMPG-pTZ57R/T recombinant plasmid [9] was transformed to *E. coli* DH5 competent cells used for immunization. The transformants were revived in LB broth in the presence of ampicillin and incubated at 37°C for 16 hrs. After 16 hrs, the cells were harvested by spun at 5,000 rpm for 5 mins and stored 4°C. Lysate PCR amplification was also performed from the *E. coli* DH5 α cells using *A. hydrophila* specific OMP primer (Figure 1a).

Source of Bacterial Outer Membrane Protein (BOMP)

A highly virulent strain of *A. hydrophila* (AHV1; GenBank: HQ331525.1) was used in this study for extracting outer membrane protein. The BOMP was extracted by using the method of Chakraborty et al. [10] with some modifications. The *A. hydrophila* cell pellets were washed twice in Phosphate-Buffered Saline (PBS) and once in 10 mM Tris-hydrochloride (pH 7.5). Cells were re-suspended in Tris-HCl and sonicated at 50 W for 30 s (4 times on ice). Following sonication, the suspension was mixed with Sarkosyl for solubilization of the OMP and incubated at 25°C for 30 min. After incubation, the suspension was centrifuged at 4000 $\times$ g (20 min), and the supernatant was collected. After centrifugation at 45 000 $\times$ g (45 min), the pellet was collected and stored at 20°C until it was used. The BOMP protein profile was analyzed by the method of Laemmli [11] using 10% SDS PAGE and the BOMP was stored at -20°C for further immunization study (Figure 1b).

Experimental set-up and vaccination

The healthy *C. auratus* (16.4 ± 1 g mean weight) were purchased from local ornamental fish hatchery, transported to laboratory and acclimatized in 1000 l FRP tanks for 10 days. The fishes were fed ad libitum thrice daily with a formulated standard diet and commercial pellet feed at a ratio of 10% of their body weight throughout the period of study. Partial water exchange was performed daily to remove waste feed and faecal materials. Only healthy fish, as determined by general appearance and level of activity, were used for the experiment. After acclimatization, triplicate tanks containing a total of sixty fishes (20 $\times$ 3=60) were maintained in each experimental and control groups. The

tanks were of 500 l capacity, flow-through aquaria with water flow rate of 1 ml/min and the fishes fed with commercial formulated feed of thrice a day in ad libitum. The vaccines including BOMPG clone and BOMP were administered to the experimental fishes by intraperitoneal injection at 1 and 15th days. The vaccine delivery dose for BOMPG was 10^7 cfu/ml bacterial cells and 200 μ g total protein of BOMP per fishes in each injection. The blank control (BC) group received no vaccine delivery and no *A. hydrophila* challenge. The control (C) group received the *E. coli* cells at the density of 10^7 cfu/ml without BOMPG-pTZ57R/T clone and *A. hydrophila* challenge.

Challenge with virulent *Aeromonas hydrophila*

After the termination of culture period, virulent *A. hydrophila* were injected to the experimental and control (C) fishes by intraperitoneally at the rate of 10^7 cfu/ml. The cumulative mortality was monitored for a group of fishes in all experiments and the rest were sacrificed and bled the blood for biochemical, haematological and immunological parameters.

Cumulative mortality and Specific bacterial count

The cumulative mortality in *A. hydrophila* was assessed after challenge for 10 days. The randomly selected fishes from each group were weighed aseptically and immersed in 50 ppm formalin solution for 5 min (to remove external bacteria present on the goldfish). They were rinsed thoroughly with sterilized water for 30 sec to remove the remaining surface bacteria and disinfectant. The washed muscle were homogenized with 5 ml of sterile distilled water and serially diluted up to 10-fold. Sample of 0.5 ml was taken with the help of sterilized pipette and poured into *Aeromonas* isolation agar medium-M884 (HIMEDIA, India). Triplicate plate count were maintained in each sample and incubated at 37°C for 48 h.

Biochemical parameters

Saturated ammonium sulphate was added to 100 μ l of serum in micro centrifuge tube, mixed well and left to stand for 1 hour at room temperature. The tubes were then centrifuged at 10,000 rpm for 10 minutes and the supernatant (albumin fraction) was collected into separate tubes. The precipitate (globulin fraction) was dissolved in 500 μ l of distilled water. The protein content of both albumin and globulin fractions were determined [12]. Total serum protein was determined at 650 nm under UV visible spectrophotometer by using bovine serum albumin (BSA) as standard protein.

Haematological parameters

Haemoglobin level was determined by haemoglobin cyanide method described by Van Kampen and Zijlstra [13]. Total erythrocyte count was performed following the method of Hendricks [14] using a haemocytometer.

Immunological parameters

The Haemagglutination assay of serum samples was carried out using the method described by Sritunyalucksana et al. [15]. This assay was performed in U shaped micro well plates. Two fold dilution of serum samples were made in 1 $\times$ tris buffered saline (TBS). An equal volume of 1.5% RBC was added to each dilution of serum sample. The plates were incubated at 37°C for 30 min. Haemagglutination titre was recorded as the reciprocal of last dilution, resulting in agglutination

after 30 minutes incubation. Negative controls comprised of equal volume of RBC and TBS. Phagocytosis was performed following the method of Park and Jeong [16] using formalin killed *A. hydrophila* at the rate of 10^7 cfu/ml with 0.1 ml of blood samples of each groups. Bactericidal activity was determined in vivo i.e., fish were injected with 10^7 cfu of live *A. hydrophila* suspension and the blood samples were collected from the fishes after 10 and 90 min intervals. One hundred micro liters of blood was serially diluted and spread plated in triplicate on TSB agar plates (HIMEDIA, India) and incubated at 34°C for 24 h. From the total number of *A. hydrophila* colonies in each plate, total colony forming unit (cfu) in each blood sample was estimated. Intra-agar lysozyme activity was performed against *Micrococcus luteus* culture by diluting the blood cells and inoculating a drop upon the agar wells. The drops were allowed to absorb onto the agar and were incubated in a inverted manner for 24-48 hrs at 37°C . The plates for the highest dilution of blood cells capable of lysing the test microbes were then scored.

Data analysis

One way and two way analysis of variance (ANOVA) were carried out using SPSS statistics data package and Ky plot respectively. Positive correlation analysis also performed to compare the data by Microsoft Excel programme. Means were compared at 0.05, 0.01 and 0.001% level.

Results

Cumulative mortality of *C. auratus* after challenge

C. auratus succumbed to 100% death within five days after being challenged with virulent *A. hydrophila*. The BOMP vaccines helped to increase the survival to 70 and 80% respectively in BOMPG and BOMP treated groups. Only 10% of cumulative mortality was varied among the vaccine treated fishes and the two way ANOVA revealed that the values significantly differed from each other's (Column: $F=50.62$; $P \leq 0.001$ and Row: $F=2.38$; $P \leq 0.05$) (Figure 2).

Specific bacterial count in blood and muscle

The specific *Aeromonas* count of the control and vaccinated fishes are given in the Table 1. The bacterial loads observed of 1.5×10^6

cfu/ml and 7.4×10^5 cfu/g after 8 days from challenge in blood and muscle respectively. After 2 days from the challenge, the load was observed to be 1.8×10^4 and 1.6×10^4 cfu/ml in the blood of BOMPG and BOMP treated *C. auratus* respectively. Surprisingly, the BOMP vaccines decreased the load to 1.1×10^2 and 3.5×10 cfu/ml respectively in the blood of BOMPG and BOMP treated *C. auratus*. The same trend of reduction was also observed in the muscle.

	Aeromonas Count					
	Blood (cfu ml-1)			Muscle (cfu g-1)		
	2 Days	4 Days	8 Days	2 Days	4 Days	8 Days
Blank Control	-	0.3×10^1 $\pm$ 0.1×10^1	0.5×10^1 $\pm$ 0.1×10^1	-	-	0.2×10^1 $\pm$ 0.01×10^1
Control	4.4×10^4 $\pm$ 0.21×10^1	8.6×10^5 $\pm$ 0.5×10^1	1.5×10^6 $\pm$ 1.4×10^1	3.6×10^2 $\pm$ 0.3×10^1	1.4×10^3 $\pm$ 0.15×10^1	7.4×10^5 $\pm$ 0.11×10^1
BOMPG	1.8×10^4 $\pm$ 0.10×10^1	4.0×10^3 $\pm$ 0.05×10^1	1.1×10^2 $\pm$ 0.3×10^1	6.3×10^3 $\pm$ 0.2×10^1	1.2×10^3 $\pm$ 0.3×10^1	8.5×10^2 $\pm$ 0.10×10^1
BOMP	1.6×10^4 $\pm$ 0.06×10^1	1.1×10^2 $\pm$ 0.02×10^1	3.5×10^1 $\pm$ 0.05×10^1	19.5×10^2 $\pm$ 0.05×10^1	8.7×10^2 $\pm$ 0.04×10^1	1.2×10^2 $\pm$ 0.01×10^1

Table 1: *Aeromonas* count from blood and muscle samples from BOMP vaccinated *C. auratus* after *A. hydrophila* challenge at different time intervals.

Biochemical parameters of *C. auratus* after challenge

Serum albumin in the blood was found to be 241.25 $\mu\text{g/ml}$ in *C. auratus* when no vaccination was given. The level was increased significantly ($P \leq 0.001$) to 252.45 and 268.22 $\mu\text{g/ml}$ in BOMPG and BOMP treated groups. The globulin level also increased in the same way as albumin in the vaccinated groups with a maximum of 245.2 $\mu\text{g/ml}$ in BOMP treated fishes. Serum protein in the blood was 466.30 $\mu\text{g/ml}$ in *C. auratus* with no vaccination and it increased significantly ($P \leq 0.001$) to 485.34 and 518.04 $\mu\text{g cfu/ml}$ respectively in the BOMPG and BOMP treated groups (Figure 3). The two vaccinated groups of BOMPG and BOMP were influenced to increase the biochemical parameters such as serum albumin, serum globulin and serum protein. The BOMP vaccines helped to increase the serum biochemical parameters around 4% than the BOMPG group by the effective antigen presentation and higher antibody production. The positive correlation analysis also revealed a significant relationship ($P < 0.05$) found among the treatment from control to experimental groups (Figures 4a-4c).

Figure 3: Biochemical changes of BOMP vaccinated *C. auratus* after *A. hydrophila* challenge Means with the same superscripts (a-d) do not differ from each other-One Way ANOVA ($P \leq 0.001$).

Figure 4: Positive correlation between control and vaccine treated fishes' serum biochemical parameters. Values are the mean of three determinations $\pm$ SEM ($P < 0.05$).

Haematological parameters of *C. auratus* after challenge

The red blood cell (RBC) count was recorded of $46.2 \times 10^2/\text{mm}^3$ in blank control fish group, whereas the count was drastically decreased to $29.28 \times 10^2/\text{mm}^3$ in control group by *A. hydrophila* interruption. Surprisingly the RBC count was significantly ($P \leq 0.01$) increased to 48.4 and $52.3 \times 10^2/\text{mm}^3$ in BOMPG and BOMP respectively. The haemoglobin level was observed to be 4.39 mg/dl in *C. auratus* challenged with *A. hydrophila* without vaccine treatment. The vaccine BOMP helped to increase the haemoglobin level significantly ($P \leq 0.001$) of 9.83 mg/dl (Figure 5).

Figure 5: Haematological parameters such as RBC and haemoglobin of BOMP vaccines treated goldfish *C. auratus* against virulent *A. hydrophila* challenge after 30 days. Means with the same superscripts (a-d) do not differ from each other – one way ANOVA * ($P \leq 0.01$); ** ($P \leq 0.001$).

Immunological parameters of *C. auratus* after challenge

Haemagglutination assay were carried out against the RBC of human A, B and O blood groups (Table 2). The vaccine treated *C. auratus* antigen reacted against the human RBC even at 1:4 and 1:16 dilutions in the BOMPG and BOMP respectively. The BOMP helps to improve the immune efficiency at one time in *C. auratus* against *A. hydrophila*. Phagocytic activity was observed to be 18.23% in control group (Figure 6), and this was increased significantly ($P \leq 0.001$) to 49.73 and 55.11% in BOMPG and BOMP treated fishes against *A. hydrophila* challenge. The serum bactericidal activity also showed the same trend about 4.15% in the control fishes whereas this was significantly ($P \leq 0.001$) increased to 14.33 and 17.07% in BOMPG and BOMP treated fishes respectively. The activity was increased more than three times than the control fishes against BOMP treated fishes (Figure 6). The intra-agar lysozyme assay against *Micrococcus luteus* revealed that less activity of 4.93 mm zone of inhibition was found in the serum of control fishes. The serum from vaccinated fishes helped to increase the activity to 14.83 and 17.72 mm of zone of inhibition against *M. luteus* in BOMPG and BOMP treated fishes respectively. One way ANOVA revealed that, the activity significantly differed from each other's ($P \leq 0.001$ -Table 3).

Discussion and Conclusion

Considerable interest has been shown in bacterial OMP vaccines since these were thought to contain some of the major antigens responsible for inducing an immune response in the host [3,17]. The outer membrane proteins of *A. hydrophila* have a role in the virulence of the organism and are potential candidates for vaccine development [18]. The present study, outer membrane proteins play a significant role in virulence as they comprise the outermost surface in contact with host cells and immune defense factors. Earlier works supported *C. auratus* vaccinated with recombinant OMP showed a higher percentage of survival after a virulent *A. hydrophila* challenge [9]. *C. auratus* vaccinated with *A. hydrophila* BOMP revealed that, the vaccinated fishes survived more than 70% after challenge. Among the two treatments such as BOMPG and BOMP in *C. auratus*, the BOMP vaccine was highly influenced to improve the survival, biochemical, haematological and immunological parameters.

Figure 6: Phagocytic activity and serum bactericidal activity (%) of BOMP vaccines treated goldfish *C. auratus* against the virulent *A. hydrophila* challenge after 30 days. Means with the same superscripts (a-d) do not differ from each other-one way ANOVA ($P \leq 0.001$).

The BOMP was helped to reduce the specific bacterial load at a higher level than the BOMPG after *A. hydrophila* challenge. The higher level reduction was found in muscle and blood of BOMP treatment than the BOMPG delivered *C. auratus* after *A. hydrophila* challenge. The BOMP vaccine helped to increase the production of higher antibody production against *A. hydrophila* outer membrane protein. This higher antibody production level may be suppressed the growth of *A. hydrophila* load. The earlier report by Thangaviji et al. [19,20] showed that the *A. hydrophila* OMP and biofilm vaccines with the herbal immunoadjuvant, *Asparagus racemosus* extracts helped to decrease the *Aeromonas* load of 1.1×10^2 and 0.5×10^2 cfu/g respectively in the muscles on the 50th day of vaccination.

Generally there is an increased serum biochemical parameters observed in immunized fishes with vaccines or immunostimulants. Vaccination of *A. hydrophila* ECP, OMP and biofilm with herbal immuno-adjuvant helped to increase the serum albumin and globulin levels in *C. auratus* [20]. The herbal immunostimulants obtained from plants such as *Lonicera japonica* and *Ganoderma lucidum* helped to increase the immunoglobulin level in *Oreochromis niloticus* against *A. hydrophila* challenge [21]. Dina Rairakhwada et al. [22] also found that the globulin level was significantly increased in the levan; an immunostimulant fed common carp fingerlings after challenged with *A. hydrophila*. Misra et al. [23] also reported that total serum protein content increased significantly in the fish fed with dietary doses of β -glucan when compared with control group. Since serum proteins include various humoral elements of the non-specific immune system, high concentrations of total serum protein, albumin and globulin might be due to the enhancement of non-specific immune response of fishes.

Treatments	Dilutions	Blood Group		
		A	B	O
Blank Control	Negative control	+	+	+
	Positive control	-	-	-
	1:1	-	-	-
Control	Negative control	+	+	+

BOMPG	Positive control	-	-	-
	1:1	-	-	-
	1:1	+	+	+
	1:2	+	+	+
	1:4	-	+	+
BOMP	1:8	-	-	+
	1:16	-	-	-
	1:1	+	+	+
	1:2	+	+	+
	1:4	+	+	+
	1:8	+	+	+
	1:16	-	+	+
	1:32	-	-	-

Table 2: *In vitro* haemoagglutinin assay of BOMP vaccine treated *C. auratus*'s blood with human A, B and O and groups RBC after 30th day immunization after challenged with *A. hydrophila*.

Treatments	Lysozyme activity (mm)			
	Dilution factor			
	Blank (DH2O)	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Control (C)	0.0 ± 0.0	4.93 ^a ± 0.04	4.06 ^a ± 0.10	2.40 ^a ± 0.03
BOMPG	0.0 ± 0.0	14.83 ^b 0.04	12.84 ^b ± 0.03	9.84 ^b ± 0.02
BOMP	0.0 ± 0.0	17.72 ^c 0.05	14.06 ^c ± 0.01	11.77 ^c ± 0.02

Table 3: Zone formations (mm) of intra-agar lysozyme activity of the blood of *C. auratus* treated with BOMP vaccine and challenged with *A. hydrophila* after 30 days immunization. Means with the same superscripts (a-c) do not differ from each other- One Way ANOVA, ($P \leq 0.001$)

Blood cells play an important role in cellular defence mechanisms. The decreased number of circulating cells in the host blood indicates that, the system is less resistance against pathogens. In the present study, RBC and haemoglobin level was found to be a maximum of $52.3 \times 10^3/\text{mm}^3$ and 9.86 mg/dl in the fishes immunized with BOMP. The BOMP vaccine helped to increase the RBC level to 78% and haemoglobin level to 124% from the control groups due to higher immune response in the vaccinated fishes. The low level of RBC found in the control fishes suffered to loss of body fluids, oxygen carrying capacity and finally leading to mortality. The decreased RBC, haematocrit, and haemoglobin levels in the infected and untreated fishes indicated that, the blood cells destroyed by leukocytosis with subsequent erythroblastosis [24]. Sahu et al. [25] reported that WBC and RBC counts were higher in *Labeo rohita* fingerlings fed *M. indica* kernel when compared to the control. In rainbow trout, *Oncorhynchus*

mykiss infected with *A. hydrophila*, had increased white blood cells leading to anemia could be attributed to increase destruction, loss, or suppression of RBCs [26].

Thangaviji [20] found that, the serum of *C. auratus* immunized with outer membrane vaccines was agglutinated at the dilutions of 1: 12 level. *A. racemosus* has been used as a good adjuvant [27] and provides an efficient and sustained immunostimulation to improve the immunogenicity of weak or low dose of antigens. The present study the *C. auratus* immunized with BOMP had higher titer value than the fish immunized with BOMPG. This may due to more antibody production by the outer membrane protein than the BOMPG. Oral administration of yeast products in channel catfish [28], chitin [29], plant extract including *Zingifer officinalis* increased the phagocytic capability of the cells in rainbow trout [30] and extracts of four Chinese herbs (*Rheum officinale*, *Andrographis paniculata*, *Isatis indigotica*, *Lonicera japonica*) were increased the phagocytosis and white blood cells in Crucian carp [31]. The BOMP had also responsible for higher phagocytic activity than the BOMPG in the vaccine treated *C. auratus*. OMP are located at host-bacterial interface and are important for host immune responses and as targets for drug therapy [32]. Fang et al. [3] showed significant protection against two isolates of *A. hydrophila* in blue gourami, *Trichogaster trichopterus* immunized with a recombinant 43 kDa OMP. Khushiramani et al. [33] isolated 3 to 4 high intensity OMP bands from 40 strains of *A. hydrophila* had the range between 25 to 45 kDa. The OMPs extracted from the *A. hydrophila* cell wall and the recombinant OMP gene cloned to the plasmid improved the survival, bacterial load reduction and improved immunological parameters including serum bactericidal activity, lysozyme activity and phagocytosis in the vaccinated *C. auratus*. The OMP may boost the immune system and activate the T cell as well the B cell memory. Once the *A. hydrophila* pathogenic interruption happened in the host system, the T cell as well the B cell are recognizing the OMP of cell wall of the *A. hydrophila* and destroy. Even nucleotide vaccines without the recombinant technology also induce the immune system in various fresh water fishes including carp and other ornamental fishes against *A. hydrophila* infection [34]. The present findings revealed the bacterial outer membrane protein (BOMP) had higher immunostimulatory effects against *A. hydrophila* infections than the delivery of BOMPG through vector. The reasons behind it is that outer membrane proteins (BOMP) helps to produce more antibodies than the BOMPG and it may have been easily degraded in vivo conditions. Another possibility is to enhance the immunity by delivering the antigens with adjuvant or microspheres.

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