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A vaccine based on biodegradable microspheres induces protective immunity against scuticociliatosis without producing side effects in turbot

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ABSTRACT

The histiophagous scuticociliate parasite *Philasterides dicentrarchi* is an emergent pathogen in aquaculture and causes significant economic losses on turbot (*Scophthalmus maximus*) farms. In this study, the surface antigens (Ag) of the parasite were encapsulated and covalently linked to a polymeric microparticle formulation composed of two biodegradable polymers (chitosan and Gantrez). The antigenicity of the formulation and the protection provided were compared in mice and turbot. This formulation induced a higher antibody (Ab) response in mice at doses of 5 mg of microspheres (MS) conjugated with approximately 230 μg of Ag (MS-Ag^c). However, Ab levels were significantly lower than in mice vaccinated with the same concentration of Ag in complete Freund's adjuvant (FCA). In turbot, the MS-Ag^c formulation induced a higher level of Abs than that induced by the same vaccine emulsified in FCA. The challenge experiments performed with *P. dicentrarchi* and vaccinated turbot also showed a clear correlation between Ab levels and survival levels. Growth was significantly affected in fish vaccinated with FCA, but not in fish vaccinated with MS. The high adjuvant capacity of MS, together with its biodegradability and low toxicity to fish, makes this new vaccine an economical, effective and safe alternative to oil-based adjuvants for the immunoprophylaxis of scuticociliatosis in turbot.

1. Introduction

In recent years, systemic scuticociliatosis caused by the histiophagous parasite *Philasterides dicentrarchi* has been recognized as an emerging parasitic disease and has become one of the most important parasitological problems causing significant economic losses in turbot culture in Europe [1]. So far, no systemic chemotherapeutic treatments have yet proven effective against the parasite when present as an endoparasite in fish [2,3]. Therefore, considerable effort has been directed at the development of a vaccine as an attractive alternative to chemotherapeutic treatments for effective prevention of this disease. We recently developed a formalin-fixed ciliate vaccine that produces a high degree of protection in turbot challenged with a virulent isolate of *P. dicentrarchi* [4–6]. Earlier studies have shown that *P. dicentrarchi* expresses surface antigens that can confer a degree of protection against this pathogen in turbot [7]. For vaccines to produce a high level of protection in fish, adjuvants must be included to increase and prolong the immune responses, ideally without any adverse effects [8–10]. Oil-based adjuvants have been widely used in association with fish vaccines [11]. However, the use of oil-based adjuvants is often associated with adverse effects such as the development of granulomas and necrosis, which cause intra-abdominal lesions, adhesions in the peritoneum, which can lead to slowed growth rate, downgrading of fish, as well as suffering and even death of fish [12].

The use of biodegradable polymeric microspheres (MS) as a platform for antigen (Ag) delivery systems has been extensively studied in mammals [13]. Biodegradable MS containing Ag have been showed to serve as adjuvants for antibody (Ab) induction [14]. The immune response is mainly produced because the MS are a similar size as the pathogens and are therefore efficiently internalized by Ag presenting cells. The uptake of MS <5 μm by phagocytic cells is likely to be important in the ability of particles to perform as vaccine adjuvants [15]. It is also well known that MS with entrapped Ag display comparable immunogenicity to Freund's adjuvant and that they are well tolerated and do not cause the inflammation and formation of granulomas associated with many of the oil-based vehicles used as Ag depots [16]. Therefore, MS can serve as vehicles and provide controlled release

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of entrapped Ag (Ag^e) or coupled Ag (Ag^c). MS have been made from a number of different types of material including chitosan, which has been shown to be a suitable vaccine carrier [17]. Chitosan easily forms MS and nanoparticles that encapsulate large amounts of Ag, and its mechanical properties can be improved by further cross linking with bifunctional reactants like glutaraldehyde [18].

Poly (methyl vinyl ether)-co-(maleic anhydride) (Gantrez® AN119; PVMMA) is a polymer belonging to the vinyl ether group that it is widely used for pharmaceutical purposes and has also been used to prepare ligand-nanoparticle conjugates for eliciting immune responses [19].

The aim of this study was to develop a versatile MS formulation, based on two biodegradable polymers (chitosan and Gantrez), with optimal physico-chemical characteristics for use as an Ag delivery system to increase the immunogenic potency and protection for use as a vaccine against turbot scuticociliatosis.

2. Materials and methods

2.1. Materials for MS preparation

Chitosan Seacure 210 HCl was obtained from Pronova (Drummond, Norway). Poly (methyl vinyl ether)-co-(maleic anhydre) (PVMMA) was kindly gifted by ISP Corporation (Barcelona, Spain). Ethyl formiate and acetone were purchased from Merck, Spain.

2.2. Parasites

P. dicentrarchi (isolate I1) [20] was isolated, under aseptic conditions, from the ascitic fluid of infected turbot obtained from a local fish farm (Galicia, Spain). The isolate was cultured at 18 °C in L-15 Leivovitz medium as previously described [21]. The ciliates were passage through fish before being used in the vaccines or to induce experimental infections [22].

2.3. Ag preparation

Prior to preparation of the Ag, ciliates (2×10^6) from the early stationary phase culture were washed two times by centrifugation at 700 × *g* at 4 °C and resuspended in PBS pH 7.4 and a third time in 0.25 M sucrose. Once the pellet was resuspended in PBS containing 1 mM phenylmethylsufonyl fluoride (PMSF, dissolved in ethanol), ultrasound was applied to the ciliates on a bed of ice, in order to totally lyse them. For separation of membrane Ag, the ciliate suspension was centrifuged at 3000 *g* for 5 min and the pellet was resuspended in PBS. To solubilise the Ag, 0.5% Triton X-114 was added and the solution was rotated end over end for 12 h at 4 °C. The solution was exhaustively dialysed in PBS to remove the excess triton, and then stored at −80 °C until use.

2.4. Preparation of Chitosan Seacure 210 HCl − PVMMA MS

Chitosan-PVMMA MS were prepared by spray drying. A Mini Spray Dryer (Mini Büchi 190 B, Büchi, Flawil, Switzerland) was used to disperse 240 mg of PVMMA in 50 ml of acetone. This solution was added drop wise to a solution of 250 mg of chitosan Seacure 210 HCl in 50 ml of 0.1 M acetic acid with 0.075% of glutaraldehyde as a cross-linking agent. The polymeric dispersion was dried at 72 °C in the mini Spray Dryer. The product was fed at 3 ml/min, at an outlet temperature of 42 °C, and a pressurized air flow of 500 l/h. Finally, MS were collected in the cyclone and kept under vacuum for 18 h.

2.5. Ag encapsulation

Ag encapsulation (Ag^e) was carried out by preparing 250 μl of the completely homogenized Ag solution (20 mg/ml) to the chitosan aqueous phase. PVMMA in acetone was added drop wise to the chitosan solution. The spray drying process was carried out as above. In this case, the atomization temperature was 69−70 °C to prevent degradation of the protein.

2.6. Ag covalent coupling

The coupling of the Ag to MS surface was performed as following: Chitosan Seacure 210 HCl and PVMMA MS were collected in the cyclone and kept under vacuum at 40 °C for 12 h to produce the optimal cross linking of polymers. After this period, 10 mg of MS were weighted and placed in an eppendorf tube, and 1 ml of the Ag solution (1 mg/ml protein) was added. This mixture was incubated in a shaker at 500 rpm for 15 min at 20 °C. The MS were collected by centrifugation 5000 × *g* for 10 min, washed twice with PBS and once with water. The MS were then freeze dried. In all cases, the incubation solutions were maintained to measure the amount of protein linked to MS.

2.7. MS characterization

2.7.1. MS morphology and particle size measurement

The MS morphology was determined by phase contrast microscopy and scanning electron (SEM) microscopy. The size and size distribution of the MS were analysed by a laser light-scattering method (Mastersizer, Malvern Instruments, UK).

Particle size distributions were calculated in the volume-weight mode and characterized as mean diameter, $D(0.5)$. $D(0.1)$ and $D(0.9)$ were used to determine the span of the distribution.

2.7.2. Zeta (ζ) potential

The ζ-potential was determined in a Zeta Plus Potential Analyzer (Brookhaven Instruments Corporation, New York, USA) and the MS were resuspended (0.05%) in 0.1 M acetic acid.

2.7.3. Encapsulation efficiency

Twenty milligrams of MS were dissolved in 0.1 M acetic acid for 18 h, and the sample was then centrifuged for 20 min at 20,000 × *g*. The protein was measured by a spectrophotometric assay, with a Quanti Pro BCA assay kit (Sigma Aldrich).

2.7.4. Coupling efficiency

The amount of protein in the supernatant was quantified with a Quanti Pro BCA assay. The amount of unbound Ag recovered by centrifuging and washing the MS was subtracted from the initial amount of protein. The fluorescence was also measured with Fluorostar Optima equipment (BMG Labtech, Biogen, Madrid, Spain) at 485 nm fluorescence excitation and 520 nm for emission and wavelength, respectively.

2.7.5. In vitro release

Protein release from MS was determined in PBS pH 7.3. Thirty milligrams of MS were incubated under agitation at 37 °C and 100 rpm (Heidolph Instruments incubator 1000) in 1 ml of PBS. After 30, 60, 120, 180 min and 24 or 48 h, samples were centrifuged at 20,000 × *g* for 20 min and the supernatant was measured by a spectrophotometric assay, with a Quanti Pro BCA assay kit.

2.7.6. Infrared studies

Infrared spectroscopy (IF) was carried out to confirm conjugation of the protein with MS. Samples were prepared in KBr discs