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Short communication

A model to study fish DNA immersion vaccination by using the green fluorescent protein

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Direct intramuscular injection of some reporter genes and their expression under fish (Rahman & MacLean 1992), frog (Amsterdam, Lin & Hopkins 1995; Amsterdam, Lin, Moss & Hopkins 1996) or viral (Hansen, Fernandes, Goldspink, Butterworth, Umeda & Chang 1991; Anderson, Mourich & Leong 1996b; Gomez-Chiarri, Livingston, Muro-Cacho, Sanders & Levine 1996) promoters has been reported. Furthermore, expression of the G and N proteins from infectious haematopoietic necrosis virus (IHNV) (Anderson, Mourich, Fahrenkrug, LaPatra, Shepherd & Leong 1996a) and viral haemorrhagic septicaemia virus (VHSV) (Heppell, Lorenzen, Armstrong, Wu, Lorenzen, Einer-Jensen, Ahrens, Schorr & Davis 1998), and production of high levels of specific neutralizing and protective antibodies against these viruses (Bourdinot, Blanco, De Kinkelin & Benmansour 1998) have been detected following intramuscular injection of plasmids containing the corresponding genes. Furthermore, it has been demonstrated that fish injected with DNA containing viral genes were protected against viral challenges. However, injection vaccination methods (Leong, Bootland, Anderson, Chiou, Drolet, Kim, Lorz, Mourich, Ormonde, Perez & Trobridge 1995; Olesen & Korsholm 1997) are restricted to medium-sized fish.

To make the use of the DNA-vaccination technologies more practical, and to enable vaccination of younger fish or fish against other pathogens, immersion methods need to be developed (Fernandez-Alonso, Alvarez, Estepa & Coll 1998). However, there are no reports of the expression of proteins following immersion of fish into water - dissolved plasmids containing the corresponding reporter or pathogen genes. A model system is described in the present study which uses DNA-liposome formulations and immersion of rainbow trout, *Oncorhynchus mykiss* (Walbaum), into plasmid coding for the green fluorescent protein (GFP) (Cormack, Valdivia & Falkow 1996). The system uses small (0.2–0.5 g) fish to make experimentation easier, enabling a range of variables and experimental conditions to be studied. Once an optimum experimental method has been selected using the small fish model, the use of 5–15 g fish in immersion vaccination could be assayed, since this is the size of fish usually vaccinated by immersion.

Plasmid constructs containing the green fluorescent protein (GFP), cloned downstream of the cytomegalovirus (CMV) promoter in $pQBI_{25}$ Quantum Biotech Inc., Montrevil-sous-bois, France, were used to transform *Escherichia coli* XL2.

The glycoprotein G gene of VHSV (French isolate 07.71) was removed from the pcDNAI (Invitrogen, Groningen, The Netherlands) vector (Dr M. Bremont, INRA, Jouy-en-Josas, Paris, France) with restriction endonucleases EcoRI and XhoI, and inserted into the pcDNAI/Amp (Invitrogen) vector to create a new recombinant plasmid called G3-pcDNAI/Amp. The construct was transferred and multiplied in the *E. coli* strain Top10F′ grown on LB. To produce recombinant *E. coli* in

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large amounts, a 17-L fermentator using JLB medium (12 g tryptone, 24 g yeast extract, 4 mL glycerol, 2.3 g of 170 mM KH_2PO_4, 12.5 g of 720 mM K_2HPO_4 L^{-1}) in the presence of 50 mg L^{-1} of ampicillin (Inbiotec, León, Spain) was used. Plasmids were prepared from *E. coli* pellets using the Wizard plus Megaprep DNA purification system (Promega, Madison, WI, USA). Plasmid solutions were adjusted to 1 mg mL^{-1} of total DNA (A 260-nm) which contained 30–60% G3-pcDNAI/Amp, as shown by agarose gel electrophoresis, the rest being other bacterial DNA.

Epithelial papillosum carp (EPC) cells were grown in 24-well plates at 26–28 °C with RPMI culture medium supplemented with 10% foetal calf serum. When cells were about 70% confluent, the material was incubated for 1 or 2 h with 0.5 μg of G3-pcDNAI/Amp complexed with 10 μL of lipofectin reagent (GibcoBRL, Life Technologies, Postfach, Germany) in 500 μL of RPMI. Complete medium (3 mL) was then added and the transfected cells grown for 3 days more (Bearzotti, Perrot, Michard-Vanhee, Jolivet, Attal, Theron, Puissant, Dreano, Kopchick, Powell, Gannon, Houdebine & Chourrout 1992) prior to FACS analysis. Transfected cells were washed and detached with 3 mL of buffer I (0.13 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 0.1% bovine serum albumin, 0.01% N_3Na, 5 mM EDTA, pH 7.4). The cells were then centrifuged and incubated with neutralizing MAb 3F1 A12 (N. Lorenzen *et al.*, personal communication) in buffer I containing 2% rabbit serum, 2% goat serum, 2% *E. coli* extract and 2% rainbow trout serum. After centrifugation, the cells were incubated with fluorescent goat anti-mouse Fab'2 fragment (Caltag, San Francisco, CA, USA). The EPC cell suspensions were again centrifuged and washed twice. On the same day as harvest and staining, 5000 cells were analysed by flow cytometry in a Beckton-Dickinson (San José, CA, USA) FACScan apparatus using the LYSYS II Version 1.0 program. Fluorescence was measured in the FL1 region (514–545 nm, green).

For the DOTAP (1,2, dioleoyl-3–4 trimethyl ammonium propane) (Avanti Polar Lipids, Alabaster, AL, USA)-DNA preparations, 20 mM DOTAP and 20 mM cholesterol sonicated liposomes were mixed and lyophilized with 20–200 μg of DNA just prior to use (Gregoriadis, Saffie & BriandeSouza 1997). Five rainbow trout (body weight = 0.2–0.5 g; Las Zayas, León, Spain) were maintained in a beaker with 20 mL of aquarium water for 15–30 min with the DOTAP-DNA preparations. As a positive control (Heppell *et al.* 1998), fish were intramuscularly injected with 1 μg of plasmid DNA using a 10-μL Hamilton syringe (1 μL $fish^{-1}$). The fish were then placed in 500-mL beakers and maintained at 4–10 °C for 1–2 weeks until analysed for GFP expression. The plasmid DNA remaining in the water after immersion of the trout was examined by agar electrophoresis and no variations in the amounts or band patterns were detectable in any of the cases (data not shown).

After immersion in the DNA preparations, the fish were anaesthetized with 20 mg L^{-1} of MSS-222 (3-aminobenzoic ac.ethyl ester, Sigma Chemical Co., St Louis, MO, USA) and the caudal fins covered with a coverslip. Caudal fins were then examined and photographed at × 40 (CCDOPS program VSSS1.09) using a Nikon Diaphot inverted microscope fitted with a mercury lamp using a FITC filter set (DM510 B-2 A) and a SBIG ST-7 camera (Santa Barbara Instrument Group, Santa Barbara, CA, USA). Identical fluorescent background and range values were chosen for comparison between the different photographs.

Before use in the DNA-vaccination attempts, expression of both GFP (not shown) and G (Fig. 1) were demonstrated by FACS after transfection with lipofectin in a fish cell line.

After treatment with the GFP-coding $pQBI_{25}$ plasmid complexed with DOTAP-cholesterol liposomes, trout caudal fins showed abundant fluorescent spots of about 10–20 μm in diameter brighter than background levels. Caudal fins from fish intramuscularly injected with $pQBI_{25}$ alone were also fluorescent, whereas non-injected and non-$pQBI_{25}$-exposed fish had no noticeable fluorescence above background levels. The fluorescence detected could first be detected 2–3 days after plasmid exposure and lasted for at least 10 days more. Fluorescence appeared not only in the caudal fins, but also in the other fins examined (i.e. ventral and dorsal). In the initial experiments, whole fish were observed to determine the easiest way to assay for fluorescence and it was found that only the fins could be observed without damaging the fish. On the other hand, some internal organs were fluorescent even in fish not exposed to plasmids. Thus, caudal fins were selected for further studies because the fish could be handled more easily. All the caudal fins were examined at × 40 under the inverted microscope and the brightest fluorescent fields were photographed for each fish examined. The photo-

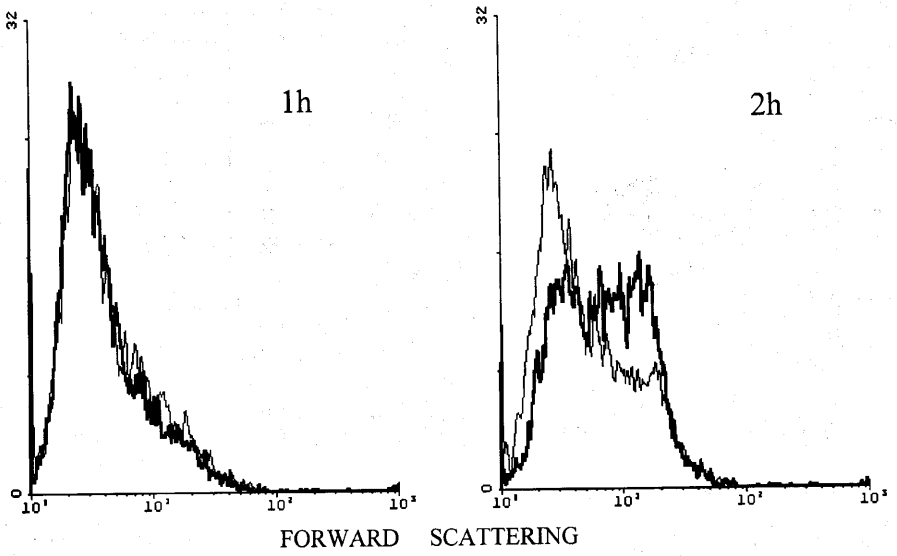

Figure 1 Flow cytometry estimation of G protein expression by staining of epithelial papillosum carp (EPC) cells non-transfected (–) and transfected with G3-pcDNAI/Amp (–). The EPC monolayers were transfected with G3-pcDNAI/Amp with lipofectin for (a) 1 or (b) 2 h. Three days later, the EPC cells were detached, stained with the neutralizing monoclonal antibody 3F1 A12 and examined by flow cytometry.

graphs obtained were then compared at the same background and range values.

Under the light microscope, caudal fins showed both long cartilage tissue and abundant melanophore cells scattered throughout the fins (Fig. 2a). When the same field was observed using blue light, the intercartilage spaces appeared filled with fluorescent spots ≈ 10–20 μm in size. The fluorescent spots were situated beneath the most superficial layer of cells in which the melanophore cells were also located. Only a few cells appeared fluorescent at or inside the cartilage (Fig. 2b).

Fluorescent fins were obtained 7 days after vaccination only when 10 μg mL^{-1} of $pQBI_{25}$ were used during immersion. As negative controls, in the absence of plasmid or with 10 μg mL^{-1} of G3-pcDNAI/Amp, fluorescence above background levels (Fig. 3) was not detected. In order to make a more reliable estimation of the observed fluorescence in each case, more than 10 different optimal photographs were taken of the caudal fin from between four and five trout per experiment, and the fluorescence of these fish was compared under the same conditions of background and range. None of the photographs were fluorescent positive in fish not exposed to plasmid or to G3-pcDNAI/Amp, whereas 56% of the photographs were fluorescent positive in fish exposed to 10 μg mL^{-1} of $pQBI_{25}$.

In an effort to obtain and compare more quantitatively the fluorescence results, a study of all the pixels in selected photographs was also made. Figure 3 shows that both the average fluorescence and the peak was maximal in the images obtained from trout exposed to 10 μg mL^{-1} of $pQBI_{25}$.

Resuming immersion in GFP-plasmid DOTAP formulations resulted in clearly visible fin fluorescence. Visible fluorescence was also obtained by injection of the plasmid in water, thus providing an important positive control to assist interpretation of results. Controls containing no plasmid, lower amounts of GFP-plasmid or other plasmid were not found to induce detectable levels of fin fluorescence. Because the G3-pcDNAI/Amp was capable of expressing protein G, the failure of this plasmid to produce fluorescence could not be a result of non-expression of the corresponding coded protein when used under the same conditions of the GFP-coding plasmid, thus providing a further control for the specificity of the fluorescence observed.

Since the fluorescence obtained was distributed in spots corresponding to the size of the cells (10–20 μm diameter) and it was not found in cartilage, it is likely that the fluorescence observed is caused by cells transfected with the GFP-containing plasmid. The present results suggest that the detected fluorescence is most probably caused by the induced

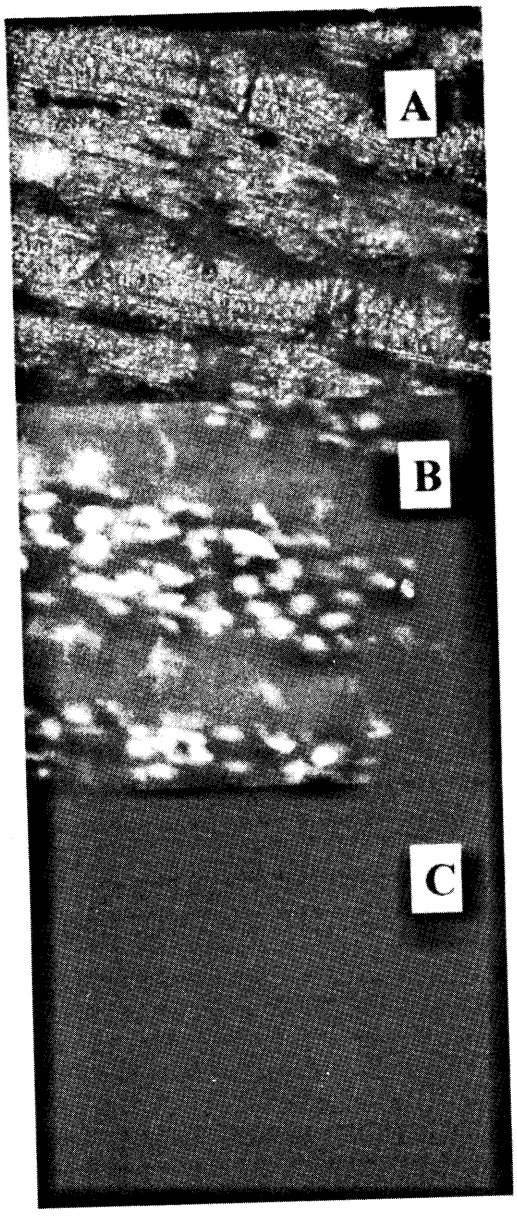

Figure 2 Caudal fins viewed by (A) clear-field or (B, C) fluorescence microscopy. Rainbow trout fry were exposed to the presence of 10 μg mL^{-1} of GFP-expressing plasmid $pQBI_{25}$, and examined 7 days later by (A) clear-field microscopy or (B) for the presence of fluorescence in the caudal fins. (C) Controls were not exposed to the plasmid.

expression of GFP in transfected trout cells. Further experiments are required to demonstrate the presence of GFP and/or G proteins by other complementary techniques to confirm the observations presented in the current preliminary report.

It is not known whether the plasmid entered the trout by the gills or went directly into the epithelial

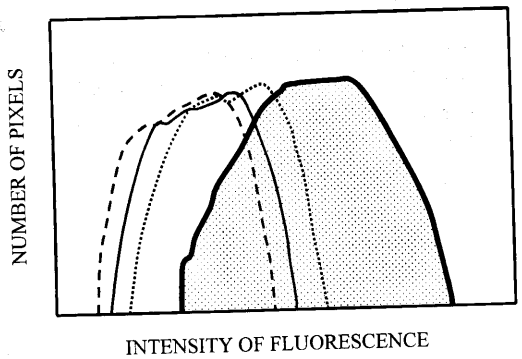

Figure 3 Distribution of levels of fluorescence in all photograph pixels. Four photographs were selected to represent negative, medium and high fluorescences, and the intensity of fluorescence represented for each pixel (CCDOPS program): (·····) not exposed to the plasmid; (- - - - -) 10 μg mL^{-1} of G3-pcDNAI/Amp; (——) 1 μg mL^{-1} of GFD-$pQBI_{25}$; (——) 10 μg mL^{-1} of GFP-$pQBI_{25}$.

skin cells. Further experiments are required to clarify this point.

Quantification of these results was difficult because of fish-to-fish variations, the selection of the photographs and the background fluorescence. It would be worthwhile using other reporter genes in which expression could be more easily quantified.

Although the level of GFP expression obtained in the present study seems to be higher than in other reported experiments with transgenic fish (Amsterdam *et al.* 1996), further improvements in methodology might lower the amount of plasmid needed to obtain measurable GFP expression by improving the data obtained. Improved data should also assist in designing novel strategies for immersion DNA-vaccination to transiently express antigens from pathogens (i.e. G3-pcDNAI/Amp).

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