Antibody production in Japanese eels, *Anguilla japonica* Temminck & Schlegel

H-W Hung, C-F Lo, C-C Tseng and G-H Kou

Department of Zoology, National Taiwan University, Taipei, Taiwan, ROC

Abstract

Adult Japanese eels, *Anguilla japonica* Temminck & Schlegel, (200–250 g, 45–55 cm) were immunized by intramuscular injection with goat IgG. After 5 weeks, eel immunoglobulin (Ig) was purified using affinity chromatography. The purified eel Ig was used to immunize rabbits to produce anti-eel Ig antibody. The highest antibody ELISA value in eels was reached 3 weeks after initial immunization with goat IgG, and then gradually decreased. The antibody could still be detected at 140 days post-immunization. The optimal temperature for antibody production was 30°C. Freund’s complete adjuvant and secondary immunization both increased antibody production in eels.

Introduction

Antibody production in fish is generally affected by the temperature, type and dose of antigen, and the use of adjuvant. For most warmwater fish, the optimal temperature for antibody synthesis is 20–30°C (Avtalion 1969). The cellular immune responses of the fish, such as macrophage activation and phagocyte function, are also affected by temperature (Van Ginkel, Van Muiswinkel, Merchant, Lizzio, Dixon & Anderson 1985; Collazos, Ortega & Barriga 1994; Hardie, Fletcher & Secombes 1994). Additionally, adjuvants are used to enhance the immune response in fish against diseases (Anderson 1992; Tyler & Klesius 1994). For example, the eastern brook trout, *Salvelinus fontinalis* (Mitchell), increased the agglutinin response when formalin-killed *Aeromonas salmonicida* bacterins were injected with Freund’s incomplete adjuvant (FIA) (Cipriano & Pyle 1985).

Moreover, fish have also been found to have enhanced serum antibody activity after secondary immunization with a homologous antigen (Cossarini-Dunier 1986). In this study, we used eel immunoglobulin (Ig) prepared according to the method described by Hung, Lo, Tseng & Kou (1996) to produce rabbit antisera against eel Ig. We also investigated the effects of adjuvant, temperature and booster injection on eel antibody production.

Materials and methods

Fish

Adult Japanese eels, *Anguilla japonica* Temminck & Schlegel, weighing 200–250 g each were obtained from a local farm in Taoyuan, Taiwan, ROC, and kept in 500-l tanks containing 100 l of water. All fish were maintained in the laboratory in a recirculating system with constant aeration at 28 ± 2°C.

Preparation of rabbit anti-eel immunoglobulin serum

Five one-year-old eels with an average weight of 200 g were used. Each fish was injected intramuscularly (i.m.) with 0.5 mg goat IgG emulsified with 0.5 ml of Freund’s complete adjuvant (FCA, Sigma) and then maintained in the laboratory in a recirculating system with constant aeration at 28 ± 2°C.
aeration at 28 ± 2°C. After 5 weeks, the eel Ig was purified using affinity chromatography and then used to produce rabbit anti-eel Ig serum. Details of this procedure are described more fully in a previous paper (Hung et al. 1996).

The effects of adjuvant on antibody production in eels

Thirty eels with an average weight of 200–250 g were separated into three groups. One group of experimental fish was injected i.m. with 1 mg goat IgG only, and a second group with 1 mg goat IgG plus 100 µl FCA. The third group was injected with phosphate-buffered saline (PBS; 15 mM phosphate buffer containing 0.15 M NaCl, pH 7.3) plus 100 µl FCA. After 3 weeks of maintenance in the laboratory in a recirculating system with constant aeration at 28 ± 2°C, the fish were anaesthetized in cold water and blood was drawn from the caudal vein of each eel once a week. Serum was separated by centrifugation at 700 g for 30 min and then analysed by the ELISA method described below.

The effects of temperature on antibody production in eels

Fifty adult eels with an average weight of 200–250 g were used. Fish were injected i.m. with 1 mg goat IgG and 100 µl FCA. After injection, fish were separated into five groups and kept in 50-l plastic tanks containing 30 l of water at different temperatures (15°C, 20°C, 25°C, 30°C and 35°C, respectively). Fish were killed and their sera tested for antibody by using ELISA techniques 25 days after immunization.

The effects of booster injections on antibody production in eels

Fifty eels were injected i.m. with 1 mg goat IgG and 100 µl FCA, and then kept in a 500-l plastic tank containing 100 l of water with a recirculating system and constant aeration at 28 ± 2°C. Blood samples were taken from the caudal vein of three eels once a week after injection and assayed for antibodies using ELISA. Six weeks after immunization, the fish were divided into two groups. One group received a second dose of goat IgG plus 100 µl FCA and the other group received an injection of PBS only. Blood samples were again taken from both groups of fish each week after the second injection and assayed for antibodies.

Duration of eel antibody production

Sixty adult eels were injected i.m. with 1 mg goat IgG and 100 µl FCA, and then kept in a 500-l plastic tank containing 100 l of water with a recirculating system and constant aeration at 28 ± 2°C. Three eels were killed and blood samples were taken from their caudal vein each week (from 0 to 6 weeks post-immunization) or every 2 weeks (from 12 to 20 weeks post-immunization). These blood samples were assayed for antibodies using the ELISA method.

Enzyme-linked immunoabsorbant assay (ELISA)

The goat IgG was bound to 96-well microtitre plates (5 µg well–1) by incubation in 100 µl per well of carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. After coupling, plates were washed three times with 0.01 M PBS containing 0.5% (v/v) Tween 20 (PBS-Tween) and blocked with PBS containing 3% (w/v) skimmed milk for 1 h at 37°C. Subsequently, non-immunized (control) or immunized fish serum diluted 1:1000 in PBS containing 3% (w/v) skimmed milk was added to each well. After incubation for 1 h at 37°C, plates were washed five times in PBS-Tween. Rabbit anti-eel Ig serum, diluted to 1:1000 in PBS containing 3% (w/v) skimmed milk was added to the wells (100 µl well–1) and incubated for 1 h at 37°C. The plates were then washed five times in PBS-Tween. Horseradish peroxidase-labelled goat anti-rabbit IgG (Sigma) was diluted 1:2000 in PBS containing 3% (w/v) skimmed milk before being added to the wells (100 µl well–1). The ELISA plate was incubated for 1 h at 37°C. After five washes in PBS-Tween, 100 µl of phenylenediamine at a concentration of 0.04% (w/v) in phosphate-citrate buffer, pH 5.0, containing 0.001% (v/v) H2O2, was added to each well. The reaction was stopped after 30 min with 2 M H2SO4, and the optical density of the wells was read at 492 nm by spectrophotometer.

Results

The effects of adjuvant, temperature and booster injection on antibody production in eels

Two weeks after injection, the eel anti-goat IgG antibody could be detected. Eels injected with goat
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Figure 1. The effect of Freund’s complete adjuvant (FCA) on eel anti-goat IgG response. Each bar represents the mean ± SE for duplicates of three fish. Each fish received an intramuscular injection of either goat IgG, goat IgG emulsified with an equal volume of FCA or phosphate-buffered saline emulsified with an equal volume of FCA.

Figure 2. A comparison of immunized eel antiserum ELISA values at different incubation temperatures. Eels were injected with goat IgG emulsified with Freund’s complete adjuvant. Blood samples were collected 25 days post-injection. Each bar represents the mean ± SE of three eels.

IgG plus FCA had ELISA values two to four times higher than those injected with goat IgG alone (Fig. 1). The optimal temperature for the eels to produce antibodies was 30°C (Fig. 2). Figures 1, 3 and 4 all show that the ELISA values of the immunized eels’ antibodies were already decreasing by about 4 weeks after immunization. Without a booster injection, this downward trend continued, but in the re-immunized eels, serum antibody levels increased once more and exceeded the ELISA value which followed the initial immunization (Fig. 3).

Long-term study of antibody production in eels

Two weeks after the eels were injected with goat IgG emulsified with an equal volume of FCA, a significant anti-goat IgG level was detected using ELISA (Fig. 4). The highest anti-goat IgG ELISA
value occurred at week 3 post-injection. The ELISA values then decreased gradually, but antibody could still be found at 20 weeks post-immunization.

**Discussion**

Commercially available materials have been used to successfully isolate polyclonal anti-goat Ig, which is about 90% pure, from the serum of *Oreochromis aureus* Steindachner (Smith, Gebhard, Housman, Levy & Noga 1993). In our previous study (Hung et al. 1996), we found that eel Ig was easily eluted from agarose beads by using glycine NaOH. Electrophoresis (SDS-PAGE) using reduced affinity-purified *A. japonica* Ig on a 10% polyacrylamide gel showed that the eel Ig was
very pure. The estimated molecular weights of the components of the reduced affinity-purified *A. japonica* for the heavy chain and light chain are very similar to those reported for Ig from European eel, *Anguilla anguilla* L. (Buchmann, Østergaard & Glamann 1992). We have used an ELISA method to detect the specificity of rabbit anti-*A. japonica* Ig serum; a strong and specific reactivity was found with serum from *A. japonica*, with no cross-reactivity with serum from distantly related species of fish, including loach and carp (data not shown). These results suggest that affinity chromatography techniques can provide fast and good quality eel Ig purification.

ELISA is a useful technique for the detection of eel antibodies (Iida, Yonekura, Izumiyama & Wakabayashi 1991). It is important to reduce the background level of non–specific interactions when using ELISA. Thuverdander, Hongslo, Jansson & Sundquist (1987) showed that DEAE-Sepharose purification of the rabbit anti-trout IgM serum can reduce these interactions. By using protein A sepharose (Pharmacia LKB Biotechnology Ltd, Uppsala, Sweden), we found that the affinity-purified rabbit anti-*A. japonica* Ig serum can successfully reduce the non-specific interactions (data not shown).

The presence of FCA in conjunction with goat IgG caused an enhanced immune response compared to that produced by goat IgG alone (Fig. 1). Moreover, under some experimental conditions, booster immunization can increase antibody production. For example, a secondary *Yersinia ruckeri* immunization was found to enhance a primary one in rainbow trout, although the anamnestic response observed with *Y. ruckeri* is only 4 log₂ higher than the primary response (Cossarini-Dunier 1986). In the present study, the highest ELISA value of secondary anti-goat Ig (Fig. 3) was higher than that of the primary value at $P < 0.05$ (ANOVA; $F = 8.34$), but not at $P > 0.01$. This secondary response compares weakly with the enhancement obtained in mammals. This may be related to the fact that fish are not able to give a typical mammalian secondary antibody response (Wilson & Warr 1992).

Many of the standard errors in this study are relatively large (e.g. Figs 2 & 4). In our previous study, the ELISA values of antiserum from eels naturally infected with *Pleistophora anguillarum* Hoshina also showed high variability (Hung et al. 1996). This may be caused by individual differences among the eels. An increased sample size would help to reduce standard errors.

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