An enhanced humoral immune response against the swimbladder nematode, *Anguillicola crassus*, in the Japanese eel, *Anguilla japonica*, compared with the European eel, *A. anguilla*

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Abstract

The humoral immune response in the two eel species, *Anguilla japonica* and *Anguilla anguilla* against two fractions of antigens in *Anguillicola crassus* were studied. Within species, both eel species showed significantly elevated titres compared with controls when immunized with antigens from *Anguillicola crassus.* In interspecific comparison, *Anguilla japonica* showed significantly elevated titres in comparison with *Anguilla anguilla*. Immunization of *Anguilla anguilla* caused a significantly decrease in the plasma levels of protein in comparison with control fish and all groups of *Anguilla japonica.* In contrast, *Anguilla japonica* showed significantly lower plasma levels of Ig in all groups compared with *Anguilla anguilla.* The different susceptibilities to *Anguillicola crassus* between the natural host, *Anguilla japonica,* and the naïve, *Anguilla anguilla,* is partly due to differences in the ability of the two eel species to mount a humoral immune response.

Introduction

The Japanese eel, *Anguilla japonica*, and European eel, *A. anguilla*, reared in fish farms contaminated with the swimbladder nematode, *Anguillicola crassus*, have demonstrated highly different infection susceptibilities (Egusa, 1979). This nematode, which was originally endemic to Asia (Yamaguti, 1935; Kuwahara *et al*., 1974) now occurs at low abundance in the native eels in Japan (Egusa, 1979). The introduction of *Anguillicola crassus* to Europe has resulted in extensive morbidity and even mass mortality in European eel stocks (van Banning & Haenen, 1990; Molnár *et al*., 1991, 1993, 1995; Haenen *et al*., 1996). The prevalence of *Anguillicola crassus* in wild eel populations of Europe can be as high as 80–100% (Køie, 1991; Nielsen, 1997) which is several times higher than the level of infection in Japan (K. Ogawa, personal communication).

The different susceptibility to *Anguillicola crassus* infection is to be expected from an evolutionary point of view. Natural selection through perhaps thousands of years will result in relatively parasite resistant stocks of eels. However, the precise mechanism behind this relative resistance is unknown. Immune mechanisms are likely to play a role in the question of susceptibility to the parasite (Wakelin, 1996).

The aim of the present work was to compare the humoral immune response to *Anguillicola crassus* antigens, in the two eel species *Anguilla anguilla* and *A. japonica*.

Materials and methods

Fish

Non-infected eels were supplied from commercial eel farms in Japan and Denmark, known to be free of

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Anguillicola crassus. The mean size of the eels was: *A. japonica* (n = 100; mean mass 25.4 ± 1.6 g; mean length 28.3 ± 2.4 cm) and *A. anguilla* (n = 84; mean mass 32.4 ± 6.6 g; mean length 29.2 ± 1.1 cm). The eels of each species were divided into four groups, each containing approximately 25 specimens and acclimated for two weeks prior to the experimentation. During the experimental and the acclimation period, the eels were kept at 20°C in well oxygenated fresh water.

Eels for collection of blood for immunoglobulin (Ig) purification were obtained from the wild. Japanese eels were collected near Hamamatsu in Japan (n = 10; mean mass 1086.3 ± 24.5; mean length 52.7 ± 6.7) and the European eels from Lake Arresø in Denmark (n =12; mean mass 1495.4 ± 125.45; mean length 56.6 ± 12.9).

Collection of parasites

Naturally infected European eels, *Anguilla anguilla*, were obtained from Lake Arresø. Eels were anaesthetized and adult specimens of *Anguillicola crassus* were collected from the swimbladders. Parasites were rinsed by washing for 2 h with nematode Ringer pH 7.2 (DeCharleroy *et al*., 1990).

Preparation of antigens

For the preparation of excretory/secretory (ES) antigens, adult specimens of rinsed *Anguillicola crassus* were placed in 15 ml glass tubes each containing 5 ml of nematode Ringer's solution and 5 g of parasites. The worms were kept at 15°C for 48 h, thereafter the nematode Ringer's solution was collected and centrifuged for 3 min at 350 *g* to remove eggs and larvae. The protein content of the supernatant was acetonitrile precipitated and stored at –20 °C for future use as excretory/secretory antigens. For the preparation of cuticlar antigen, the overlayer of the body wall was dissected from adult specimens of rinsed *Anguillicola crassus*. The cuticle was rinsed in nematode Ringer's solution, and subsequently sonicated and frozen at –20°C for future use as antigens.

Immunization

At the beginning of the experiment, eels from two of the four groups were intraperitoneally immunized with 100 μl cuticle-antigen or ES-antigen from *Anguillicola crassus* at a concentration of 0.1 mg protein ml^{-1} diluted in Freund's complete adjuvant (Sigma Immuno Chemical, F-5881). Eels from the two remaining groups were injected only with Freund's complete adjuvant and used as controls. After 28 days, all eels were re-immunized with the same concentration of the appropriate antigen in Freund's incomplete adjuvant (Sigma Immuno Chemical, F-5506). The control groups were re-injected with Freud's incomplete adjuvant only.

Blood sampling

Four weeks after re-immunization, eels were anaesthetized and killed in an overdose of benzocaine (Sigma Chemicals, E-1501). Blood was collected via the caudal vessels in heparinized syringes and dispensed into 1.5 ml microcentrifuge tubes. After 5 min of centrifugation at 14,500 *g*, the plasma was stored at –20°C until required.

Gel filtration of total eel Ig

In order to obtain Ig for standard series in the total Ig analysis, pooled sera from the European and Japanese eels were fractionated separately on a Sephacryl-300 Superfine column (Pharmacia, Umeå, Sweden). The fractions from the gel filtration were collected and frozen at –20°C.

Sodium-dodecyl-sulphate polyacrylamide-gel electrophoresis (SDS-PAGE)

SDS-PAGE (10% polyacrylamide) was performed on the different gel fractions of plasma from both the European and Japanese eel under reducing conditions according to Laemmli (1970). The fractions only containing putative heavy and light chains were selected for further concentration of Ig.

Concentration of eel Ig and protein determination

The eel Ig was concentrated with an ultrafiltration device (Centiprep-10, Amicon, Beverly, USA) according to the manufacturer's recommendations. The purity of the concentrated Ig was determined using SDS-PAGE and the protein concentration determined.

The concentration of total plasma protein was measured in triplicate by a commercial Coomassie Protein Assay (No 23200, Pierce Chemical Company, USA) according to the manufacturer's recommendations.

Antibodies

Antibodies used in these experiments were: rabbit anti-eel Ig (Buchmann *et al*., 1992) and monoclonal antibody against eel Ig (Mab) (van der Heijden *et al*., 1995). These were tested for use against Ig from the Japanese and European eels using dot blot tests. Secondary antibodies used in this experiment were: goat anti-rabbit IgG conjugated to alkaline phosphatase (A-3687, Sigma Immuno Chemicals Company, USA), swine anti-rabbit IgG conjugated to peroxidase (P-0217, DAKO A/S, Denmark) and rabbit anti-mouse IgG conjugated with peroxidase (P-0161, DAKO A/S, Denmark).

Dot blot test

From either Japanese or European eel 3 μl of purified Ig were placed onto 1 × 1 cm nitrocellulose 0.45-μm membranes and allowed to air dry. The filters were blocked with blocking buffer (1% non-fat dry milk in phosphate buffered saline, PBS pH 7.2). Filters were subsequently washed 3 × 10 min in washing buffer (PBS pH 7.2 with 0.05% Tween-20) and incubated for 1 h with either rabbit anti-eel Ig (diluted 1:1000 in dilution buffer (1% non-fat dry milk in PBS pH 7.2)) or mouse anti-eel Ig (diluted 1:1000 in dilution buffer). Control filters were only incubated with dilution buffer. After incubation the filters were washed as described above. All filters were then incubated with either swine anti-rabbit IgG or rabbit anti-mouse IgG conjugated with peroxidase for 1 h and

then washed. Finally, enzyme substrate (Sigma Chemicals, D-4293) was added and the enzyme reaction was stopped by washing with distilled water when a colour reaction appeared.

Antibody titre determination using a capture ELISA

Polystyrene microtitre plates (Sero-Wel, UK) were coated with 200 μl 0.02 mg of antigen ml^{-1} in a coating buffer (4.29 g $Na_2CO_3 \cdot 10H_2O$ and 2.93g $NaHCO_3$ diluted to 1 l with deionized water, pH 9.6) for 12 h at 4°C. Unbound antigen was removed by five successive washings with a washing buffer (PBS pH 7.2 with 0.05% Tween-20). After washing, the antigen-uncoated sites were blocked with 200 μl blocking buffer (PBS pH 7.2 containing 0.5% bovine serum albumin and 0.01% sodium azide) for 45 min at room temperature. The wells were washed five times with washing buffer between each of the following steps. From each plasma sample, a ^{2}log serial dilution was made in a dilution buffer (PBS pH 7.2 containing 0.1% bovine serum albumin and 0.01% sodium azide). The serial dilution was made for all immunized and control samples from both species. Duplicates of 200 μl of each concentration of the serial dilution were added to the coated wells. The microtitre plate was then incubated during gentle shaking for 2 h at room temperature. Thereafter 200 μl secondary antibody rabbit anti-eel Ig (diluted 1:500 in dilution buffer) was added to the wells and the plate incubated with gentle shaking for 2 h at room temperature. Each well was then supplied with 200 μl goat anti-rabbit Ig alkaline phosphatase conjugate (diluted 1:2000 in dilution buffer) and incubated with gentle shaking for 90 min at room temperature. After incubation with goat serum, the plate was washed five times in washing buffer. 200 μl p-nitrophenyl phosphate enzyme substrate (Sigma Chemicals, N-2765) was added to the wells, which were incubated for 15 min at room temperature. The enzyme reaction was stopped by adding 50 μl of sodium hydroxide (3M). The resulting colour reaction was measured spectrophotometrically at 405 nm using a microplate reader (Multiscan RC, type 351, Labsystems, Finland).

Ig determination using a sandwich ELISA

All buffers used in this ELISA correspond to the buffers in the previously described ELISA. Polystyrene microtitre plates (Sero-Wel, UK) were coated with 200 μl monoclonal mouse anti-eel Ig antibody diluted 1:500 in coating buffer and incubated for 12 h at 4°C. Unbound antigen was removed by five successive washings with a washing buffer, and then the antibody-uncoated sites were blocked with 200 μl blocking buffer for 45 min at room temperature. Between each of the following steps the wells were washed five times with washing buffer. For each microtitre plate a standard series of purified Ig 100 μg from either *A. japonica* or *A. anguilla* was made. In duplicates, 200 μl of the standard series and plasma samples were added to the microtitre plates and incubated with gentle shaking for 2 h at room temperature. Hereafter 200 μl secondary antibody rabbit anti-eel Ig (diluted 1:500 in dilution buffer) was added to the wells and the plate incubated with gentle shaking for 2 h at room temperature. Hereafter the ELISA was performed as previously described. The concentration of Ig in the plasma samples from *A. japonica* were determined using the standard series made from Ig purified from *A. japonica* and likewise for *A. anguilla*.

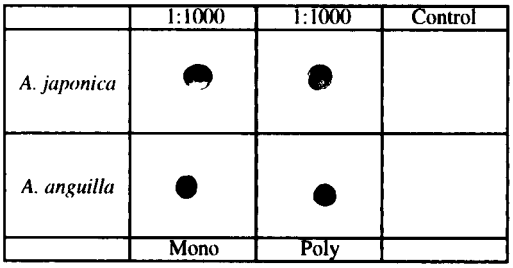

Fig. 1. Dot blot of using purified Ig from *Anguilla anguilla* and *A. japonica* as antigens and the dilution buffer as control. Mono, monoclonal antibody raised in mice against Ig from *A. anguilla* (diluted 1:1000); Poly, polyclonal antibodies raised in rabbit against Ig from *A. anguilla* (diluted 1:1000).

Statistical analysis

Data were analysed using two-way analysis of variance (ANOVA, Sheffé *F* test) with species and antigens as factors. Student's *t*-test were used to compare values within. Significant differences were accepted at a probability level of 0.05. Data are given as arithmetic means ± SEM.

Results

Antibodies

The monoclonal and polyclonal antibodies reacted strongly against Ig from both *A. anguilla* and *Anguilla japonica* in the dot blot (fig. 1). In the negative control neither the polyclonal nor the monoclonal antibody showed any reaction (fig. 1).

SDS-PAGE

The SDS-PAGE of Ig from the two eel species showed two distinct bands for both *A. anguilla* and *A. japonica* around the 25 kDa (light chain of Ig) and 70 kDa (heavy chain of Ig) (Buchmann *et al*., 1992; van der Heijden *et al*., 1995). Data not shown.

Total plasma Ig

The total plasma immunoglobulin concentration in *A. japonica* did not vary between the experimental groups. In the experiment with *A. anguilla*, the ES-immunized group had significantly higher plasma immunoglobulin concentrations when compared with control fish and fish immunized with cuticlar antigen. The total plasma immunoglobulin concentration was significantly lower in *A. japonica* compared with *A. anguilla* for all experimental groups (fig. 2).

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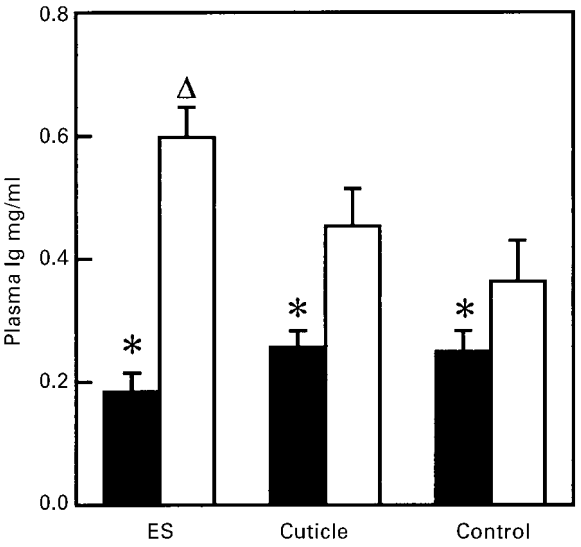

Fig. 2. Total plasma Ig concentration in mg ml^{-1} (mean ± SEM). ■, *Anguilla japonica*; □, *A. anguilla*; ES, eels immunized with ES-antigens; Cuticle, eels immunized with cuticle antigens; Control, eels immunized only with adjuvant; *, *A. japonica* significantly different from *A. anguilla*; Δ, ES significantly different from cuticle and control groups of *A. anguilla*; n = 25 for all groups of *A. japonica*; n = 21 for all groups of *A. anguilla*.

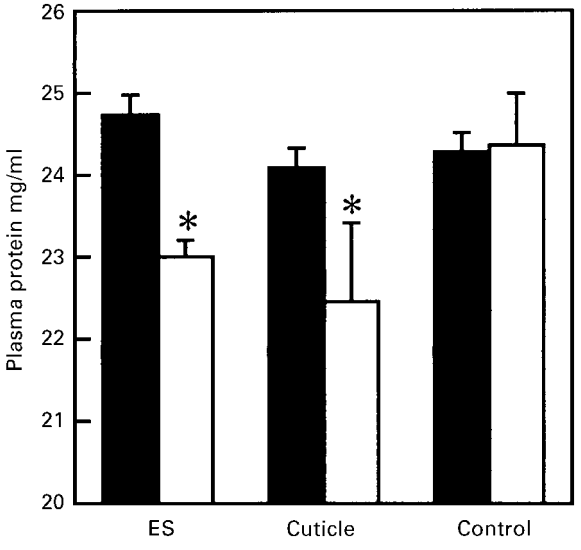

Fig. 3. Total plasma protein concentration in mg ml^{-1} (mean ± SEM). ■, *Anguilla japonica*; □, *A. anguilla*; ES, eels immunized with ES-antigens; Cuticle, eels immunized with cuticlar antigens; Control, eels immunized only with adjuvant; *, significantly different from the control group of *A. anguilla* and all groups of *A. japonica;* n = 25 for all groups of *A. japonica*; n = 21 for all groups of *A. anguilla*.

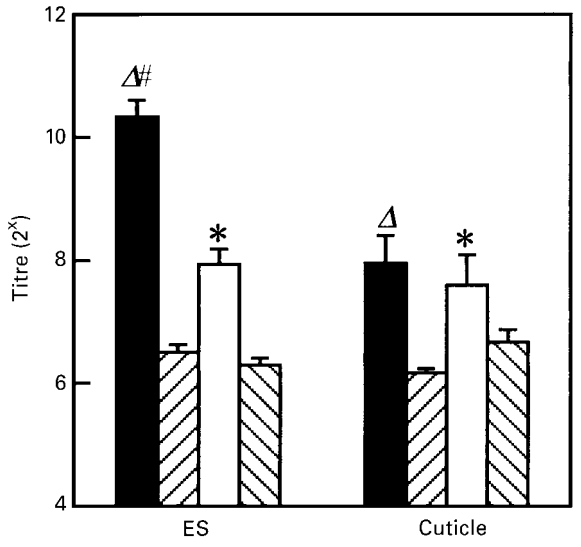

Fig. 4. Antibody titre (mean ± SEM). ■, *Anguilla japonica*;, ▨, *A. japonica* control; □, *A. anguilla*; ▧, *A. anguilla* control; ES, eels immunized with ES-antigens; Cuticle, eels immunized with cuticlar antigens; Control, eels immuinized only with adjuvant. *, immunized *A. anguilla* significantly different from control *A. anguilla*; Δ, immunized *A. japonica* significantly different from control *A. japonica*; #, ES immunized *A. japonica* significantly different from cuticle immunized *A. japonica;* n = 25 for all groups of *A. japonica*; n = 21 for all groups of *A. anguilla*.

Total plasma protein

The total plasma protein concentration decreased significantly in *A. anguilla* for both the immunized groups compared with controls and all groups of *A. japonica*. The plasma protein level of *A. japonica* did not show any significant differences between the immunized groups and the control group (fig. 3).

Antibody titres

For both species, the immunized groups showed significantly elevated titres compared with the their respective control groups. For *A. japonica*, the group immunized with ES-antigen had significantly higher titres than ES-immunized *A. anguilla*. The latter species showed no intraspecific differences in titre elevation between the two immunization regimes. This is in contrast to *A. japonica* where the group immunized with ES-antigen showed highly significantly elevated titres compared with the group immunized with cuticular antigens (fig. 4).

Discussion

In the present study antibodies raised against Ig from European eel, *A. anguilla*, were able to recognize Ig from both *A. japonica* and *A. anguilla*. This accords with Buchmann & Pedersen (1994) who found that rabbit antisera raised against *A. anguilla* could recognize Ig from *A. japonica* and vice versa. This justifies the use of the antibodies raised against *A. anguilla* in experiments with *A. japonica*.

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There are many examples of the devastating effects observed after introduction of a pathogenic parasite into an immunologically naïve host population (Hoffman, 1970; Heggberget & Johnsen, 1982). The mechanisms involved in the reduced resistance to *Anguillicola crassus* infections in *Anguilla anguilla* have never been properly elucidated. The present study suggests at least one of the many factors causing this susceptibility: the efficiency by which *A. japonica* is able to produce a specific humoral immune response against important antigens in *Anguillicola crassus* compared to *A. anguilla*. The involvement of this immune mechanism allows the European eel to mount a humoral immune response against *Anguillicola crassus* (Buchmann *et al*., 1991; Höglund & Pilström, 1994, 1995; Nielsen & Buchmann, 1997).

The response to the ES-antigens was particularly high and the high titre produced in the Japanese eel could contribute to the rapid elimination of the majority of invading nematode larvae from this host. Correspondingly, a lower immune competence in *A. anguilla* would render a higher susceptibility to an infection with *Anguillicola crassus* compared to *A. japonica.* To what extent non-specific factors as complement and cellular reactions are involved in this differential immune response is as yet unknown. However, high numbers of inflammatory cells, mainly phagocytes has been found associated with *Anguillicola crassus* infections in the European eel (Haenen *et al*., 1989; Molnár *et al*., 1993). Therefore, several other factors are likely to be involved in the differing susceptibilities of these anguillids. Specific receptor ligand interactions between the parasite and the host may also be involved as in the case of virus infections (Nomoto *et al*., 1994).

The higher non-specific Ig level in found European eel (all groups) as compared with the Japanese eel is not readily explained. However, it may indicate that the immune system in the Japanese eel is better adjusted to produce specific immunoglobulin to this helminth. This could explain the lack of increase in the total concentration of Ig following immunization with antigens from *Anguillicola crassus* in *Anguilla japonica*.

The low plasma protein level in the immunized groups of *Anguilla anguilla* compared with all other groups could be explained as an effect of stress, which is known to influence the osmotic balance (Eddy, 1981; Nielsen *et al*., 1994; Postlethwaite & McDonald, 1995). Thus, a decreased ability to resist the osmotic pressure in a hypo-osmotic environment will result in an influx of water followed by the dilution of the plasma, followed by a reduction in the plasma protein concentration. Thus, the significant decrease in the concentration of plasma protein observed in the two immunized groups of *Anguilla anguilla* may indicate that the eels are more stressed in these groups compared to the other groups. This suggests that *A. anguilla* are stressed not due to the injections *per se,* but due to the injected antigens.

In conclusion, *A. japonica* possesses a higher specific humoral immune response against antigens from *Anguillicola crassus* compared with *A. anguilla* and this may explain in part why the European eel is more susceptible to infections with *Anguillicola crassus* than the Japanese eel. The results from this study also raise the question of how will the American eel, *Anguilla rostrata,* cope with the parasite, the occurrence of which has recently been reported in the southeastern United States (Fries *et al*., 1996).

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