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Humoral immune response of European eel *Anguilla anguilla* experimentally infected with *Anguillicola crassus*

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ABSTRACT: A humoral immune response of the European eel *Anguilla anguilla* elicited by an experimental infection was demonstrated for the first time against the swimbladder nematode *Anguillicola crassus*. Eels were experimentally infected once or repeatedly and the antibody response was observed over a period of 325 d. Specific antibodies against *A. crassus* in the peripheral blood of the eels were measured using an ELISA and the immunoblot technique. Anti-*A. crassus* antibodies were first observed 8 wk post infection, and appeared to be independent of both the number of infective third stage larvae (L_3) administered and the frequency of administration. However, individual eels showed great differences in the course of the antibody response. The late appearance of antibodies in the peripheral blood supports the hypothesis that not the invading L_3 but rather the adult parasites elicit the production of specific antibodies. A stage-specific antibody response against the L_3 was not observed. Main antigens are located in the body wall, especially in the gelatinous outer cuticle, of adult *A. crassus*.

KEY WORDS: *Anguilla anguilla* · *Anguillicola crassus* · Humoral immune response · Experimental infection

INTRODUCTION

Nematodes of the genus *Anguillicola* are specific parasites of eels belonging to the genus *Anguilla* (Sures et al. 1999). Whereas infective third stage larvae (L_3) invade the hosts' swimbladder wall and feed on tissue, the haematophagous adult worms dwell in the swimbladder lumen (Kuwahara et al. 1974, Würtz & Taraschewski 2000). *Anguillicola crassus* was originally a parasite of the Japanese eel *Anguilla japonica* endemic in the Far East (Nagasawa et al. 1994). However at least the congeneric European eel *Anguilla anguilla* and the American eel *Anguilla rostrata* can also serve as final hosts of this nematode (Taraschewski et al. 1987, Johnson et al. 1995, Fries & Williams 1996). Introduced into Europe, *A. crassus* proved to be a very successful coloniser within the indigenous eel populations (Kennedy & Fitch 1990). Within 10 yr of its first record in northern Germany in 1982 it had spread over almost the whole of Europe (Moravec 1992). Prior to its introduction into Europe *A. crassus* had already been reported to cause morbidity among European eels cultivated in Japan, whereas Japanese eels were less affected by this parasite (Egusa 1979). Severe histopathological alterations and a malfunction of infected swimbladders were observed in *A. anguilla* captured in European waters (Van Banning & Haenen 1990, Molnár et al. 1993, Molnár 1994, Haenen et al. 1996, Würtz et al. 1996, Würtz & Taraschewski 2000). Additionally, an infection with *A. crassus* seems to favour secondary bacterial infections (Van Banning & Haenen 1990).

In aquaculture, infected European eels show a reduced growth and increased mortality (Køie 1991). A mass mortality of eel coinciding with a very high

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prevalence of *Anguillicola crassus* was reported from Lake Balaton, Hungary (Molnár et al. 1991), though additional adverse factors, such as a low water oxygen content or secondary bacterial infections, were also considered as the primary cause of the observed mortality (Békési et al. 1997). Accordingly *A. crassus* is not only a possible threat to eel farming, but also a serious danger to the continued existence of wild European eel populations, since eels with severe swimbladder lesions may be incapable of migrating to their spawning grounds (Würtz et al. 1996).

So far investigations concerning the resistance of the eel to *Anguillicola crassus* have mainly focused on the humoral immune response of the European eel against the parasite. In naturally infected eels specific antibodies against antigens of the adult nematode were detected using the immunoblot technique (Buchmann et al. 1991, Höglund & Pilstöm 1995, Haenen et al. 1996). More recently, some of the adult worm antigens were described to be subunits of a glutathione-s-transferase (Nielsen & Buchmann 1997). An enzyme-linked immunosorbent assay (ELISA) for the detection of specific antibodies against *A. crassus* in eel serum was developed by Höglund & Pilström (1994, 1995). However, all these examinations were carried out with sera of wild-caught, i.e. naturally infected, eels, or with sera of eels that were injected intraperitoneally with an adult whole-worm homogenate together with Freund's adjuvant (Höglund & Pilström 1994, 1995). Up to now it was not possible to prove the presence of any specific antibodies in experimentally infected eels (Haenen et al. 1996, Van der Heijden et al. 1996). However, only the experimental approach allows the exclusion of cross-reacting antibodies against other parasites (Knopf et al. in press), and it has the additional advantage that the history of the infection is known. This study describes the course and the quality of the humoral immune response following an experimental infection of European eels with *A. crassus*.

MATERIAL AND METHODS

Source and maintenance of eels. For the experimental infection non-infected European eels *Anguilla anguilla* weighing 69.0 ± 20.9 g (mean ± SD) were obtained from an eel farm free of *Anguillicola crassus* (Limnotherm, Bergheim, Germany). The eels were kept individually at 20°C. Each tank, with a volume of 40 l, was aerated and equipped with a polypropylene tube serving as hiding place. A flow through system with non-chlorinated tap water preserved a constant water quality. The eels were fed twice a week with pelleted food at a rate of 0.7 g per eel. Prior to the experiment fish were allowed to acclimatise for 2 wk.

Infective third-stage larvae of *Anguillicola crassus* and infection technique. Third stage larvae (L_3) were produced and administered to the eels as previously described (Knopf et al. 1998). Second stage larvae (L_2) were collected from the swimbladder lumen of naturally infected eels and were fed to wild-caught planktonic copepods. After 20 d at 20°C L_3 were isolated from the intermediate hosts by the potter method described by Haenen et al. (1994). The larvae intended for the infection of the eels were stored in RPMI-1640 medium (Sigma, Deisenhofen, Germany) containing 0.2% kanamycin (v/v) at 4°C until use, but not longer than 8 wk. The required number of L_3 was counted, suspended in approximately 100 µl RPMI-1640 and administered through a stomach tube (1.5 mm diameter).

The L_3 used for the antigen preparation were thoroughly cleaned by multiple centrifugation over Histopack 1017 (Sigma), washed in phosphate-buffered saline (PBS), pH 7.2, and stored in a small volume of PBS at −70°C.

Sampling of sera. Blood samples of 150 µl were drawn from the caudal vein of the eels. At the beginning of the experiment (Day 0) and at Day 142 the sample volume was increased to 400 µl. Blood was allowed to clot for 1 h at 20°C, and centrifuged for 5 min at $2000 \times g$; sera were collected and stored at −70°C.

Experimental design. Three randomly formed groups of 9 eels were experimentally infected with *Anguillicola crassus*. Group 1 received 40 L_3 per eel once, at the beginning of the experiment (Day 0). In the other 2 groups, a repeated infection was intended to simulate natural conditions with a high infection pressure. Therefore eels were infected with 3 L_3 twice a week (Group 2) or with 20 L_3 once a week (Group 3). The repeated infection was stopped at Day 140 of the experiment, followed by a final administration of 25 L_3 at Day 185. An additional group of 9 eels, Group 4, serving as control, was sham-infected with the RPMI-1640 medium twice a week. All eels were bled every second week from Day 0 to Day 325. The individual course of the antibody response was investigated with an enzyme-linked inmmunosorbent assay (ELISA) and immunoblotting.

Preparation of crude antigen extracts. Crude antigen extracts for the assays were prepared from L_3 and adult *Anguillicola crassus* as described in Knopf et al. (in press). Adult nematodes collected from naturally infected eels were washed in PBS, pH 7.2, and dissected. The worms were divided into entire body wall, outer cuticle (cf. Taraschewski et al. 1988), male and female gonads, intestinal content and intestinal wall. The intestinal wall was thoroughly rinsed in PBS to remove the intestinal content.

For ELISA, body walls of adult *A. crassus* were sonicated in Sarcosyl-TE-buffer (10 mM Tris, 1 mM ethyl-

ene diamine tetracetic acid disodium salt [EDTA], 2% N-lauroylsarcosine-sodium salt [w/v] in double-distilled water, pH 8.0) and centrifuged for 20 min at 16 000 × *g*. L_3 somatic antigen was prepared out of total L_3 in the same way. For immunoblotting adult nematode tissues and intestinal content as well as total L_3 were suspended in sample buffer containing 50 mM Tris, 10% (v/v) glycerol, 7% (w/v) sodium dodecyl phosphate (SDS), 1 mM EDTA, 1 mM phenylmethyl sulfonylfluoride (PMSF) and 3% (w/v) dithiothreitol (DTT), heated for 3.5 min at 95°C and centrifuged for 20 min at 16 000 × *g*. The resulting supernatants were used as crude antigens.

Enzyme-linked immunosorbent assay (ELISA). The ELISA procedure was recently described extensively. Non-specific binding of secondary and tertiary antibodies could be excluded and the specificity of the assay was determined to 97.7% (Knopf et al. in press). Briefly, polystyrene microtitre plates (Nunc, Kamstrup, Denmark) were coated with the crude antigen extracts at a concentration of 1.5 µg ml^{-1} in carbonate coating buffer (10 mM Na_2CO_3, 35 mM $NaHCO_3$ in double-distilled water, pH 9.6) and blocked with 1% (w/v) bovine serum albumin (Fluka, Buchs, Switzerland) in PBS containing 0.05% (v/v) Tween 20 (PBS-T-BSA). Controls for non-specific binding antibodies were performed with BSA-blocked plates without *Anguillicola* antigen.

Eel sera were tested in duplicate at a dilution of 1:100 in PBS-T-BSA. As secondary and tertiary antibodies monoclonal mouse IgG specific for the heavy chain of eel immunoglobulin (WEI 1, Van der Heijden et al. 1995; 1:500 in PBS-T-BSA) and sheep anti-mouse IgG conjugated with horseradish peroxidase (AP271, The Binding Site, UK; 1:1000 in PBS-T-BSA) were used, respectively. As substrate *o*-phenylenediamine (Abbott, Wiesbaden, Germany) was used and results were read at 492 nm.

If absorbance values of highly positive sera exceeded 2.0, measurements were repeated with a higher serum dilution. The effect of the higher dilution was mathematically compensated with a correction factor determined by the use of less reactive reference sera measured in the same trial.

In order to eliminate the inter-assay variation, absorbance values were related to the absorbance value of a defined negative control-serum by calculation of the quotient *Q*:

$$Q = \frac{\text{Absorbance of test-serum}}{\text{Absorbance of negative control-serum}}$$

A positive reaction was defined for values exceeding a cut-off value calculated by adding the mean and the 3-fold standard deviation of all *Q* measured at the beginning of the experiment (Day 0).

The relative increase of *Anguillicola*-specific antibodies in the peripheral blood of individual eels as shown in Figs. 1 to 4 was calculated by dividing *Q* determined at any time during the experiment (Q_x) by the *Q* determined for the respective eel at Day 0 (Q_0):

$$\text{Relative increase of antibodies} = \frac{Q_x}{Q_0}$$

Immunoblot. The immunoblotting procedure was described in detail in Knopf et al. (in press). Briefly, SDS-PAGE was performed under reducing conditions in the Phast-System (Pharmacia LKB, Uppsala, Sweden) with purchasable homogenous gels containing 12.5% polyacrylamide (Pharmacia LKB). Semi-dry Western blotting on a polyvinyldiene fluoride (PVDF) membrane (Pall Gelman Sciences, Roßdorf, Germany) was also performed in the Phast-System.

Strips of the membrane were successively incubated with eel sera diluted 1:100 in PBS containing 0.05% Tween 20 (PBS-T), with WEI 1 (1:500 in PBS-T) and with sheep anti-mouse IgG conjugated with horseradish peroxidase (1:1000 in PBS-T). Bands were visualised by adding the substrate diaminobenzidine (DAB, Sigma).

Statistical analysis. The time of onset and the intensity of the antibody response were compared between different treatments using the Kruskal-Wallis test with a significance level of $p \leq 0.05$.

RESULTS

For all 3 groups of eels infected with *Anguillicola crassus* the production of *A. crassus*-specific antibodies was demonstrated by ELISA. Controls, inoculated with only RPMI-1640, showed no increased absorbance values over the entire experimental period of 325 d. Values obtained using the antigen preparation of the adult worm body wall were generally higher than those obtained using the somatic L_3 antigen. However, the temporal course of the detected antibodies in the peripheral blood of individual eels was identical for both antigen preparations. Controls with BSA-blocked microtiter plates, but without *Anguillicola* antigens, were negative.

The course as well as the intensity of the antibody response of the individual eels showed a high heterogeneity (Figs. 1 to 4). Six of the 9 eels in the group infected repeatedly with 3 L_3 (Group 2) showed a detectable humoral response during the experimental period of 325 d (Fig. 2) as against 4 out of 9 eels in each of the groups infected only once with 40 L_3 (Group 1) or repeatedly with 20 L_3 (Group 3) (Figs. 1 & 3). There were marked differences in the onset and intensity of the response in these eels. A first increase of specific

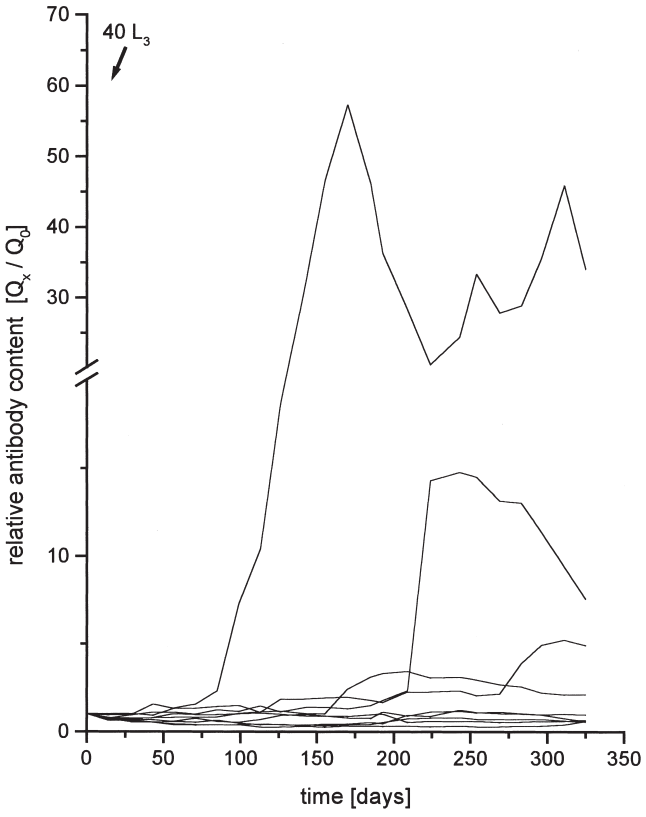

Fig. 1. *Anguilla anguilla.* Courses of the antibody response of 9 individual eels in Group 1: orally administered with 40 L_3 at the beginning of the experiment as measured by ELISA using a crude antigen from the body wall of adult *Anguillicola crassus*. The graphs show the antibody content relative to the start of the experiment

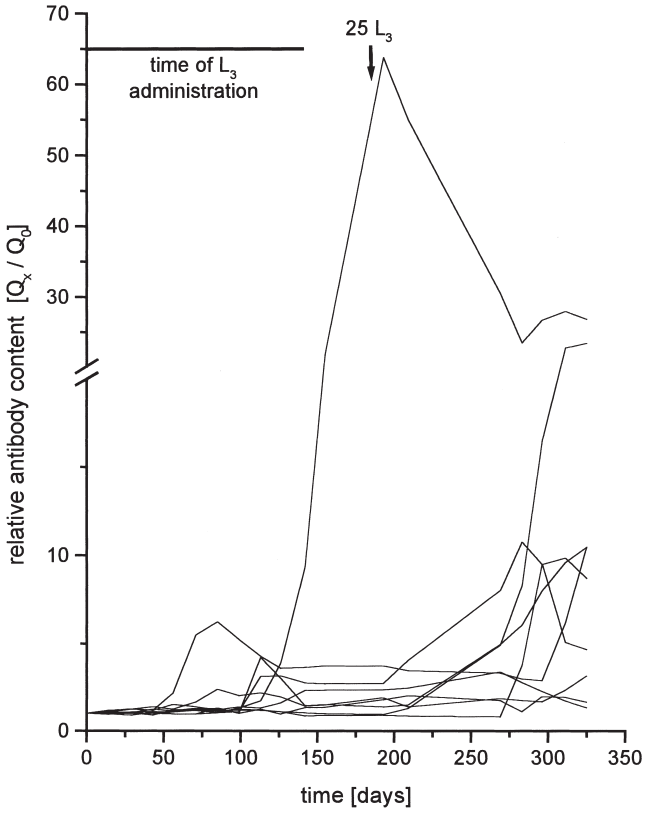

Fig. 2. *Anguilla anguilla.* Courses of the antibody response of 9 individual eels in Group 2: orally administered twice a week with 3 L_3 for 140 d and 25 L_3 at Day 185 as measured by ELISA using a crude antigen from the body wall of adult *Anguillicola crassus*. The graphs show the antibody content relative to the start of the experiment

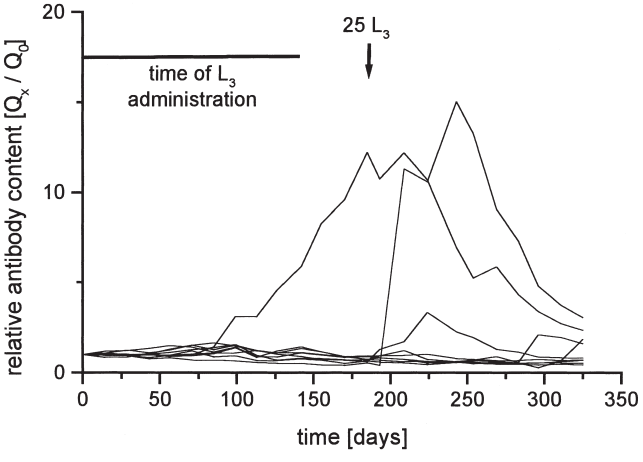

Fig. 3. *Anguilla anguilla.* Courses of the antibody response of 9 individual eels in Group 3: orally administered weekly with 20 L_3 for 140 d and 25 L_3 at Day 185 as measured by ELISA using a crude antigen from the body wall of adult *Anguillicola crassus*. The graphs show the antibody content relative to the start of the experiment

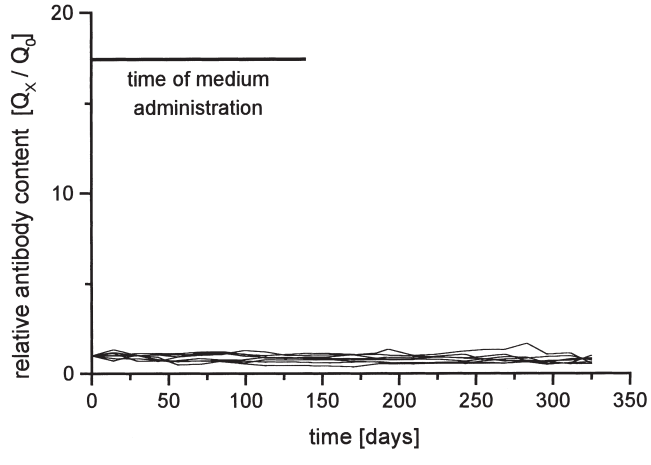

Fig. 4. *Anguilla anguilla.* Courses of the antibody response of 9 individual eels in Group 4 (control): orally administered with RPMI-1640 medium twice a week over 140 d as measured by ELISA using a crude antigen from the body wall of adult *Anguillicola crassus*. The graphs show the antibody content relative to the start of the experiment

antibodies in the peripheral blood was detected at Day 56. However, other specimens of each of the infected groups reacted much later, even as late as Day 296. Statistical analysis did not reveal a difference for the time of onset or the intensity of the antibody response between the 3 infected groups (Kruskal-Wallis test, $p > 0.5$).

Immunoblot analyses showed that the eels produced mainly antibodies against antigens associated with the body wall and outer cuticle of adult *Anguillicola crassus*. Typical patterns of the antibody response of 3 eels are presented in Fig. 5. At the beginning of the experiment some weak bands with a molecular weight about 100 kDa and higher were detected, but the intensity of these bands clearly increased during the experiment. Using the crude antigen of the outer cuticle, a strong, blurry band at about 100 kDa appeared. Immunoblots using the antigen preparation of the entire body wall showed at least 2 bands in this region. At the same time the intensity of the reaction with 3 antigens of about 120 to 135 kDa increased and further bands appeared at about 71 and 38 kDa. Antigens of about 83 and 43 kDa reacted already prior to the infection and the intensity of these bands increased during the first 2 mo in the early phase of the infection. However, these bands were not detected when the somatic L_3 antigen was used (Fig. 5).

Of 5 eels tested, only 2 produced antibodies against antigens of the intestinal wall. One of them also showed a reaction to an antigen of the intestinal content and a very weak reaction to antigens of the female reproductive tract (Fig. 5). Stage-specific antigens of the L_3 were not detected. Each band that appeared using the somatic L_3 antigen was also observed using the body wall antigen preparation of adult *Anguillicola crassus* (Fig. 5).

DISCUSSION

The course of the antibody response of European eels elicited by an experimental infection with *Anguillicola crassus* appeared to be highly heterogeneous in both a quantitative and a qualitative way. Although the eels were used for a subsequent reinfection experiment and thus it was not possible to check by dissection the success of infection, it can be assumed that failed infection is not the cause of a missing immune response. Previous experiments (Knopf et al. 1998) revealed that a recovery rate of about 40% can be achieved by the applied infection technique. Therefore, with a total number of 40 to 400 L_3 administered, the probability of successful infection is high.

One possible reason for the marked individuality of the humoral immune response is the genetic variability of the eels. As breeding of eels is still not possible, the experimental eels were caught from a natural population. Hung et al. (1996) described similarly pronounced individual differences in the reaction pattern of serum antibodies to spore proteins of *Pleistophora anguillarum* in eels naturally infected with this microsporidean. Dunier (1985) assumed a high genetic variability within a population of rainbow trout *Oncorhynchus mykiss* to be the cause of a heterogeneous immune reaction against synthetic antigens (DNP-Haemocyanin and DNP-Ficoll).

In mammals, qualitative and quantitative differences in the individual immune response to parasites are well known, and generally it can be stated that differences in the susceptibility or resistance to a variety of parasitic infections result from the genetic variability within the host population (Wakelin 1996). For example, humans within an endemic region show highly different immunological and clinical reactions to an infection with filariae (Soboslay et al. 1994). In sheep farming the genetic variation in resistance to nematode parasites is exploited in breeding programs selecting the most resistant hosts (Gay 1997). Furthermore, the course of the infection itself might cause or contribute to the individual differences in antibody responses. It is plausible that many parasite antigens contained in the crude extracts are not presented to the hosts' immune system until the parasite has died (Voller & De Savigny 1981). Therefore, in some cases the strong serological response to crude extracts appearing several months after the inoculation might be causally related to the presence of dead, decomposing worms.

The course of the humoral immune response of the experimentally infected eels indicates that the antibody response is more likely to be directed against antigens of the adult worms than against antigens of the invasive L_3. Firstly, ELISA measurements showed no differences in the temporal course of the antibody response against larval and adult worm antigens, indicating that the antibodies measured by ELISA cross-react with larval and adult worm antigens or react with antigens which are common to both larvae and adult worms. However, the results indicated a higher proportion of the main antigens in the body wall antigen preparation of adult worms than in the somatic L_3 antigen.

Secondly, the initial increase of specific antibodies in the peripheral blood was observed 8 wk after the first inoculation with L_3. In contrast, an intraperitoneal injection of homogenised *Anguillicola crassus* at the same temperature (20°C) resulted in antibody production within 5 to 12 d (unpubl. data). Therefore, the late onset of the antibody response in the present study seems to be an immunological characteristic of the living parasites. Obviously the L_3, migrating in the host tissues, are not sufficiently immunogenic, or the dose

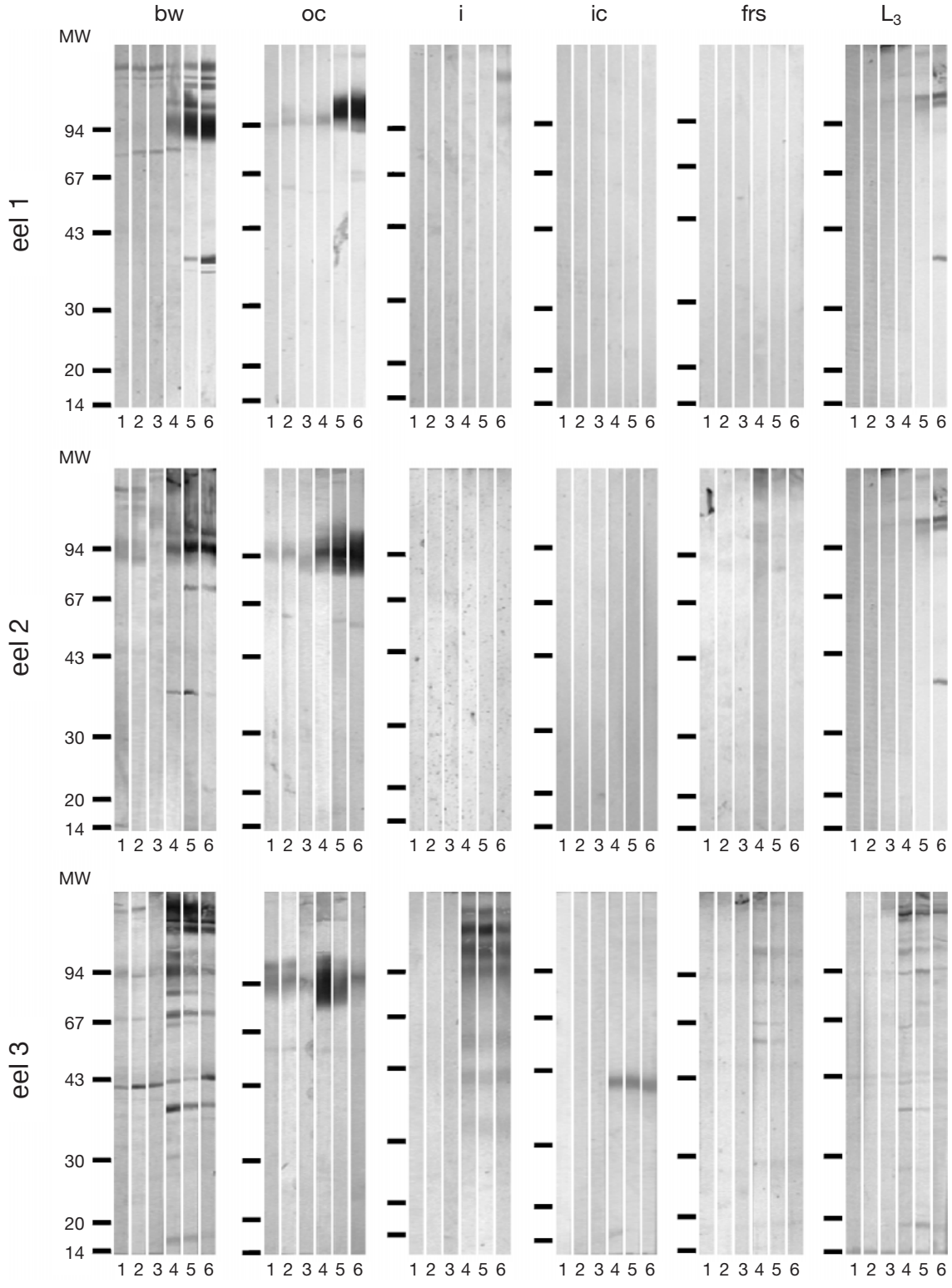

Fig. 5. *Anguilla anguilla*. Pattern of the antibody response of 3 experimentally infected eels. Immunoblots were performed using crude antigen extracts of the body wall (bw), outer cuticle (oc), intestinal wall (i), intestinal content (ic) and female reproductive system (frs) of adult *Anguillicola crassus* and a somatic L_3 antigen. Strips 1 to 6 represent Days 0, 56, 113, 224, 283 and 325. Molecular weight (MW) in kilodalton

was still too low to elicit a detectable antibody response. Similar results were obtained by Ramakrishna et al. (1993) investigating the cell-mediated immune response of rainbow trout to larval *Pseudoterranova decipiens*. They found striking differences in the immunogenicity of living parasites and parasite extract. They suggested that either the larvae release antigens slowly and thus the immune response is delayed or antigens are hidden from specific immune detection. The onset of the antibody response, about 8 wk after the first infection, seems to be elicited by the first adult worms appearing in the swimbladder lumen, because the development of *A. crassus*, from the infection of the eel until first worms appear in the swimbladder lumen, takes about 50 d at 20°C (Nagasawa et al. 1994, Haenen et al. 1996, Knopf et al. 1998) and about 1 wk must be calculated for the induction of a primary immune response.

Thirdly, the immunoblot analyses revealed strongest reactions with antigens of adult *Anguillicola crassus*. Major antigens were located in the body wall of the adult worms. Similar to immunoblots with sera from naturally infected eels (Knopf et al. in press), a strong, broad and blurry band appeared using the antigen preparation of the gelatinous outer part of the cuticle. Using the somatic L_3 antigen no or only very weak bands were detected, and these seem to be identical to some of the bands detected with the crude antigen from the body wall of adult *A. crassus*. However, it must be considered that crude extracts were used in this study and possibly important antigens might have been destroyed during the preparation process or could not be detected because their concentrations were too low in the complex mixture of antigens.

Immunoblot analyses in this study indicate that the *Anguillicola crassus*-specific antibodies produced by the experimentally infected eels correspond with those of naturally infected eels (Knopf et al. in press). However, sera of naturally infected eels contained antibodies against L_3 antigens (Knopf et al. in press), which could not be proved for the experimentally infected eels. In principle it is possible that antibody production was suppressed by the unavoidable stress during the experiment (Roberts 1985, Van Muiswinkel 1995). Nevertheless, a strong antibody response against adult worm antigens was detected, and it is unlikely that a possible immunosuppression selectively affects the antibody response against the larvae. More probably, antibodies in the sera of wild eels elicited by other parasites cross-reacted with larval antigens of *A. crassus*.

A comparison of the 3 experimentally infected groups of eels did not reveal a difference in antibody response. This implies that the main characteristics of the antibody response in the present study, namely the late onset of the antibody production and the absence of detectable L_3-specific antibodies, are independent of the pressure of infection over a wide range.

The absence of a measurable immune response against the migrating L_3 can be explained by a certain ability to escape the hosts' immune response. Other nematodes, such as filariae, are known to be able to modify their surface structure, to secrete immunosuppressive substances and to mask their surface with serum albumins of the host and thus evade the hosts' immune defence (Soboslay et al. 1994). Probably the larvae of *Anguillicola crassus* protect themselves by similar evading mechanisms. In contrast to the adult worms, which live comparatively protected in the swimbladder lumen, the larvae are in close contact with the host tissues during migration from the intestine to the swimbladder and the subsequent development in the swimbladder wall (Haenen et al. 1989, De Charleroy et al. 1990). Therefore they are directly exposed to attacks by the hosts' defence mechanisms. Thus an effective evading mechanism of the larval stages to escape the hosts' immune defence would represent an ingenious survival strategy determining the success of an infection.

It is still in question, though, why no antibodies against any excretory or secretory substances of the larvae could be detected, although nematodes generally possess a variety of such highly immunogenic substances (Wakelin 1994). Moreover, a trypsin-like penetration proteinase was described for the L_3 of *Anguillicola crassus* (Polzer & Taraschewski 1993). For *Trichinella spiralis* it is known, however, that secreted proteins are not necessarily immunogenic (Parkhouse et al. 1987), and this might also be true for the larvae of *A. crassus*. It is also possible that the proportion of excreted/secreted substances in the somatic L_3 antigen is simply too low to be detected.

Adult *Anguillicola crassus* living in the swimbladder lumen do not have direct contact to the host tissues except while feeding. Thus, one should expect an immune response of the eel against excretory/secretory substances passing the swimbladder epithelium or being secreted while the parasites are sucking blood.

In contrast to naturally infected eels (Knopf et al. in press) those experimentally infected showed at best a very weak antibody reaction to antigens of the female reproductive tract. It must be assumed that the uterus tissue of the worms only becomes accessible to the hosts' immune system once the parasites have died. Probably the eggs or the emerged L_2 only develop immunogenic capacity when accidentally enclosed by the pathologically altered swimbladder tissue as described for naturally infected eels (Van Banning & Haenen 1990, Molnár et al. 1993, 1995).

Although in the present study a few eels produced antibodies reacting with antigens of the intestinal con-

tent or the intestinal wall of adult *Anguillicola crassus*, it seems — in accordance with the findings obtained with sera of naturally infected eels (Knopf et al. in press) — that the humoral immune response was mainly directed against antigens located in the body wall and especially the gelatinous outer cuticle. This indicates that immunogenic substances of the body wall pass through the swimbladder epithelium after being secreted. Remarkably, several important parasitic nematodes of mammals, such as *Onchocerca volvolus*, *Trichinella spiralis* and *Parafilaria bovicola*, secrete highly immunogenic and specific antigens from their cuticle (Carbrera & Parkhouse 1987, Parkhouse et al. 1987, Sundquist et al. 1988, 1989). Philipp et al. (1980) proved that *T. spiralis* secretes stage-specific cuticle proteins into the surrounding medium when cultured *in vitro*.

Further studies should therefore focus on the characterisation of the immunogenic substances harboured in the body wall of adult *Anguillicola crassus* and their relevance for the host-parasite interaction. In view of the results of this study, a closer look into the suggested evasion mechanisms of the invading L_3 will also be of great interest.

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