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Humoral immune response of flounder to *Edwardsiella tarda*: the presence of various sizes of immunoglobulins in flounder

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ABSTRACT: Antisera obtained from flounder *Paralichthys olivaceus* immunized with *Edwardsiella tarda* were fractionated using Sephacryl S-300 gel filtration. The flounder was found to contain various sizes of immunoglobulins (Igs) ranging from <200 to >700 kDa. These Igs were combined into 3 pools according to molecular weight; pool I consisted of high molecular weight (HMW) Igs of >700 kDa, pools II and III consisted of low molecular weight (LMW) Igs of 230 to 700 kDa (pool II) and <230 kDa (pool III). After being further purified by hydroxyapatite column chromatography and isoelectric focusing, HMW Ig in pool I and LMW Ig in pool III were compared with each other by SDS-PAGE under reducing conditions. They were found to be composed of 1 size of heavy chain and 2 sizes of light chain. While HMW Ig in pool I contained 68 kDa H chains and 22 kDa and 24 kDa L chains, LMW Ig in pool III contained 69 kDa H chains and 22 kDa and 26 kDa L chains. A set of 5 monoclonal antibodies (MAbs) against HMW Igs was prepared. The reactivities of these MAbs with Igs in the 3 pools were determined by enzyme linked immunosorbent assay (ELISA) and dot immunoassay. All 5 MAbs reacted strongly only to the HMW Igs. Results suggest that flounder Igs comprise LMW Igs as well as HMW Igs and the physicochemical and antigenical characteristics of LMW Igs are different from those of HMW Igs.

KEY WORDS: Flounder *Edwardsiella tarda* · Immunoglobulin · LMW Ig · HMW Ig

INTRODUCTION

Immunoglobulin (Ig) synthesized in response to antigenic stimulation is one of the key components of the humoral immune system in vertebrates and has been an object of study for a long time.

In amphibians and higher vertebrates, more than 2 classes of Ig are present and a molecular weight shift from high molecular weight (HMW) to low molecular weight (LMW) Ig commonly occurs during the immune response. In general, 19S pentameric HMW IgM first appears in the early immune response, followed by 7S monomeric LMW IgG (Nossal et al. 1964, Rabbitts et al. 1980). Sharks appear to have only 1 class of Ig, i.e. IgM, but a molecular weight shift from 19S pentameric to a 7S monomeric form of IgM is thought to occur during the immune response (Marchalonis & Edelman 1965, Clem & Small 1967). Recently, some elasmobranchs have been reported to contain a second class of Ig other than IgM (Kobayashi et al. 1984, Kobayashi & Tomonaga 1988) having more than 2 distinctly different heavy chain isotype genes (Kokubu et al. 1988, Harding & Amemiya 1990, Harding et al. 1990).

Many teleosts have been reported to possess only 16S tetrameric HMW IgM which has a molecular weight of about 700 kDa (Shelton & Smith 1970, Litman 1975, Amemiya & Litman 1990, Pilstroem & Petersson 1991), and a molecular weight shift during the immune response has not been reported. However,

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some teleosts possess LMW Igs which are found to be either a monomeric form of HMW Ig or to be a physicochemical form distinct from HMW Ig. For example, goldfish (Uhr et al. 1962, Marchalonis 1971), plaice (Fletcher & Grant 1969), margate (Clem & McLean 1975), sheepshead (Lobb & Clem 1981), and toad fish (Warr 1983) possess LMW IgM, a 7S monomeric form of IgM. Other teleosts such as giant grouper (Clem 1971), rainbow trout (Elcombe et al. 1985), channel catfish (Lobb & Olson 1988), and carp (Rombout et al. 1993) are reported to possess LMW Igs whose H chain is structurally and/or antigenically distinct from that of HMW Ig. However, at present, it is not clear whether or not the LMW Igs of those fishes are a second class of Ig distinct from IgM and are the result of a molecular weight shift during the immune response.

In this paper, we investigated the humoral immune response in flounder *Paralichthys olivaceus* to *Edwardsiella tarda*, a causative agent of edwardsiellosis in fish (Egusa 1976, Nakatsugawa 1983), reported the presence of LMW Ig as well as HMW Ig during an immune response in flounder and concluded that LMW Ig is structurally and antigenically different from HMW Ig.

MATERIALS AND METHODS

Flounder antisera. Healthy flounders *Paralichthys olivaceus* weighing about 110 g were maintained in filtered seawater at 20 to 23°C. Five fish were intraperitoneally injected once with formalin-killed *Edwardsiella tarda* prepared from a strain (FSW-910410) isolated from a flounder in Korea (Bang et al. 1992). The bacterin dose contained about 4×10^9 bacterial cells per fish. These fish were bled periodically from the caudal artery; the serum was collected after centrifugation and stored at –20°C.

Agglutination test. Antiserum or purified antibody solutions were 2-fold serially diluted with phosphate-buffered saline (PBS) and mixed with an equal volume of formalin-killed bacterial cells (approximately 2.0×10^8 cells ml^{-1}) in a round bottom multiwell plate (Green Cross, Korea) and incubated for 2 h at room temperature. The end point of titrations were indicated by macroscopic settling patterns of the bacterial cells.

Column chromatography. The proteins in 3 ml of sera were precipitated with sodium sulfate (18% w/v) at room temperature and collected by centrifugation at $3000 \times g$ for 30 min at 25°C. The precipitates were dialyzed using PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) at 4°C. Sephacryl S-300 (Sigma, USA) gel in a 1.5 × 120 cm column was equilibrated with PBS, and flounder serum proteins were eluted with the same buffer at a flow rate of 20 ml h^{-1}. The samples were collected at a volume of 1.0 ml per tube and analyzed for protein by measuring optical density at 280 nm (OD_{280}) and Ig quantity using the agglutination test and ELISA. In order to estimate the approximate molecular weights of the proteins fractionated on gel-filtration, standard size marker proteins such as blue dextran (2000 kDa), thyroglobulin (669 kDa) and catalase (232 kDa) (Pharmacia, Sweden) were eluted under the same conditions as described above. The fractions with agglutination titers were pooled and dialyzed using 0.01 M K_2HPO_4 (pH 8.0) and injected into a hydroxyapatite column (1.0 × 25 cm) equilibrated with 0.01 M K_2HPO_4 (pH 8.0). Elution was carried out using a linear gradient from 0.01 to 0.5 M K_2HPO_4 at a flow rate of 15 ml h^{-1}; 1.5 ml fractions were collected and analyzed for protein (OD_{280}) and agglutination titer.

Isoelectric focusing. Flounder Igs were further purified by isoelectric focusing. The Igs were separated in pH 3.5 to 10 isoelectric focusing gel and purified by electroelution with a gel eluter (Hoeffer, USA).

Monoclonal antibodies (MAbs). MAbs against HMW Igs were prepared by a modification of the methods described by Lane (1985). Briefly, balb/c mice were immunized intraperitoneally 4 times with purified HMW Igs mixed with Freund's adjuvant (Sigma) and received an additional intravenous injection 3 to 4 d before fusion. Spleen cells from immunized mice were fused with SP-2/0 myeloma cells in 50% polyethylene glycol 1500 (Sigma). The cells were resuspended in RPMI-1640 medium containing hypoxanthine, aminopterin, thymidine and 10% fetal bovine serum (Gibco BRL, USA) and plated in 96-well culture plates. Hybridoma supernatants were screened using ELISA. Selected hybridomas were cloned a minimum of 2 times by limiting dilution on macrophage feeder cells and then expanded in a culture flask for *in vitro* production of monoclonal antibodies.

MAb class was determined by using a mouse monoclonal sub-isotyping kit (GibcoBRL, USA).

Enzyme-linked immunosorbent assay (ELISA). The reactivities of MAbs with flounder Igs were assayed by an ELISA. Microtiter plates (Nunc, Denmark) were coated with 2 µg protein ml^{-1} of flounder Igs in coating buffer (0.5 M carbonate-bicarbonate, pH 9.6) at 4°C for 16 h. The plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. After washing, MAbs diluted 1/50 in PBS containing 0.05% Tween 20 were added, incubated for 2 h and washed 3 times with PBS-Tween 20 (0.05%). Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) was added and was detected by the addition of alkaline phosphatase substrate, *p*-nitrophenyl phosphate (1 mg ml^{-1} in 0.1 M $NaHCO_3$ and 1.0 mM $MgCl_2$, pH 9.8). The absorbance was read at 405 nm.

For the quantification of anti-*Edwardsiella tarda* Igs in flounder sera, microplates were coated with 2 mg proteins ml^{-1} of *E. tarda* cells lysate. Then 2-fold serially diluted flounder sera was added to microtiter plates and was reacted successively with rabbit anti-flounder Igs and goat anti-rabbit IgG conjugated with alkaline phosphatase. The bound alkaline phosphatase was detected by the addition of *p*-nitrophenyl phosphate.

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (1970). Serum proteins were electrophoresed using the discontinuous buffer system in 5% polyacrylamide gel under non-reducing conditions and 10% polyacrylamide gel under reducing conditions. The gels were stained with 0.1% Coomassie Brilliant Blue, destained and then dried. The molecular weights of the polypeptides were calculated from their electrophoretic mobilities relative to standard proteins (Bio-Red, USA) run in parallel. Bovine IgG and IgM (Sigma) were used as reference standards.

Western blots and dot immunoassay. Electrophoretically separated polypeptides were transferred to nitrocellulose (NC) paper at 40 mA for 16 h using a transfer buffer of 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. The NC paper was blocked with 1% BSA in PBS. After incubation of mouse MAbs for 2 h, bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies followed by the addition of BCIP/NBT phosphatase substrate, consisting of 5-bromo-4-chloro-3-indolyl phosphate sodium salt (0.15 mg ml^{-1}) and *p*-nitro blue tetrazolium chloride (0.3 mg ml^{-1}) in carbonate buffer.

For the dot immunoassay, 1 µg protein of flounder Ig was dotted to NC paper. After the dots had been allowed to dry, the NC paper was blocked and incubated with MAb and alkaline phosphatase-conjugated goat anti-mouse IgG as described above.

RESULTS

Immune response

The humoral immune response of flounder immunized once with formalin-killed *Edwardsiella tarda* was examined by testing the anti-*E. tarda* agglutination titers in the sera collected from 5 fish once a week (Fig. 1). The agglutination titer was detected in the sera collected 1 wk post-immunization and increased continuously reaching maximum levels by Week 5. The agglutination titers remained at maximum levels until Week 7 after immunization, at which time they started to decrease gradually for the remainder of the experiment. However, the anti-*E. tarda* agglutination titer remained relatively high after 16 wk had passed and did not return to background levels until 1 yr after the start of the experiment.

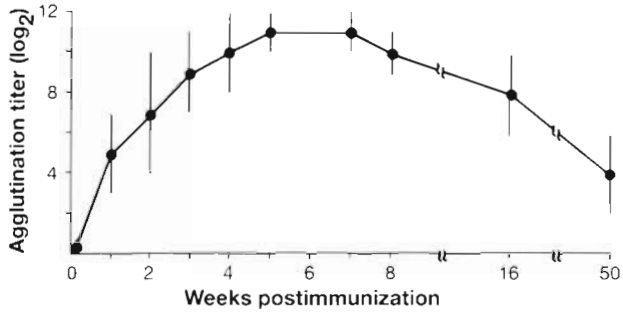


Fig. 1 Anti-*Edwardsiella tarda* agglutination titers of sera taken from flounder immunized once with formalin-killed *E. tarda* cells. Each data point represents the mean ± SE agglutination titer from 5 fish

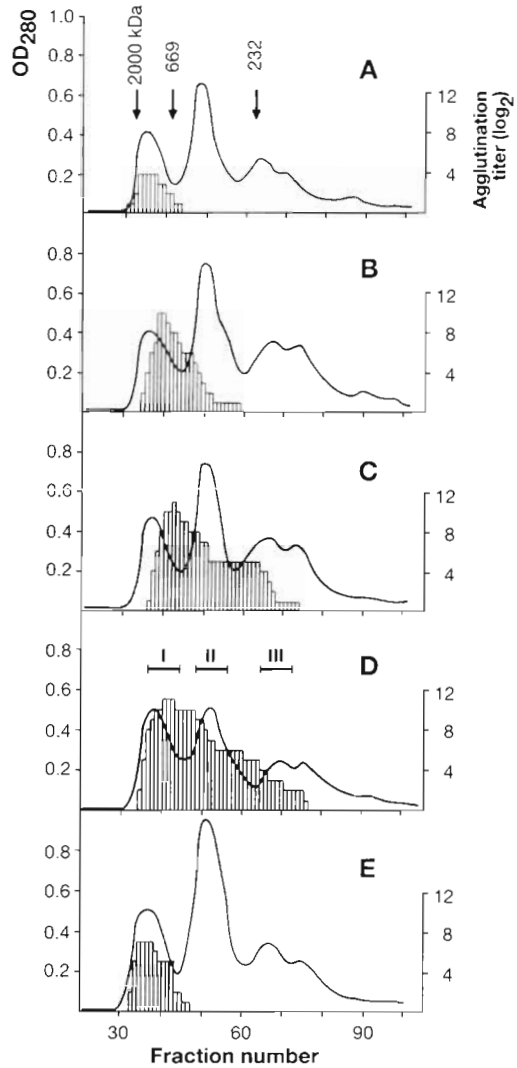
Elution profile of gel filtration

Flounder sera were fractionated by using gel filtration in order to ascertain whether various sizes of Igs, from HMW Ig to LMW Ig, appeared during the immune response.

Flounder sera from 5 fish were collected and pooled at 1, 2, 4, 5 and 16 wk post-immunization, and 3 ml of each sera pool was passed over a Sephacryl S-300 column. The elution profile and the agglutination titers are shown in Fig. 2. All the sera collected at different periods post-immunization were separated into 4 peaks. Based on the elution of the molecular weight standards, the 4 peaks were ascribed molecular weight ranges (Fig. 2A). The first peak contained molecular weights greater than 700 kDa, the second ranged from 230 to 700 kDa, and the third and the fourth peaks were less than 230 kDa.

The distributions of the anti-*Edwardsiella tarda* Igs were not constant among the antisera collected at different periods. In antiserum collected at 1 wk post-immunization, *E. tarda* agglutinating activities were detected only in the first peak (Fig. 2A). This seemed to be HMW Ig with a molecular weight of more than 700 kDa. But with time, even though most of the flounder *E. tarda* agglutinating activity involved HMW Ig, LMW Ig with *E. tarda* agglutinating activity could be detected, and by 4 to 5 wk post-immunization the agglutination activity was distributed among the first, second and even the third peak (Fig. 2B, C & D). However, in the serum collected at 16 wk, the agglutination activity was again only detectable in the first peak (Fig. 2E).

Using agglutination assay, LMW Igs were not detected during the early (1 wk post-immunization) and the late (16 wk post-immunization) immune response.



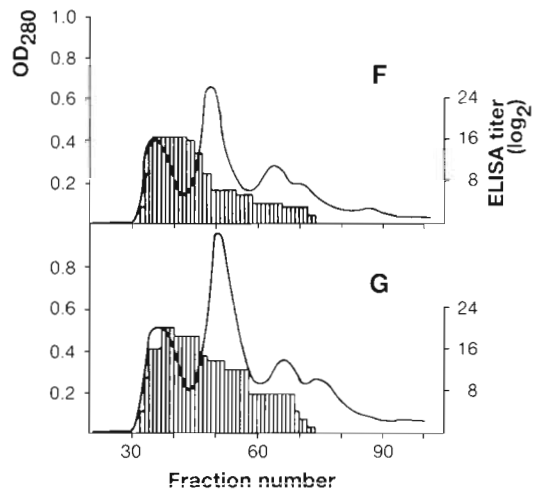


Fig. 2. Elution profile of flounder anti-*Edwardsiella tarda* serum from a Sephacryl S-300 column. Flounder sera were collected from the tail vein of flounders at (A) 1 wk, (B) 2 wk, (C) 4 wk, (D) 5 wk, and (E) 16 wk post-immunization with formalin-killed *E. tarda* cells. Each fraction was analyzed for proteins and Ig quantity using the agglutination test (A, B, C, D, and E) or ELISA (F and G). Lines indicate absorbance at 280 nm and bars indicate the antibody titer. To determine the approximate molecular weight of the eluted proteins, 3 standard size marker proteins (blue dextran, 2000 kDa; thyroglobulin, 669 kDa; catalase, 232 kDa) were used and their positions indicated with arrows in (A). In D, I, II, III indicate pools I, II and III (see 'SDS-PAGE of flounder Igs)

However, it was probable that LMW Igs might be produced during the entire response but that agglutination assay was not sensitive enough to detect the low concentration of LMW Igs during the early and the late immune response. A more sensitive assay, ELISA, was employed to detect any LMW Igs present at a low concentration during the early and the late immune response. Using ELISA, LMW Ig with *Edwardsiella tarda* binding activities in the second and the third peaks as well as HMW Ig in the first peak were detected in the sera collected during the early and the late immune response (Fig. 2F & G). This result supports the idea that LMW Ig and HMW Ig are present during the entire immune response.

SDS-PAGE of flounder Igs

The eluted fractions of flounder serum at 5 wk showing agglutination activity were combined into 3 pools according to their molecular weight: pool I contained the first peak; pool II, the second peak; pool III, the first portion of the third peak (Fig. 2D). Then, each pooled fraction was further purified by hydroxyapatite gel column chromatography and elution from the gel by isoelectric focusing.

In order to elucidate the purity and to compare the relative size of Igs in each pool, these pools were analyzed by SDS-PAGE under non-reducing conditions. As shown in Fig. 3A, only 1 band was observed from each pool and the sizes were different from each other, pool I > pool II > pool III. Then, the polypeptides of the HMW Igs in pool I and the LMW Ig, in pool III were analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 3B, Igs in each pool were composed of 1 H chain and 2 L chains and the sizes of these chains of LMW Ig were different from those of HMW Ig. While the HMW Ig in pool I contained a 68 kDa H chain and 22 kDa and 24 kDa L chains, the LMW Igs in pool III contained a 69 kDa

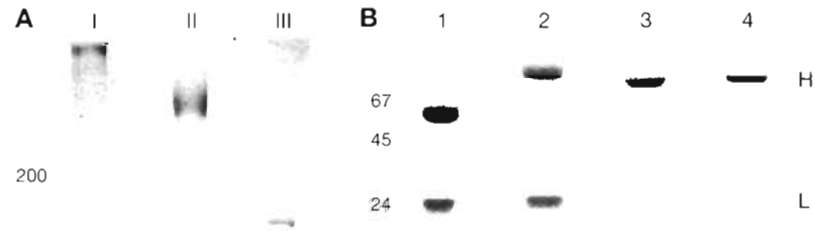


Fig. 3. Electrophoretical analysis of flounder immunoglobulins. Flounder immunoglobulins were purified by hydroxyapatite column chromatography and isoelectric focusing. Purified immunoglobulins, HMW Igs (pool I) and LMW Igs (pools II and III), were analyzed by (A) 5% SDS-PAGE under non-reducing conditions and (B) 10% SDS-PAGE under reducing conditions, and visualized by Coomassie staining. Values on *y*-axis are kDa. Lanes 1 and 2 of (B) are bovine IgG and IgM respectively. H: H chain; L: L chain

H chain and 22 kDa and 26 kDa L chains. This result implies that HMW Ig and LMW Ig are composed of different sizes of H and L chains.

Reactivities of different-sized Igs with MAbs

In order to compare the antigenicity of Igs in the 3 pools, a panel of 5 MAbs was prepared from a balb/c mouse immunized with HMW Igs in pool I. These MAbs were named AE10, AF7, AG9, BH8, and BH11 Among these 5 MAbs, 1 MAb, BH11, reacted with both H and L chains of HMW Ig but the other 4 reacted only with the H chain of HMW Ig (Fig. 4). The reactivities of these MAbs with different-sized Igs in the 3 pools were determined by ELISA and dot blot immunoassays. In ELISA, even though 1 MAb, BH11, showed equal reactivity with both HMW Ig in pool I and LMW Ig in pool II, the other 4 MAbs showed strong reactivities only with HMW Ig (Fig. 5A). In dot immunoassay, all the 5 MAbs reacted strongly only with the HMW Ig in pool I (Fig. 5B). The results revealed that the epitopes recognized by these MAbs were specific to the HMW Ig.

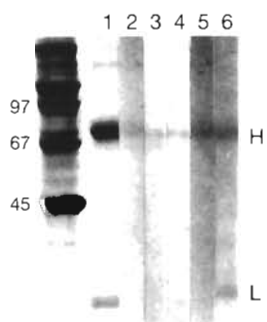


Fig. 4. Immunoglobulin polypeptides recognized by monoclonal antibodies (MAbs). Five MAbs against HMW Ig (pool I) were prepared and the reactivities of these MAbs with the HMW Ig were analyzed by Western blots. Lane 1: polypeptides of HMW Ig stained with Coomassie Blue; lane 2: MAb AF7; lane 3: MAb AE10; lane 4: MAb AG9; lane 5: MAb BH8; lane 6: MAb BH11. The first lane is the standard. Values on y-axis are kDa. H: H chain; L: L chain

DISCUSSION

The present study was conducted to determine whether or not various sizes of Igs were present in flounder during an immune response and, if so true, whether or not these different-sized Igs were the same class with different molecular weights.

In order to answer the first question, sera collected at various times post-immunization with *Edwardsiella tarda* were fractionated by gel filtration and each of the fractions was analyzed for the presence of Igs using an agglutination test. The fractions with agglutination activities were assigned to 3 pools based on size: pool I > 700 kDa, 230 kDa < pool II < 700 kDa and pool III < 230 kDa. Early and late sera, collected at 1 and 16 wk, contained HMW Igs only in pool I. However, by 4 and 5 wk post-immunization, LMW Igs in pools II and III were detected even though the HMW Igs were at a consistently higher concentration than the LMW Igs. When these HMW and LMW Igs were analyzed by SDS-PAGE under non-reducing conditions, they were detected as distinct bands of different sizes with pool I > pool II > pool III, as could be predicted from the results of gel filtration. This indicated the presence of various sizes of Igs in flounder during the immune response.

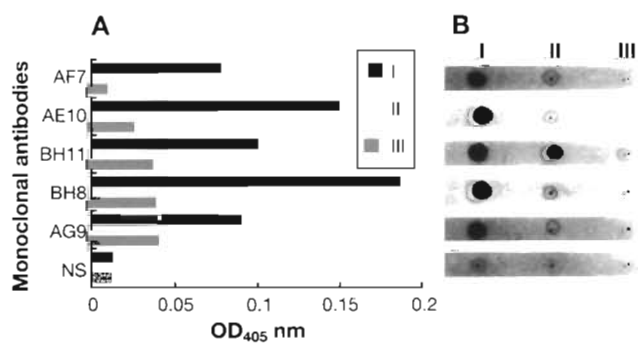


Fig. 5. Reactivity of 5 MAbs with flounder immunoglobulins. Flounder immunoglobulins, HMW Ig (I) and LMW Ig (II and III), captured on the microtiter plates were reacted with 5 MAbs and the amount of protein-bound MAbs were determined by (A) ELISA and (B) dot immunoassay. I, II, III (B) indicate pools I, II and III. Normal mouse serum (NS) was used as a control

In flounder, the LMW Igs with agglutination activity appeared approximately 4 wk post-immunization. These results suggested that LMW Ig could be produced only during active immune response. However, it is more probable that both HMW and LMW Igs coexist during the entire response but that the concentration of the LMW Igs during the early and late immune response would be too low to be detected by agglutination assay. To clarify this, a more sensitive assay, ELISA, was employed and, as a result, the LMW Igs in pool II and III as well as HMW Ig in pool I were detected in the sera collected during the early and the late immune response. This result supports the coexistence of HMW and LMW Igs during the entire immune response. However, even though most of the antibody activity throughout the immune response involved HMW Ig, the concentration of LMW Ig seemed to increase more rapidly than HMW Ig during the immune response. This suggests the possible presence of a molecular weight shift.

The possibility that the LMW Igs may be the products of *in vitro* degradation of HMW Igs could be ruled out due to the fact that the H and L chains of LMW Igs were larger than those of HMW Igs and the antigenic determinants of HMW Igs were distinct from those of LMW Igs. This is discussed below.

Many fishes are known to possess LMW Igs. Some LMW Ig is found to be a monomeric form of HMW IgM. However, some fishes possess LMW Igs structurally and antigenically distinct from HMW IgM. For example, skate, a cartilaginous fish, possesses LMW Igs whose H chains are structurally and antigenically distinct from a μ chain (Kobayashi et al. 1984, Kobayashi & Tomonaga 1988) and have more than 2 distinctly different H chain isotype genes (Harding & Amemiya 1990, Harding et al. 1990). Among teleosts, giant grouper (Clem 1971), rainbow trout (Elcombe et al. 1985), channel catfish (Lobb & Olson 1988), and carp (Rombout et al. 1993) possess LMW Ig whose H chain is structurally and/or antigenically distinct from that of HMW Ig

Our next question is whether or not LMW Igs in flounder are the cleavage products of HMW Igs or are physicochemically distinct from HMW Ig. The evidence presented here demonstrates that, in flounder, the LMW Igs are structurally and antigenically distinct from HMW Ig on the basis of 2 criteria. Firstly, LMW Igs possess H and L chains whose sizes are different from those of HMW Ig. While the HMW Ig in pool I contained a 68 kDa H chain and 22 kDa and 24 kDa L chains, the LMW Ig in pool III contained a 69 kDa H chain and 22 kDa and 26 kDa L chains. The presence of multiple L chains in flounder Igs is not unique, but several fishes have previously been reported to possess multiple L chains with a molecular weight of 22 to 26 kDa (Lobb & Clem 1983, Warr 1983, Lobb et al. 1984, Rast et al. 1994). In channel catfish, 3 molecular weight L chain variants with molecular weights of 22, 24 and 26 kDa were observed and grouped into 2 classes based on their reactivities with MAbs (Lobb et al. 1984), and several C region gene segments of the L chain were found (Ghaffari & Lobb 1993). At present, there is no data comparing the similarities between the L chains of flounder and those of other fishes. Secondly, the H chains were antigenically different. A set of 5 MAbs was prepared from a balb/c mouse immunized with HMW Ig in pool I. They were found to recognize the H chains of the HMW Igs. When the reactivities of these MAbs with different-sized Igs in the 3 pools were determined by ELISA and dot immunoassay, they were immunoreactive with HMW Ig but were not or hardly immunoreactive with other LMW Igs in pool II and pool III. Even though it is not now certain whether the MAbs recognize the determinants in the constant region or variable region of H chain, this clearly shows that LMW Igs are somehow antigenically different from HMW Ig.

Although the physicochemical studies reported here on flounder Igs are somewhat limited, it seems reasonable to suggest the gross architecture of flounder Igs. During an active immune response, flounder possess various sizes of Igs which are detected as bands on polyacrylamide gel under non-reducing conditions. The Igs in pool III are considered to be a bivalent monomeric type of Ig composed of 2H-2L chains with an approximate molecular weight of 182 kDa and/or 190 kDa, since this type of Ig is able to agglutinate bacterial cells (monovalent Ig is unable to agglutinate cells), had H chains of 69 kDa and L chains of 22 and 26 kDa as determined by SDS-PAGE, and was smaller than 230 kDa as determined by gel filtration. Similar reasoning suggests that the Igs in pool II are dimer (4H-4L) and/or trimer (6H-6L) with a molecular weight of about 360 kDa and/or 540 kDa respectively and that the largest Igs in pool I are tetramer (8H-8L) with a molecular weight of 720 kDa. Although there is no data which allow direct comparisons, the tetrameric HMW Ig of flounder seems to belong to the same class as that seen in other teleosts, IgM.

In conclusion, even though the tetrameric form of IgM made up most of the Igs throughout the immune response of flounder against *Edwardsiella tarda*, LMW Igs with monomeric, dimeric and trimeric forms consistently coexisted during the immune response. HMW Ig and LMW Ig were composed of different sizes of H and L chains and were antigenically distinct from each other, which suggests the possible presence of a different class or subclass of Igs. To further examine this possibility, peptide mapping and amino acids sequencing studies are presently being conducted.

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