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*J Immunol* 2012; 188:1341-1349; Prepublished online 28 December 2011; doi: 10.4049/jimmunol.1101938

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**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/12/29/jimmunol.1101938.DC1

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Discovery and Characterization of Secretory IgD in Rainbow Trout: Secretory IgD Is Produced through a Novel Splicing Mechanism

Francisco Ramirez-Gomez,* Whitney Greene,* Katherine Rego,* John D. Hansen,†,‡ Greg Costa,* Priti Kataria,* and Erin S. Bromage*

The gene encoding IgH δ has been found in all species of teleosts studied to date. However, catfish (Ictalurus punctatus) is the only species of fish in which a secretory form of IgD has been characterized, and it occurs through the use of a dedicated δ-secretory exon, which is absent from all other species examined. Our studies have revealed that rainbow trout (Oncorhynchus mykiss) use a novel strategy for the generation of secreted IgD. The trout secretory δ transcript is produced via a run-on event in which the splice donor site at the end of the last constant domain exon (D7) is ignored and transcription continues until a stop codon is reached 33 nt downstream of the splice site, resulting in the production of an in-frame, 11-aa secretory tail at the end of the D7 domain. In silico analysis of several published IgD genes suggested that this unique splicing mechanism may also be used in other species of fish, reptiles, and amphibians. Alternative splicing of the secretory δ transcript resulted in two δ-H chains, which incorporated Cα1 and variable domains. Secreted IgD was found in two heavily glycosylated isoforms, which are assembled as monomeric polypeptides associated with L chains. Secretory δ mRNA and IgD* plasma cells were detected in all immune tissues at a lower frequency than secretory IgM. Our data demonstrate that secretory IgD is more prevalent and widespread across taxa than previously thought, and thus illustrate the potential that IgD may have a conserved role in immunity. The Journal of Immunology, 2012, 188: 1341–1349.

Immunglobulin δ (IgD) homologs have been discovered in all jawed vertebrates with the exception of aves (for review, see Refs. 1, 2). The structure of the δ-chain isotypes demonstrates considerable diversity in size, with as few as two constant domains observed in mice and as many as 16 constant domains in zebrafish (Danio rerio) (2). Teleosts demonstrate remarkable plasticity in their δ genomic arrangement, with many species possessing repeated blocks of exons encoding Cδ2–4 (up to four repeats have been described) as well as duplicated δ loci (3–5). In Atlantic salmon (Salmo salar), a salmon-specific genome duplication event (IGH-A and IGH-B) resulted in parallel δ loci that have diverged from each other, resulting in an additional δ2–4 repeat in the IGH-B locus (6). In channel catfish (Ictalurus punctatus), there are two functional copies of the δ gene (7) on the same IGH locus. One is found in the typical location downstream of Cμ, whereas the other copy is found upstream of the functional Vδ1-Dδ1-Jδ1 region. The downstream gene encodes the membrane-bound form of δ, and the upstream gene has a dedicated exon for the secretory product. This within-IGH δ duplication and specialization (membrane and secretory) have not been observed in other teleosts and have led to the speculation that secretory IgD is rare in fish (1).

As recently as 1997, the absence of IgD in serum led scientists to believe that it was a newly evolved Ig that was exclusive to primates and rodents (7). Research during the genetics revolution of the 1990s reinforced the “newly evolved” hypothesis, as the IgD gene was found in mice and primates, but not found in the genome of aves. Birds, especially chickens, were regarded as a model species for B cell studies; the absence of IgD suggested that it would not be present in lower vertebrates. However, after compelling comparative research using catfish (7) and Xenopus (8), it was determined that an IgD gene was present in these primitive species. The discovery of a novel chimeric Ig in channel catfish set the foundation for all future studies on teleost IgD (7). The chimeric molecule demonstrated homology with the mammalian IgD, but its genetic structure was much longer than what had been seen in human and mouse IgD. The research with Xenopus and catfish completely opened the panorama for future findings, allowing the discovery of other IgD genes in nonmammalian species. The δ loci have been described in primates, rodents, cattle, sheep, pigs (9), horses (10), giant pandas (11), and dogs (12), as well as more exotic species, including the platypus (13), the leopard gecko (14), Chinese soft-shelled turtle (15), and the green anole lizard (16). IgD is now considered an ancient Ig that was present in vertebrates ancestral to both the mammals and the ray-finned fishes (7).

Despite IgD’s widespread prevalence, the secreted form of IgD has only been identified in mammals and catfish, and we are be-
beginning to understand its significance. Recently, Chen et al. (17) determined that IgD secreted from activated B cells binds to basophils and mast cells and activates these cells to produce antimicrobial factors for participation in respiratory immune defenses. In turn, the stimulated basophils release B cell homeostatic factors further enhancing the B cell response (17). This group also found that, in addition to antimicrobial factors, IgD-armed basophils produce opsonizing, inflammatory, and immune-stimulating factors. It was suggested that although IgD is part of the adaptive response, the role of IgD may be to coordinate an immune surveillance system at the crossroads of the innate and adaptive systems. In support of the dual nature of the IgD molecule, Edholm et al. (18) found that catfish secretory IgD lacked a V region. It was concluded that IgM-IgD B cells most likely expand in response to certain pathogens, and the secreted V-less IgD Fc region may function as an innate pattern recognition molecule. The study concluded that the V-less IgD may represent an early transition of the innate pattern recognition receptors to adapt losing Ig molecules.

The original goal of this research was to examine IgD expression on rainbow trout (Oncorhyncus mykiss) B cells using a newly developed mAb, which is specific to the repeated β2 domain of trout membrane IgD. Fortuitously, we applied the Ab reagent to trout serum, and, in doing so, identified a putative secretory IgD product. Further molecular and serological studies have confirmed the presence of secretory IgD in rainbow trout. Based upon these findings, we scanned available genomic resources and found other examples of secretory IgD H chain mRNAs, suggesting that secretory IgD may be more widespread than previously anticipated.

Materials and Methods

Peptide and Ab development

The peptide (C-DGKDPTSRTPVNQA) used for IgD immunizations was synthesized with an additional cysteine added to the N terminus for keyhole limpet hemocyanin conjugation (Genscript). This peptide sequence is found in the repeated β2 domain of the membrane HC δ sequence (Fig. 1). Six-week-old BALB/c mice were immunized with 50 μg (peptide weight) keyhole limpet hemocyanin-peptide conjugate emulsified in complete Freund’s adjuvant. A 10 μg booster shot in IFA was given at weeks 4 and 8 postimmunization. A final i.v. injection (5 μg in PBS) was given 4 d before the fusion (week 12). A standard 50% polyethylene glycol fusion was performed, and resultant hybridoma supernatants were screened against the fusion (week 12). A standard 50% polyethylene glycol fusion was performed, and resultant hybridoma supernatants were screened against the fusion (week 12). A standard 50% polyethylene glycol fusion was performed, and resultant hybridoma supernatants were screened against the fusion (week 12). A standard 50% polyethylene glycol fusion was performed, and resultant hybridoma supernatants were screened against the fusion (week 12). A standard 50% polyethylene glycol fusion was performed, and resultant hybridoma supernatants were screened against the fusion (week 12).

**mAb validation**

The specificity of the resultant mAbs was determined via Western blot and affinity-purification experiments. Affinity purification of trout serum IgD was performed using the Pierce direct IP kit (Thermo Scientific) following the manufacturer’s TBS buffer protocol. The bound protein was eluted from the column and run on a 4–15% SDS-PAGE gel. The predominant 165- and 100-kDa proteins were excised from the gel and sent for amino acid sequencing via liquid chromatography-tandem mass spectrometry (ProtTech). The remainder of the eluted protein was used as a standard for quantitative Western blotting and deglycosylation studies.

**Western blot analysis**

Peripheral blood leukocytes (PBL) were lysed using the Pierce IP lysis buffer (Thermo Scientific) following the manufacturer’s directions supplemented with Halt protease inhibitor mixture (Thermo Scientific). Ten micrograms of PBL lysate was resuspended in an equal volume of reducing sample buffer and boiled for 10 min. Ten microliters of the lysate suspension was loaded and run on a 4–15% gradient gel. The gel contents were transferred to polyvinylidene difluoride (PVDF)-FL (Millipore), and the membrane blocked with 3% casein. The membrane was probed with the anti-IgD reagent, 1 μg/ml; LiCor) and SA-IR800 to detect surface-biotinylated membrane proteins. Trout serum and affinity-purified trout IgD were analyzed via SDS-PAGE and SDS-composite agarose gel electrophoresis (SDS-CAGE). One microcrill of trout serum was added to either 200 μl reducing sample buffer or nonreducing sample buffer and boiled. Reduced serum samples were loaded and run on a 4–15% gradient gel, whereas nonreduced proteins were processed on 3.1% SDS-CAGE gels (19) at 100 V and 4 °C. The proteins were subsequently transferred to PVDF-FL membranes (Millipore) and blocked with 3% casein. Following blocking, 5 μg/ml anti-IgD was added and allowed to incubate for 1 h. The blot was washed three times, and then the bound Ab was detected with goat anti-mouse IR680 (1:10,000; LiCor). For in-blot comparison, IgM was detected using biotinylated anti-trout IgM, followed by a 1:10,000 dilution of SA-IR800. To determine whether the detected proteins were on the surface of the lymphocytes, PBL were surface biotinylated using sulfo–normal human serum–biotin (Pierce) and used for Western blot. The membrane was probed with the anti-IgD reagent, 1 μg/ml; LiCor) and SA-IR800 to detect surface-biotinylated membrane proteins. Trout serum and affinity-purified trout IgD was enzymatically deglycosylated following the manufacturer’s instructions (GlycoPro; Prozyme) and analyzed via SDS-PAGE and Western blot, as described above.

**Chromatography and ELISA**

One hundred microliters of trout serum was size separated on a Sephacyrl s300 column (100 cm, 16 mm internal diameter), and 1-ml fractions were sequentially collected. Each fraction was tested via capture ELISA for the presence of IgM or IgD. The ELISA plates were coated with either 2 μg/ml anti-IgD or anti-IgM overnight and blocked with Tween 20 and TBS plus 1% BSA. Size-separated fractions were added to individual wells and serially incubated for 1 h. The plates were washed two times, and the bound Ab was detected by the addition of either biotinylated anti-IgD or biotinylated anti-IgM. Development was performed using SA-HRPO and ABTS.

**Trough leukocyte analysis**

Trough leukocytes were isolated from the spleen, anterior kidney, posterior kidney, and peripheral blood using histopaque 1077, as previously described (20). Leukocytes were isolated from the gills by homogenizing gill filaments using a glass/Teflon Potter Elvehejen homogenizer with 3 ml ice-cold RPMI 1640 supplemented with 100 μg/ml collagenase. Following homogenization, cells were allowed to sit for 15 min on ice, large debris was removed, and the cells were washed three times before being overlaid on histopaque 1077. Recovered leukocytes were resuspended in cold RPMI 1640 at a density of 10⁷ cells/ml, and the resulting cell suspensions were used for flow cytometry, ELISPOT, and Western blot analysis.

**Flow cytometric analysis of trout leukocytes**

IgD-positive lymphocytes were identified via flow cytometry by incubating 100 μl trout leukocyte preparations with 5 μg/ml anti-IgD mAb diluted in FACS buffer (HBSS + 3% FBS + 0.02% sodium azide). After a 15-min incubation, the cells were washed three times and subsequently incubated with a goat anti-mouse γ-chain–specific secondary Ab conjugated to PE (Southern Biotechnology Associates). Cells were washed and then dual
stained with a directly labeled (AlexaFluor 647) anti-trout IgM Ab [1.14 (21)].

ELISPOT analysis

ELISPOT was used to quantify the number of IgD-secreting B cells, as previously described (20). In brief, PVDF membranes (Millipore) were placed in a BioDot apparatus and coated with either anti-trout IgD (2 μg/ml) or anti-trout IgM (1 μg/ml) mAb and blocked. Serial dilutions of leukocytes starting at 5 × 10^5 cells were added to the wells and incubated for 16 h at 18°C. After incubation, the membrane was removed from the apparatus, blocked with casein, and incubated with biotinylated anti-trout IgD (5 μg/ml) or biotinylated anti-trout IgM (2 μg/ml). The membrane was then developed using streptavidin-HRP and aminothioethylcarbazole. Once the membranes had dried, they were digitally scanned, and spot counts and spot intensity were determined using ImageJ software.

3′ RACE

Cytoplasmic RNA was isolated from PBL using the RNeasy mini kit (Qiagen) following the manufacturer’s instructions. Poly A+ RNA was isolated from the cytoplasmic RNA using the Oligotex mini kit (Qiagen). The sequence encoding the 3′ end of the IgD cDNA (3′ RACE; Invitrogen) following the manufacturer’s instructions. The cDNA was synthesized using the adapter primer from the 3′ RACE kit, and the subsequent PCRs were performed using secretory IgD-specific forward primers (D7secF5 or D7secF; Supplemental Table 1) and the universal primers (AUAP) from the kit.

Secretory IgD transcript size heterogeneity

To detect various HC β transcripts, poly-A-tailed cytoplasmic RNA from PBL was used as the template with Cβ1 (CM-F) and Cβ1/Cε1 (CMD1-F) forward primers and a 67/ssec reverse primer (D7sec-R; Supplemental Table 1). The two dominant PCR products observed in both primer combinations were cloned using the pGEM-T Easy Vector System (Promega) according to manufacturer’s instructions. After cloning, the resulting products were bidirectionally sequenced.

Secretory IgD is associated with V_H

To determine whether HC δ contains a variable domain, gene-specific cDNA was made from poly-A-tailed cytoplasmic RNA using a secretory δ-specific reverse primer (D7secR1; Supplemental Table 1). The secretory δ-specific cDNA was synthesized with the Improm II reverse transcription system (Promega) using 100 ng poly-A cytoplasmic RNA and 20 pmol D7secR1 primer, following the manufacturer’s instructions. After the secretory δ-specific cDNA synthesis was completed, PCR amplification was performed using a conserved forward primer located in the framework region 2 (FR2-F2) of the variable segment of membrane IgD (AY870260). The reverse primer was designed in the δ1 domain of HC δ (D1-R2). The PCR conditions used were as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 65°C for 45 s, and 72°C for 45 s, and a final extension step at 72°C for 10 min. The PCR product was purified (EZ-10 spin column PCR purification kit; BioBasicInc) and cloned using the pGEM-T easy cloning kit (Promega). After cloning, the resulting product was bidirectionally sequenced and analyzed.

IgD mRNA expression in tissues

Cytoplasmic RNA was isolated from trout immune tissues using the RNeasy mini kit (Qiagen) following the manufacturer’s instructions. Primer and probe sequences used for quantitative RT-PCR (qRT-PCR) are provided in Supplemental Table 1. Unique primer pairs for secreted and membrane IgD differentiate the two transcripts, but use a common IgD probe. cDNA synthesis and qRT-PCR methodology using the ABI 7900HT format (Applied Biosystems) have been previously described (22). qRT-PCR efficiency was assessed using serial dilutions of a mixture of spleen and PBLcDNA and standard curves were generated using 10-fold serial dilutions of cloned PCR products. Gene-specific amplicons for standard curves were amplified using the qRT-PCR primer sets for IgD, IgM, and acidic ribosomal phosphoprotein (ARP) and cloned into pCR4-TOPo (Invitrogen). Expression levels of membrane and secretory IgD and IgM were evaluated in the specified tissues and normalized against ARP, as previously described (22).

In silico analyses

Exon/Intron organizations for IgD genes were assessed by ENSEMBL (www.ensembl.org) and Geneious Pro. Putative secretory IgD sequences were initially identified by tBLASTn-based analysis within the “other vertebrate EST” section of GenBank at National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/). Following the identification of a putative secretory IgD expressed sequence tag for salmon (BG934529), a detailed analysis was performed on the translated genomic sequences of other species. Sequence translation of the final delta domain was continued beyond the splice donor site until a stop codon was reached. Putative N-linked glycosylation sites for trout Ig H chains were predicted using NetNGlyc v1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

Results

Discovery of secretory IgD in rainbow trout using IgD-specific mAbs

Three mAbs (3A6, 5A7, 3C5) were generated that recognized the IgD peptide and positively stained a lymphocyte population via flow cytometry, while failing to bind to IgM via ELISA. The amino acid sequences of the affinity-purified protein confirmed the specificity of the mAb for trout IgD (Supplemental Fig. 1). All the mAbs created were an IgG isotype and demonstrated the same protein specificity via Western blotting.

Lysates from PBL revealed a dominant membrane IgD H chain isoform at 125 kDa and another less prominent 165-kDa isoform (Fig. 2A, Lys.). The 125-kDa band was also positive for surface biotinylation, indicating that this protein species represents the membrane-bound form of IgD. The membrane form of IgM was observed to be 68 kDa. Probing of serum revealed three IgD H chain isoforms (165, 125, and 100 kDa), with the 165- and 100-kDa proteins accounting for >95% of the staining intensity (Fig. 2A, lanes 1–12). However, individual fish displayed different combinations of IgD isoforms (Fig. 2A, lanes 1–12). When affinity-purified IgD from lane 4 was processed under nonreducing conditions (Fig. 2B), two proteins of ~370 and 400 kDa were observed. Nonreducing analysis of affinity-purified IgD from a sample expressing predominantly the 100-kDa IgD isoform (Fig. 2A, lane 2) resulted in single 240-kDa protein (Fig. 2B). Excision of the 370- and 240-kDa proteins and subsequent processing in a reduced second dimension demonstrated that they dissociate into the prominent 165- and 100-kDa proteins, respectively. Both IgD isoforms were associated with a 25-kDa protein species that was determined to be Ig L chain based upon liquid chromatography-tandem mass spectrometry analysis (Supplemental Fig. 1). Enzymatic deglycosylation of affinity-purified IgD (lane 4) resulted in the dominant immunoreactive protein decreasing in size from 165 kDa to ~95 kDa (Fig. 2C). Similarly, the 100-kDa protein lost 30% of its mass as it decreased to ~70 kDa (data not shown).

Quantitative densitometry of sera from 12 individual, naive trout (Fig. 2A) compared with an affinity-purified IgD standard revealed that IgD concentrations varied from 2 to 80 μg/ml in these fish (incorporating all immunoreactive proteins). IgM concentrations varied from 800 to 9000 μg/ml. No relationship was observed between serum IgM and serum IgD concentrations. Size fractionation of the serum revealed IgD ELISA reactivity peaking at ~430 kDa; however, reactivity was observed over the range of 650–180 kDa (Fig. 2D).

Membrane IgD is coexpressed with membrane IgM on trout B cells

Consistent staining patterns were observed in all fish when purified trout PBL were analyzed via flow cytometry (Fig. 3). Analysis of total PBL (R1) revealed an IgD_{esp}/IgM_{esp} population (R3; ~52%), and another population of IgD_{esp}/IgM_{esp} cells (R2; ~1–5%). Back gating these cells revealed that both IgD+ populations were in the lymphocyte gate. Analysis of leukocytes from other tissues (spleen, anterior kidney, and posterior kidney) revealed the
same pattern of IgD/IgM staining, although the number of positive cells differed between tissues (peripheral blood > spleen > posterior kidney > anterior kidney).

**Secretory IgD plasma cells are primarily located in the trout anterior kidney**

The presence of IgD- and IgM-secreting plasma cells was quantified by ELISPOT from lymphocytes isolated from four naive trout (Fig. 4A). The IgM plasma cells outnumbered IgD plasma cells ~5 to 1 in the kidney and spleen, whereas in the gills the ratio approached parity (Fig. 4B). Even though IgM plasma cells were detected in the peripheral blood, IgD plasma cells were virtually absent. In three of the four fish sampled, the kidney displayed higher percentages of IgD and IgM plasma cells than the spleen. The fact that the total number of lymphocytes isolated from the kidney greatly exceeded the number recovered from the spleen (~50-fold more; data not shown) indicates that the kidney is the predominant reservoir for IgD and IgM plasma cells. It is estimated that in these four fish, the anterior kidney housed 69% of the IgD plasma cells, followed by the posterior kidney (21%), spleen (7%), blood (2%), and gills (1%). IgD spot intensity was highest in the spleen, followed by the gills, posterior kidney, and the anterior kidney.

*The transcript for secretory IgD is produced by a novel splicing event in trout*

The discovery of secreted IgD in sera (Fig. 2) led us to look for a secretory δ message using 3′ RACE. Based upon the presence of a putative secretory δ expressed sequence tag for Atlantic salmon (EG934529), we used a trout δ gene-specific forward primer that spanned the δ7 exon/intron splice site with 3′ RACE reverse primers, resulting in the amplification of two products (800 and 850 bp; Fig. 5A). Subsequent sequencing of these PCR products resulted in the identification of novel δ transcripts that ignore the splice donor site (used for splicing the δ7 exon to the δTM1 exon), continuing until a stop codon is reached 33 nt downstream (Fig. 5C; GenBank accession numbers JN173048 and JN173049; http://www.ncbi.nlm.nih.gov/genbank/). Translation of the 33-nt coding sequence predicts an 11-aa tail (GVCLSVHSILI). Interestingly, the 3′ untranslated region (UTR) of the secretory δ transcript used the TM1/TM2 donor acceptor sites and continued through until it reached the polyadenylation site used for the membrane form of δ.

**Figure 2.** Membrane and serum profile of trout IgD. A, Trout PBL lysates (Lys.) and naive trout serum (lanes 1–12 = fish 1–12) were separated under reducing conditions (4–15% SDS-PAGE) and subsequently subjected to Western blotting with an anti-IgD mAb and detected with goat anti-mouse IR680 (red). The blot was additionally probed with a biotinylated anti-IgM mAb and detected with SA-IR800 (green). Molecular masses are shown on the left. B, Affinity-purified IgD from lane 2 (2 aff.) and lane 4 (4 aff.) (above) as well as affinity-purified IgM were processed on a SDS-CAGE gel under nonreducing conditions. The membrane was probed with anti-IgM (green) and anti-IgD (red). C, Affinity-purified IgD (4 aff.) from lane 4 was deglycosylated (4 degl.) and separated on a 4–15% SDS-PAGE gel and probed with anti-IgD. D, Trout serum was size separated using a sephacryl S300 column, and each fraction was tested for the presence of IgM or IgD via capture ELISA.

**Figure 3.** IgD and IgM flow cytometry profile of trout PBL. Scatter profiles for total PBL (A), probed with anti-IgM and anti-IgD (B). Gates R2 (D) and R3 (E) were back gated to show the scatter profiles of the IgD-positive populations. Histograms of IgD (C) and IgM (F) staining.
The relative levels of IgH transcript expression in rainbow trout lymphoid tissues were as follows: secretory μ >> membrane μ >> secretory δ (Fig. 7A-D).

In silico analysis of other secreted IgD sequences

In silico analysis predicted eight N-linked glycosylation sites in rainbow trout IgD as shown in Fig. 1. Putative secretory IgD sequences were identified for Atlantic salmon (GU129139), Atlantic cod (AF155201), grass carp (GQ480796), and leopard gecko (EU312156). The hypothetical translation and alignment of the genomic sequences (Fig. 8) revealed strong identity between the rainbow trout IgD secretory tail and the sequence from Atlantic salmon and, to a lesser degree, to Atlantic cod, grass carp, and leopard gecko.

Discussion

The results from this study demonstrate that rainbow trout possess a secretory IgD product that is associated with a C3′ UTR observed in trout are hypothesized to be a result of differences between the IGHA-A and IGHB loci.

Secretory IgD size heterogeneity and association with Cμ1 and VH

Channel catfish is the only other species of fish in which a secretory IgD product has been molecularly and serologically characterized. However, catfish secretory IgD does not possess Cμ1 or variable domains (1, 7). To address size heterogeneity of secreted IgD and the possible inclusion of the Cμ1 coding region, primers were designed to amplify from the Cμ1 exon to the δ secretory tail, as well as from the Cμ1/C81 splice junction to the δ secretory tail (Fig. 6; Supplemental Table 1). Sequencing of the two major PCR products revealed that Cμ1 was associated with trout secretory δ. The larger PCR product (1800/1900 bp) encoded the same pattern as membrane δ (Cμ1-δ1-δ2a-δ3-δ4-δ2b-δ7) with the addition of the secretory tail. Sequencing of the smaller PCR product (900/1000 bp) revealed an alternative δ splicing pattern (Cμ1-δ1-δ2b-δ7).

To determine whether trout secretory δ is associated with a variable domain, PCR was performed using secretory δ-specific cDNA as the template in conjunction with a VH-framework 2-specific forward primer and either Cμ1 or C81 exon-specific reverse primers. The sequencing of the resultant products confirmed that a V region is associated with secretory δ (Fig. 6). To determine whether the variable domain was present in both IgD splice variants, PCR was performed using the VH-framework 2-specific forward primer and the D7sec-R reverse primer. The two major PCR products were cloned and sequenced, confirming that both IgD splice variants were associated with a variable domain (GenBank accession numbers JQ003979 and JQ034605; http://www.ncbi.nlm.nih.gov/genbank/). The predicted molecular masses of the two secretory δ splice variants, including an entire V region and secretory tail, are 88 and 62 kDa.

Tissue-specific expression of IgD mRNA in naive trout

Secretory IgD mRNA was found to be expressed at the highest levels in the spleen, followed by the blood and gills (Fig. 7B). The expression of secretory δ mRNA in the kidney was ~47-fold less than the levels measured in the spleen, and the secretory μ to δ ratio in the anterior portion of the kidney was 5500:1 (Fig. 7E).
mental Fig. 2). Together, these findings suggest that secretory IgD may be more common than previously anticipated, but occurring through a mechanism that is more similar to secretory IgT production (23) rather than a dedicated exon as seen in most other vertebrates.

The membrane form of IgD in trout was determined to have a molecular mass of 125 kDa. The second IgD protein species observed in PBL lysates was not surface associated, and due to its similarity in size to secreted IgD, we hypothesize that it is secretory IgD. Importantly, the serological analysis of 12 naive trout revealed two dominant secreted IgD isoforms, 165 and 100 kDa (Fig. 2). Nonreducing electrophoresis of affinity-purified secretory IgD from fish number 4 revealed two proteins with estimated molecular masses of 370 and 400 kDa. Reduction of the 370-kDa protein resulted in a 165-kDa $\kappa$ chain and 25-kDa $\lambda$ chain. These results strongly indicate that this particular IgD isoform is assembled as an H2L2 monomer ($2 \times 165 + 2 \times 25 = 380$ kDa). The smaller IgD variant also appeared to be assembled as an H2L2 monomer to produce a native $\kappa$ chain of 240 kDa ($2 \times 100 + 2 \times 25 = 250$ kDa). To the best of our knowledge, secreted IgD has always been described as a H2L2 monomer, although it demonstrates considerable diversity in H chain size (1, 24). Furthermore, deglycosylation of the two major serum IgD isoforms revealed that both these proteins were highly glycosylated, with $N$-linked sugars accounting for $\sim 30–40\%$ of their molecular mass. The predicted molecular masses of these two splicing variants, including a complete V region, would be 62 and 88 kDa, closely matching the 70- and 95-kDa deglycosylated protein species that we observed.

The $\delta$ splicing variants have also been documented in cartilaginous fish, including the nurse shark (Ginglymostoma cirratum), horn sharks (Heterodontus francisci), and the clean-nosed skate (Raja eglanteria) (8, 25, 26). The $\delta$ genes in these species contain six $C\delta$ exons, and alternative splicing results in both long and short forms of both secreted and membrane $\delta$. The studies on the leopard gecko also suggested splice variants (14), which may

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**FIGURE 5.** The 3' region of the secretory Ig $\delta$ (secIgD) mRNA. A, PCR products of the 3' RACE from four different fish amplified with the D7SecF-specific primer and the adapter reverse primer. B, Diagram showing the organization of the secIgD 3' region, by comparison with the IgHD locus (AY872256) and the membrane $\delta$ (AY870260). Dotted boxes represent the 3' UTRs. C, Sequence alignment of the two secIgD 3' regions with the corresponding region on the IgHD locus. The underlined bases show the annealing site of the IgDSecF primer. The asterisk denotes the skipped splice site that gives rise to the secretory tail (represented with a dashed bracket). The solid brackets represent the annotated exons in the genomic sequence.

**FIGURE 6.** Secreted $\delta$ transcript variability and $V_{H}$ association. To reveal the presence of an IgD $V_{H}$ region, the secretory IgD-specific cDNA was analyzed using a forward primer designed in the $V_{H}$ framework region ($V_{H}$FR2) of the membrane IgD sequence (AY870260) paired with primers designed in $C_{\mu}$ or $C_{\delta}$ domains. Alternative secretory IgD splicing transcripts were amplified from secretory IgD-specific cDNA, using primers targeting either the $C_{\mu}$ I or the $C_{\mu}$I/61 forward primers paired with a secIgD reverse primer (sec). The secreted IgD domain sequence observed for the long and short PCR products is shown.
result in variation in the protein product. The specific functional role(s) of the splice variants in the immune system has yet to be revealed. Recently, it was hypothesized that these variants may increase IgD diversity to compensate for the lack of class switch recombination in fish (24). Alternatively, in mammals it has been demonstrated that the carbohydrates found on IgD may facilitate specific receptor binding to T cells (27, 28) or granulocytic cells (17). Thus, the two splice variants characterized in this study, which contain different numbers of N-linked glycosylation sites, may facilitate different effector functions in trout.

To our knowledge, our analysis of Ab-secreting cells via ELISPOT demonstrated for the first time the presence of IgD-secreting plasma cells in immune tissues of teleost fish. Whereas an IgD secretory product has been observed in Western blots of catfish serum (4), the visualization of secretory IgD cells has not been possible. Our results suggest that IgD-secreting plasma cells in trout are common in the kidney and spleen, although they are rare in peripheral blood (Fig. 4). The kidney of rainbow trout contained ~90% of all the IgD plasma cells we recovered from the tissues sampled. Although the number of IgD plasma cells recovered from the gills was lower than in the peripheral immune tissues, the ratio of IgM to IgD plasma cells was almost equivalent. qRT-PCR data also demonstrated that secretory IgD transcription was high in the gills (Fig. 7). Taken together, these two findings may indicate a significant functional role for IgD at the gill surfaces. The tissue-specific distribution of IgD plasma cells in trout is analogous to the findings in mammals. It has been found that secretory IgD cells in the peripheral blood are rare (~3%), but make up a substantial population of the human respiratory tract mucosal B cells (17, 29). In contrast to the upper respiratory tract, there are few IgD plasma cells in the digestive system mucosa, with only trace amounts of IgD in digestive fluids (30–33). This indicates that IgD plasma cells are highly localized, and therefore may be related to the function of their secretory product.

One puzzling observation derived from this study was that despite secretory IgD transcription being low in the kidney, the percentage of IgD-secreting plasma cells found in this tissue was substantial (Fig. 4D). The difference between the qRT-PCR and the ELISPOT data may be explained by the use of different fish for each of these experiments. However, as the fish were derived from the same naive stock and the percentage of kidney IgD plasma cells regularly exceeded those found in the spleen, an alternative theory is possible. The prospect exists that secretory IgD transcription rates, on a per cell basis, may differ significantly between locations. Differences in Ab transcription rates have been shown to occur in different tissues in mammals (34, 35) and during different stages of B cell maturation and activation (36). Image analysis of our ELISPOTS revealed that the mean IgD spot intensity in the spleen was higher than the mean spot intensity observed in the kidney (Fig. 4A), suggesting that IgD plasma cells in the kidney secrete less Ab than those found in the spleen. This observation may also clarify why the high number of total IgD plasma cells observed (Fig. 4) did not translate into higher serum IgD concentrations. As the trout kidney harbors the majority of IgM and IgD plasma cells, the rate of Ab production in this tissue certainly influences serum Ab concentrations. Therefore, the high

![FIGURE 7. Normalized copy number analysis of secretory and membrane \( \mu \) and \( \delta \) in various tissues using real-time quantitative PCR. Data represent mean absolute values (±SEM) of \( n = 3 \) for all tissues. All values were normalized to ARP. A, sIgD (secretory \( \delta \)); B, mIgD (membrane \( \delta \)); C, sIgM (secretory \( \mu \)); D, mIgM (membrane \( \mu \)); E, secretory \( \mu \) to secretory \( \delta \) ratio. The \( \mu/\delta \) transcript ratios were calculated using the mean relative expression levels of the respective gene transcripts.](http://www.jimmunol.org/)

![FIGURE 8. Amino acid comparison of the trout secretory \( \delta \) final domain with other putative secretory \( \delta \) sequences from Atlantic salmon (from GU129139), Atlantic cod (from AF155201), grass carp (from GQ480796), and leopard gecko (from EU312156). Gray scale shading denotes the level of similarity.](http://www.jimmunol.org/)
concentration of serum IgM and the low concentration of serum IgD may be a direct result of the marked differences in secretory μ to secretory δ transcription rates that we observed in the kidney (Fig. 7E). We also cannot discount that secreted IgD may be bound by IgD-specific receptors (17) or may be more readily degraded in the serum, which would result in lower serum IgD concentrations than would be anticipated by plasma cell numbers alone.

An alternative theory is needed to explain why IgD plasma cells in the blood were rare, whereas secretory δ mRNA was high. We feel that there are three theories that could explain these findings. PBL are heavily skewed toward B cells, containing on average 55% membrane IgM/IgD+ cells (Fig. 3) and a small percentage of plasma cells (Fig. 4). Possibly, these B cells are producing small quantities of secretory IgD, which if released from the cell, must be below our ELISPOT detection limit. Alternatively, translational control of secretory δ mRNA or posttranslational regulatory events may result in delta not being translated or released from the cell, as happens with secretory IgM and membrane IgM in plasma cells (37, 38). We are inclined to reject the translational control hypothesis as Western blot analysis of our PBL lysates revealed an IgD isoform similar in size to secreted IgD. This would indicate that secretory IgD is being produced inside PBL, suggesting that IgD secretion in PBL is below our detection limit or under posttranslational regulation, which stops it from being released from the cell. Clearly, experiments examining secretory δ transcription parallel to quantitative secretory IgD protein expression will be vital to resolving the nature of the secretory IgD production and its tissue-specific secretion.

In humans and mice, IgM and IgD are expressed on the surface of naive mature B cells by alternative splicing of a long primary RNA transcript. They are double-positive B cells (IgM+/IgD+) and make up the majority of the peripheral B cells. Upon Ag binding, they will downregulate their IgD expression (39). Similar to these findings, we observed only IgM+/IgD+ B cells via flow cytometry, with a smaller population of IgDlow/IgM+ (Fig. 3). It is unknown whether the later cells represent activated, or partially activated, B cells, as have been described previously in peripheral blood of trout (40). In the fish we examined, there was no evidence for a membrane IgM+/membrane IgD+ lineage in trout. These findings are in contrast to those by Edholm et al. (18), who in a comprehensive study of channel catfish found at least three different B cell populations, as follows: IgD+, IgM+, and IgM+/IgD+. They found that IgD+ B cell expression varies among individuals, and that IgD functions as a typical BCR. Ig chains associate with CD79α and CD79b molecules, and all membrane IgD transcripts from sorted IgM+/IgD+ B cells contain viable VDJ rearrangements. The study concluded that catfish IgM+/IgD+ B cells most likely expand in response to certain pathogens. It is unknown whether these findings represent a fundamental difference between catfish and trout or whether study of larger numbers of trout subject to pathogenic pressure will reveal these unique B cell populations in the future.

Clearly, more research is required to examine the distribution and function of secretory IgD as well as the other Ab isotypes in trout (IgM and IgT). We first must resolve whether the inclusion of a V region allows IgD Ag-specific responses to develop in trout. The recent discovery that IgT B cells are recruited to the intestinal mucosa following challenge with an intestinal parasite (41) may indicate that IgT-specific responses are tissue specific. Similarly, we may find that IgD plasma cells are specialized to function in certain tissues, possibly the gills of fish, to provide a barrier to infection. Our discovery of secreted IgD in rainbow trout supports Chen et al.’s (17) hypothesis that secretory IgD has been pre-served throughout evolution as a system of surveillance and defense. As rainbow trout secretory IgD is produced from a novel splicing event, rather than from a dedicated secretory exon, our findings may also help others reveal that secretory IgD may be more widespread than previously imagined.

Acknowledgments

We thank Drs. M. Wilson, E. Bengten, and P. Zwollo for helpful review and discussion of this manuscript.

Disclosures

The authors have no financial conflicts of interest.

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