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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 $\delta$ 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 $\delta$-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 $\delta$ 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 $\delta$ transcript resulted in two $\delta$-H chains, which incorporated C$\mu$1 and variable domains. Secreted IgD was found in two heavily glycosylated isoforms, which are assembled as monomeric polypeptides associated with L chains. Secretory $\delta$ 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: 000–000.

Abbreviations used in this article: ARP, acidic ribosomal phosphoprotein; PBL, peripheral blood leukocyte; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative RT-PCR; SDS-CAGE, SDS-composite agarose gel electrophoresis; UTR, untranslated region.
Six-week-old BALB/c mice were immunized with 50 μg of keyhole limpet hemocyanin–peptide conjugate emulsified in complete Freunds adjuvant. A total of 2 standard 50% polyethylene glycol fusions were performed, and resultant hybridoma supernatants were screened against the fusion (week 12). A standard 50% polyethylene glycol fusion was given 4 d before the final i.v. injection (5 μg/ml) was given 20 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 membranes (Millipore) and blocked with 3% casein. Following blocking, 5 μg/ml anti-IgD antibody 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, followed by goat anti-mouse IR680 (0.1 μg/ml; LiCor) and SA-IR800 to detect surface-biotinylated membrane proteins.

Trout serum and affinity-purified trout IgD was analyzed via SDS-PAGE and SDS-composite agarose gel electrophoresis (SDS-CAGE). One microliter 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 membranes (Millipore) and blocked with 3% casein. Following blocking, 5 μg/ml anti-IgD antibody 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. Blots were visualized with the LiCor Odyssey detection system. Serum IgD concentration was determined via quantitative densitometry using a known amount of affinity-purified IgD as the standard on gels.

To determine the contribution of glycosylation to the molecular mass of serum IgD, 10 μg affinity-purified 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 Sephacryl 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 incubated for 1 h. The plates were washed, and bound Ab was detected by the addition of either biotinylated anti-IgD or biotinylated anti-IgM. Development was performed using SA-HRPO and ABTS.

**Trout leukocyte analysis**

Trout 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 Elvehjem 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 10^6 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 (1% 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-IgD Ig2 (2 μg/ml) or anti-IgM IgM (1 μg/mI) mAb and blocked. Serial dilutions of leukocytes starting at 5 × 10^6 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, blocked with biotinylated anti-IgD (5 μg/ml) or biotinylated anti-IgM (2 μg/ml). The membrane was then developed using streptavidin-HRP and aminoethylcarbazole. 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 RNaseasy 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 67/60sec through the poly-A tail was obtained by performing rapid amplification of 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 8 transcripts, poly-A-tailed cytoplasmic RNA from PBL was used as the template with Cμ1 (CM-F) and Cγ1/Cε1 (CmDM-F1) forward primers and a 87/60sec 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 Vх12**

To determine whether HC 8 contains a variable domain, gene-specific cDNA was made from poly-A-tailed cytoplasmic RNA using a secretory β-specific reverse primer (D7secR1; Supplemental Table 1). The secretory β-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 β-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 81 domain of HC 8 (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 RNaseasy 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 PBL cDNA 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 disassociate 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 2000 to 4300 μg/ml in these fish (∼5%). Back gating these cells revealed that both IgD+ populations contained, on average, 52% of the staining intensity. When IgM+ populations were gated, 20% of the staining intensity was observed (data not shown).

**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+IgMhigh population (R3; ∼52%), and another population of IgD+IgMhigh 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 IgD transcript using 3′ RACE. Based upon the presence of a putative secretory IgD expressed sequence tag for Atlantic salmon (EG934529), we used a trout IgD gene-specific forward primer that spanned the IgD 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 IgD transcripts that ignore the splice donor site (used for splicing the IgD exon to the TM1 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, 3′ untranslated region (UTR) of the secretory IgD transcript used the TM1/TM2 donor acceptor sites and continued through until it reached the polyadenylation site used for the membrane form of IgD.

FIGURE 2. Membrane and serum profile of trout IgD. A, Trout PBL lysate (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.
**FIGURE 4.** Analysis of Ab-secreting cell profiles in trout tissues. Lymphocytes were isolated from peripheral blood, spleen, gills, anterior kidney, and posterior kidney of trout (n = 4). ELISPOT assays (A) revealed the prevalence of IgM and IgD plasma cells in the kidney (50,000 cells/well), spleen (50,000 cells/well), and gills (500,000 cells/well). Normalized ELISPOT counts for IgM (C) and IgD (D) are plotted (gills not shown due to scale; IgD and IgM plasma cells in the gills average 20 and 24 per 500,000 cells, respectively). The ratio of IgD to IgM plasma cells for each tissue was determined (B).

The two size variants of the secretory δ 3′ UTR observed in trout are hypothesized to be a result of differences between the IGH-A and IGH-B loci.

**Secretory IgD size heterogeneity and association with Cψ1 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 Cψ1 or variable domains (1, 7). To address size heterogeneity of secreted IgD and the possible inclusion of the Cψ1 coding region, primers were designed to amplify from the Cψ1 exon to the δ secretory tail, as well as from the Cψ1/C81 splice junction to the δ secretory tail (Fig. 6; Supplemental Table 1). Sequencing of the two major PCR products revealed that Cψ1 was associated with trout secretory δ. The larger PCR product (1800/1900 bp) encoded the same pattern as membrane δ (Cψ1-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 (Cψ1-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 Cψ1 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). 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 (GQ48079), 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 Cψ1 and a variable domain. This product is produced via transcription through the splice site at the end of the δ7 domain (normally splicing to the transmembrane exon) and continuing into the intron until a stop codon is reached, resulting in an 11-aa secretory tail. This secretory δ arrangement differs from the dedicated secretory exon observed in channel catfish. Although the dedicated δ-secretory exon appears to be the dominant transcriptional arrangement in IgD-possessing species (2), the novel RNA splicing process discovered in trout may be widespread in bony fish and possibly other animals. This is supported by examining other fish species in which the IGH locus and corresponding cDNAs (ESTs) have been sequenced. In Atlantic salmon, directly 3′ of the δ7 exon is a virtually identical sequence to the trout secretory tail (GVCLSVHSLI for trout versus GVCLSVHSLIQ for Atlantic salmon), suggesting evolutionary pressure to maintain this sequence (Fig. 8). Analysis of the final δ exon of species including Atlantic cod (Gadus macrocephalus) and grass carp (Ctenopharyngodon idella) also suggests that there is a putative secretory tail in these species (Fig. 8). Direct evidence for this transcriptional arrangement being evolutionarily conserved comes from the recent publication revealing that the leopard gecko may also produce a secretory IgD via the same arrangement as observed in trout (14) (see Supple-
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 d-H chain and 25-kDa L chain. These results strongly indicate that this particular IgD isoform is assembled as an H2L2 monomer (2\times165 + 2\times25 = 380 kDa). The smaller IgD variant also appeared to be assembled as an H2L2 monomer to produce a native /C\underline{2}5 -240-kDa protein (2\times100 + 2\times25 = 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 ∼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 d splicing variants have also been documented in cartilaginous fish, including the nurse shark (Ginglyostoma cirratum), horn sharks (Heterodontus francisci), and the clearnose skate (Raja eglanteria) (8,25,26). The d genes in these species contain six C\underline{d} exons, and alternative splicing results in both long and short forms of both secreted and membrane d. The studies on the leopard gecko also suggested splice variants (14), which may

**FIGURE 5.** The 3’ region of the secretory Ig d (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 (AY872256S2) and the membrane d (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 d transcript variability and VH association. To reveal the presence of an IgD VH region, the secretory IgD-specific cDNA was analyzed using a forward primer designed in the VH framework region (VHFR2) of the membrane IgD sequence (AY870260) paired with primers designed in C\underline{p}1 or C\underline{d}1 domains. Alternative secretory IgD splicing transcripts were amplified from secretory IgD-specific cDNA, using primers targeting either the C\underline{p}1 or the C\underline{p}1/61 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 mRNA 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 μ and δ 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, slgD (secretory δ); B, mgD (membrane δ); C, slgM (secretory μ); D, mgM (membrane μ); E, secretory μ to secretory δ ratio. The μ/δ 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 δ final domain with other putative secretory δ 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/)
IgD secretion in PBL is below our detection limit or under post-IgD isoform similar in size to secreted IgD. This would indicate hypothesis as Western blot analysis of our PBL lysates revealed an cell, as happens with secretory IgM and membrane IgM in plasma events may result in delta not being translated or released from the naive mature B cells by alternative splicing of a long primary RNA tissue-specific secretion. parallel to quantitative secretory IgD protein expression will be control of secretory plasma cells (Fig. 4). Possibly, these B cells are producing small findings are in contrast to those by Edholm et al. (18), who in a comprehensive study of channel catfish found at least three V region allows IgD Ag-specific responses to develop in trout. We first must resolve whether the inclusion of individuals, and that IgD functions as a typical BCR. Ig chains and function of secretory IgD as well as the other Ab isotypes in B cell populations in the future.

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/IgDmed (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 CD79a 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 preserved 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.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Table 1. Primers design.

<table>
<thead>
<tr>
<th>Primers Type</th>
<th>Primer Sequence</th>
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<td>3' RACE primers</td>
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Rainbow trout IgD constant region

MTLGCIATGF TPASLTFKV WNDDEGGNSLTDF VQYPAVQTGG SYMGVSQLRV KRADWDSKKF
ECAVEHSAGS KKVVPKQLQ RVPIPNITLY PLWEELKVGVS KVGLCILGSP FYPDKLSVWEN
LLDDKTIVTS PVQRKLQSVE QLELDRSQT WQSEVTCKAT HNAAQGPTTG
TPVSNSTIC_SAPLSSTPSI DELETPRFRTV MTQETVTATC VVHSAAYAKW SLWLDGKDP
SRTPVQAQR LPPSSSPLIT LPSSWKTTLN TTICRAEHRC FNSTEKSNV KGPAVSSTTT
VLIRRLPEL LKGDASVLEC AITQLSSSDL YVTFQANGVD FFEKQYYVDVP ASKGPHTLCTR
LFSIPKSHWK TDNFTCKVN QGFSNLVSS SKVKLFGELS MELLLVFSEE SSGSTQKLM
ČSGWGFNPKI KWLSGFQGRS AADNEISMGEG DHVAALTSHI TTVQPEMWEG AVDICEVDDK
DLQKTVRKST SLCTAFFST PTYHLETPRF RTVMTQETVT ATCVVHSAYD AKVSWLLDK
DTPSRTVPVQ ASR TTQSVSS NLTPSQQWK TLNTITCRAE HRCFNSTEKT SNVK GSVTPS
APRATLLQGP SELVCLVLGF SPSDINITML LDVNTELWNNT NTSTYPARPG GKFGIRSHLS
LAHQDWTGAV VYTCRVTHTTT QTLALNISKP GVCLSIVSIL

Rainbow trout light chain 1 constant region

NSAPTLTVLP PSSEEELSSTT TATLTCLANK GFPSDWTRW KVDTGSKQGG TSSVLEKDG
LYSWSSTTLTL TGLEWTKAGE VTFCEAQNQT FVTKTRRAD C

Rainbow trout light chain 2 constant region

DSTLPPPPVL ILPPSSEDLEK SSKVTLVCLA SQMGAMYADV SWTAGGNPVT GGIATSGVPV
QADKTFTQSSL CLTVDTSEWNN QDK FSCKVT VGSKFSKDEI KKSECSTE

Rainbow trout light chain 3 constant region

SIRPTVSSLIP PSSEQLFGGS ATLACLGLSGY SPQGAMVSWE VDGEEVKDGV LTSTEEKKG
HYSRSTTLTL SKARWEGEV YTCRHANEDT SDSSAIRKSH CSV

Supplemental Figure 1. Affinity purified IgD and peptide analysis. Lane 1. Affinity purified IgD processed under non-reducing conditions on a 4-20% SDS-PAGE gel. The two prominent protein bands at 370kDa and 240 kDa were excised from the gel and processed under reducing conditions on a 4-20% SDS-PAGE gel (Lane 2 = 370kDa & Lane 3 = 240kDa). The (boxed) protein bands were excised from the gel and analyzed individually via LC-MS/MS. The peptide sequences obtained are highlighted on the amino acid sequence of rainbow trout IgD and the three rainbow trout antibody light chains. Peptide sequences from lane 2 are underlined, peptide sequences from lane 3 are bolded italicized.
Supplemental Figure 2. The amino acid residues of the final IgD constant domain (grey) of various fish, reptiles and amphibian species were aligned to the terminal amino acid of the final delta domain (underlined). In-frame translation was continued past the GT splice site until a stop codon was reached (*), revealing putative secretory tails. Accession numbers: *Salmo salar* (GU129139), *Gadus morhua* (AF155201), *Danio rerio* (BX510335), *Ctenopharyngodon idella* (GQ480796), *Ictalurus punctatus* (AF363448), *Eublepharis macularis* (EU312156), *Anolis carolinensis* (NW_003339038), *Xenopus tropicalis* (NW_003164254).