Universal Assay of Vitellogenin as a Biomarker for Environmental Estrogens

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Vitellogenin (VTG), the serum phospholipoglycoprotein precursor to egg yolk, is potentially an ideal biomarker for environmental estrogens. This study was undertaken to develop antibodies against conserved regions on the VTG molecule that could form the basis for establishing bioassays to detect estrogen exposure in any oviparous vertebrate. We developed monoclonal antibodies (mAbs) generated against purified rainbow trout (*Oncorhynchus mykiss*) VTG and selected for the property of specifically recognizing VTG purified from two phylogenetically distant vertebrates, trout and striped bass (*Morone saxatilis*). Results of enzyme-linked immunosorbent assay and Western blotting indicated that these mAbs specifically recognize purified VTG and VTG or other estrogen-inducible proteins in plasma or serum from representative species of four vertebrate classes (fish, amphibians, reptiles, and birds). All of the mAbs generated were IgM class. A polyclonal antiserum was raised against a synthetic consensus peptide representing the conserved N-terminal amino acid sequence of VTG. The results of Western blotting indicate that this antiserum specifically recognizes VTG in plasma or serum from teleost fish of diverse families. It was used to detect VTG in Western blots of serum from brown bullhead (*Ameiurus nebulosus*) with cancer (hepatocellular and cholangio-carcinoma) collected from a contaminated industrial site outside of their normal vitellogenic season. Our results indicate that it is feasible to generate antibodies capable of recognizing VTG without regard to species and that development of a universal VTG assay is an achievable goal. — Environ Health Perspect 103(Suppl 7):9–15 (1995)

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Introduction

Environmental estrogens, chemicals with bioactivity similar to endogenous estrogen, are known to affect development and sexual maturation of vertebrates and are implicated as cancer promoters (*1*). They include breakdown products of detergents, pesticides, plasticizers, and a variety of chlorinated compounds. Ideally, such chemicals would be evaluated for estrogenicity before their use or release into the environment. However, their notoriously weak structure–activity relationship requires that the identity and potency of environmental estrogens be defined by bioactivity. The current lack of effective bioassays is considered to be a proximal cause of the lack of regulatory action regarding these compounds (*2*). An effective bioassay needs to be developed to assess the estrogenicity of synthetic compounds or their derivatives and their potential effects on the reproductive biology of vertebrates.

Vitellogenin (VTG) is potentially an ideal biomarker for the estrogenicity of chemicals. It is a large serum phospholipoglycoprotein that serves as the major precursor to the egg-yolk proteins of oviparous vertebrates (*3*). VTG is synthesized and secreted by the liver in response to circulating estrogens in maturing females (*4,5*) and is normally undetectable in the plasma of immature animals and males. The presence of this estrogen-inducible protein in the serum of an animal can be taken as evidence of exposure to endogenous or exogenous estrogens or estrogen mimics. This potential of VTG as a biomarker has already been explored using several fish species, for which both *in vivo* and *in vitro* assays have been developed (*6–10*). However, immunological and structural properties of VTG can vary greatly even among closely related species (*11–14*), which limits broad scale application of these assays across vertebrate taxa.

Recently it has become apparent that VTG is an ancient protein belonging to a multigene family that includes a variety of lipoproteins, including various vertebrate and invertebrate VTGs as well as the human serum proteins, apolipoprotein

B-100 and von Willebrand factor (15–18). Coupled with prior evidence for conservation of some functional and immunological properties of the molecule (19–22), these findings suggest that VTG may possess conserved epitopes that could be exploited to develop antibodies and immunoassays of a more universal nature.

This study was undertaken to develop antibodies against conserved regions on the VTG molecule that could form the basis for establishing bioassays to detect estrogen exposure in any oviparous vertebrate. We attempted to develop both monoclonal antibodies generated against purified VTG and polyclonal antisera raised against the conserved N-terminal amino acid sequence of VTG (23). Using these two approaches we demonstrated that it is feasible to generate antibodies capable of recognizing VTG without regard to species of origin and that development of a universal VTG assay is an achievable goal.

Methods

Balb/C mice were immunized ip with either 100 µg of rainbow trout (*Oncorynchus mykiss*) VTG purified as described by Hara et al. (24) or 100 µg of striped bass (*Morone saxatilis*) VTG purified as described by Tao et al. (25) mixed 1:2 with Freund's complete adjuvant. Mice were boosted twice at 2- to 3-week intervals with 50 µg of antigen mixed 1:2 with Freund's incomplete adjuvant. Four days before fusion, they were again boosted with 100 µg of antigen in incomplete adjuvant. Monoclonal antibodies (mAbs) were then generated using standard methods (26). This entire procedure was replicated 3 times. In an attempt to induce antibody class shift and increase the affinity of the antibodies, the final set of immunizations was modified by increasing the time between boosts to a minimum of 3 weeks and incorporating a third boost prior to fusion.

Hybridoma colonies were screened for VTG cross-reactivity using an antibody capture enzyme-linked immunosorbent assay (ELISA) (27) against purified VTGs, blood plasma from immature or male striped bass and trout injected with 17β-estradiol (E$_2$) to induce vitellogenesis (E$_2$-plasma) (15), or plasma from normal male bass and trout as the reference preparations. The mAbs were harvested by collecting the tissue culture supernatant (TCSN), which was stored at 4°C in the presence of 0.01% NaN$_3$ for up to 3 months until use. TCSN (100 µl) was mixed 1:2 with 10% normal goat serum and added to the wells of a 96-well plate that had been previously coated with antigen. The second antibody was a goat antimouse IgM, µ-chain specific antibody (Sigma Chemical Co, St. Louis, MO) conjugated to horseradish peroxidase, and antibody binding was visualized with O-phenylenediamine/H$_2$O$_2$ in 0.1 M citrate-phosphate buffer, pH 5.0.

Hybridoma colonies whose TCSN demonstrated immunoreactivity in the ELISA to VTG and E$_2$-plasma from both bass and trout but did not react to normal male plasma were selected for cloning. Rainbow trout and striped bass were chosen as the species for initial screenings because they represent distinct vertebrate lines that have evolved independently over a considerable period of time (~200×10^6 years), methods for purifying their respective VTGs were well established (24,25), and they were available because they were subjects of other endocrine investigations in our laboratory. Positive colonies were subjected to limiting dilution to produce monoclonal cell lines secreting mAbs with desired characteristics. The mAbs from the monoclonal cultures were isotyped using commercial kits (Life Technologies, Gaithersberg, MD, or Sigma).

Western blots were used for a second series of mAb screens. Samples of purified VTG, E$_2$-plasma, or male plasma from trout and bass were normalized for protein concentration, separated by sodium dodecyl sulfate 4 to 15% polyacrylamide gradient gel electrophoresis (SDS-PAGE) on a PhastSystem electrophoresis apparatus (Pharmacia, Piscataway, NJ) and then electroblotted onto Immobilon-P PVDF membranes (Millipore, Marlborough, MA) using the PhastSystem immunoblot apparatus (25). Membranes were blocked in phosphate-buffered saline (PBS) (0.01 M Na$_x$H$_x$PO$_4$, 0.15 M NaCl, pH 7.3) with 3% bovine serum albumin overnight, incubated with TCSN diluted in PBS with 0.5% Tween-20 (wash buffer; range 1:3–1:10, v/v) for 2 hr, and then probed with secondary antibody (1:4000 in wash buffer) and avidin-biotin complex in wash buffer (ABC Elite Kit, Vector Laboratories, Burlingame, CA) for 30 min each. Antibody binding was visualized with diaminobenzidene (DAB Kit, Vector Laboratories).

Preliminary ELISA and Western blot screens of the ability of the mAbs to differentially bind to vitellogenic plasma and nonvitellogenic plasma from a variety of other species were also done. Additional species tested by ELISA included several fishes (white perch, *Morone americana*; white croaker, *Genyonemus lineatus*; brown trout, *Salmo trutta*; and pollock, *Theragra chalcogramma*) as well as representative reptiles (tuatara, *Sphenodon punctatus*; and rattlesnake, *Crotalus molossus*) and a bird (chicken, *Gallus domesticus*). Additional species screened by Western blot included the bullfrog (*Rana catesbeiana*), the black rat snake (*Elaphe obsoleta*), and the chicken.

To initiate generation of polyclonal antisera against known conserved regions on the VTG molecule, we microsequenced amino acids in the N-terminal regions of a number of VTGs purified from several phylogenetically diverse teleost fish (23). We obtained sequences for several teleost fish including the striped bass, brown bullhead (*Ameiurus nebulosus*), pinfish (*Lagodon rhomboides*), yellow perch (*Perca flavescens*), and medaka (*Oryzias latipes*), as well as sequences deduced from the cDNA of mummichog (*Fundulus heteroclitus*) and white sturgeon (*Acipenser transmontanus*). The striped bass was used as the template for comparison because it is the most highly evolved fish in our study. To determine an optimal peptide segment for antibody targeting, we analyzed a consensus N-terminal sequence with a protein structure program (28), which provides an antigenicity index at each position of the peptide. Using this information we designed a consensus peptide fifteen amino acids long.

The consensus peptide was assembled on a Model 431A automated peptide synthesizer (Applied Biosystems, Foster City, CA) from the C-terminus towards the N-terminus with the α-carboxyl group of the amino acid attached to the solid support, using Rink resin and 9-fluorenylmethoxy-carbonyl (FMOC) protected amino acids. An additional Cys residue was added at the N-terminus for coupling the peptide to activated carrier proteins (29). The peptide was cleaved from the resin with trifluoroacetic acid (TFA) (30), purified by reverse-phase high performance liquid chromatography (rpHPLC) on a Whatman partisil 10 ODS-2 column fitted to an HPLC system (Waters, Milford, MA) and equilibrated with 20% solvent A (0.1% TFA in CH$_3$CN) and 80% solvent B (0.1% TFA in dH$_2$O) followed by a 20-min linear gradient to 35% solvent A at a flow rate of 1 ml/min. Compositional and quantitative analysis of the peptide was conducted on a Pico Tag work station (Waters) after a 22-hr hydrolysis and phenyl isothiocynate (PITC) derivitization (31).

Aliquots of purified peptide (2 mg) were conjugated (1:2, w/w) to maleimide-activated carrier proteins, keyhole limpet

hemocyanin (KLH; mw 450–13,000 kDa), or ovalbumin (OVA; mw 45 kDa) using an Imject Activated Immunogen Conjugation kit (Pierce Chemical Co., Rockford, IL). The KLH peptide conjugate was used to immunize two female New Zealand white rabbits sc with 200 μg protein in 0.5 ml sterile distilled water mixed 1:2 with Freund's complete adjuvant. Subsequent immunizations ($n = 8$), done 2 weeks later and then at approximately 3- to 4-week intervals, used 100 μg protein and Freund's incomplete adjuvant. Rabbits were bled periodically through the marginal ear vein and serum samples were collected, diluted 1:100 in ELISA buffer, and evaluated for antibody titers using the ELISA with the OVA-peptide conjugate as the reference protein. When the titer was judged to be sufficient, the rabbits were exsanguinated and their serum was isolated and frozen at –80°C until use. Purified VTGs and serum samples obtained from striped bass males or E$_2$-treated females were separated by SDS-PAGE (32) and Western blots (33) were used to test the immunoreactivity of the antisera to the VTGs and other serum proteins. Western blots used the antiserum diluted 1:3000 and were visualized using goat anti-rabbit IgG conjugated to alkaline phosphatase as the secondary antibody.
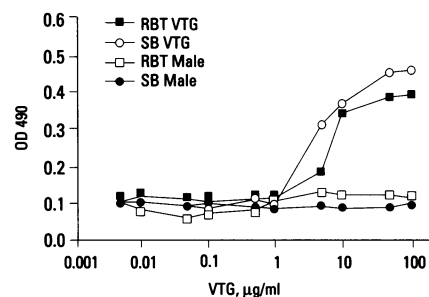
Results

Monoclonal Antibodies

Three sets of fusions resulted in the generation of seven mAb cultures. The first set of fusions gave rise to two hybridoma lines from which one monoclonal culture was developed. The mAb produced by this culture, designated R1, was isotyped as an IgM class antibody with kappa light chains. R1 demonstrated strong cross reactivity to VTG from striped bass and rainbow trout in the ELISA and was used to develop the Western blot procedure. Initial ELISA screens that reacted R1 with plasma from vitellogenic females or males of several vertebrate species from diverse taxa showed specific reactivity to vitellogenic plasma for all species tested. R1 was lost due to an equipment malfunction. The second set of fusions resulted in one mAb designated T2, but T2 lost specific immunoactivity to vitellogenic plasma samples or VTGs shortly after its development, and it was never isotyped or used for screening. The third set of fusions resulted in one positive hybridoma colony from which five monoclonal cultures were derived. The mAbs produced by these

cultures were designated 2C11, 2D8, 3E8, 5G7, and 8G7. All five were isotyped as IgMs. Because 2D8 gave the most consistent results in initial screens, it was chosen for use in subsequent analyses. All of the hybridoma cultures that were cloned were derived from mice immunized with trout VTG. None of the hybridoma colonies derived from mice immunized with bass VTG gave significant levels of cross-reactivity to both trout and bass VTG, the criterion used to select colonies for cloning.

The R1 mAb showed an excellent dose–response profile of binding to VTG purified from trout and bass and showed very low levels of reactivity to male plasma from both species (Figure 1). When the ELISA was used to screen plasma from naturally vitellogenic female or male white perch, white croaker, brown trout, pollock, snake, tuatara, and chicken, the reactivity of R1 to female plasma was a minimum of 2 to 3 times higher than to male plasma (Table 1). 2D8 immunoactivity in the ELISA to plasma from vitellogenic and nonvitellogenic striped bass was similar to that of R1 but exhibited substantially higher background levels (Figure 2). Similar results were obtained for trout plasma and a variety of purified control proteins including OVA, bovine serum albumin, and KLH, which gave the same profile as male trout or bass plasma over the same range of total protein concentration (data not shown). The higher background for 2D8 was not accompanied by any detectable nonspecific binding to plasma proteins from nonvitellogenic bass or trout in Western blots. The 2D8 mAb was also screened by ELISA against plasma from vitellogenic and nonvitellogenic black rat snakes with similar results (Figure 3).

SDS-PAGE of purified trout or bass VTG or plasma samples from fish injected

Table 1. Reactivity in the antibody capture ELISA of the R1 monoclonal antibody (OD 490) to plasma samples from vitellogenic females and males of several vertebrate species. Plasma samples were diluted 1:100.

Plasma source	Female	Male
Brown trout, Salmo trutta	1.613	0.163
Pollock, Theragra chalcogramma	0.768	0.213
White croaker, Genyonemus lineatus	0.745	0.219
White perch, Morone americana	1.123	0.143
Tuatara, Sphenodon punctatus	0.473	0.160
Snake, Crotalus molossus	0.492	0.107
Chicken, Gallus domesticus	0.464	0.186
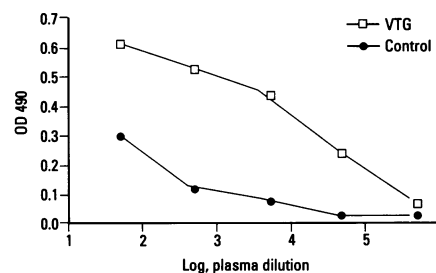
Figure 2. Binding of the 2D8 monoclonal antibody to serial dilutions of plasma from vitellogenic (VTG) and nonvitellogenic (control) immature striped bass in the antibody capture ELISA. Vitellogenesis was induced in the fish by injection with E$_2$. Note that even with high background levels for nonvitellogenic fish there is still a distinct increase in binding to plasma from E$_2$-treated fish.
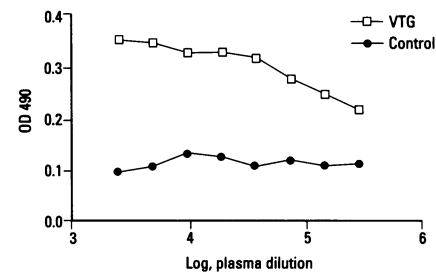

Figure 1. Binding of the R1 monoclonal antibody (OD 490) to purified striped bass (SB) and rainbow trout (RBT) VTG in the antibody capture ELISA. Dilutions of male plasma (RBT male and SB male) are serial from 1:10 to 1:1280 (scale not shown).


Figure 3. Binding of the 2D8 monoclonal antibody to serial dilutions of plasma from vitellogenic (VTG) and nonvitellogenic (control) black rat snakes in the antibody capture ELISA. Vitellogenesis was induced in the snakes by injection with E$_2$.

with E_2 revealed distinct high molecular weight bands (M_r 170,000–200,000) not present in plasma from male trout or bass (Figure 4A). We identified these bands as the primary VTG subunit and its degradation products (24,25,34). Western blots of the SDS-PAGE gels done using the 2D8 mAb revealed immunoreactivity to the purified VTGs and the high molecular weight bands for VTG in plasma, but no binding to plasma proteins from male trout or bass was detected (Figure 4B). This pattern in SDS-PAGE and Western blots is identical to that obtained using homologous antisera raised in rabbits against purified trout or bass VTG [(24,25,34); A Hara and CV Sullivan, unpublished data] with the exception that the homologous antisera reacted only very weakly with the heterologous VTG, producing a barely visible band in Western blots (35). SDS-PAGE and Western blots using the 2D8 mAb and plasma from E_2-treated and control bullfrogs, black rat snakes, and chickens also showed immunoreactivity to high molecular weight, E_2-inducible proteins, presumably VTG, but not to plasma proteins in control samples (data not shown).
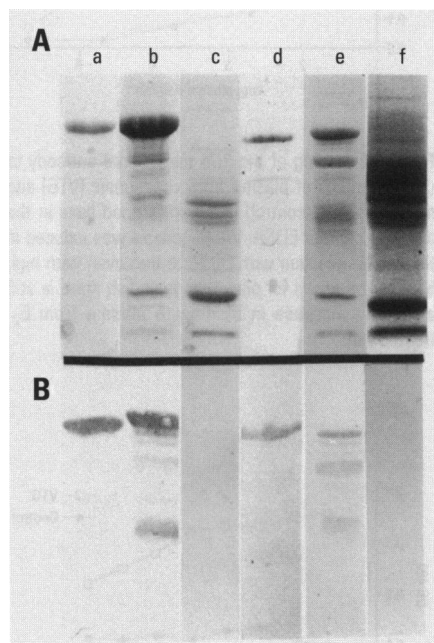
Figure 4. (A) SDS-PAGE of rainbow trout and striped bass VTG and blood plasma proteins. *Lane a,* purified striped bass VTG; *lane b,* plasma from striped bass injected with E_2; *lane c,* male striped bass plasma; *lane d,* purified rainbow trout VTG; *lane e,* plasma from rainbow trout injected with E_2; *lane f,* male rainbow trout plasma. The gels were stained with Coomassie Brilliant Blue. (B) Western blots of a parallel set of the same samples shown in A done using the 2D8 monoclonal antibody.

Negative controls for the Western blots involved using fresh culture media in place of TCSN from the monoclonal cell cultures producing the 2D8 mAb or substituting TCSN from a monoclonal culture producing an IgM class mAb directed against a recombinant hepatitis B core antigen. These substitutions eliminated immunoreactivity on the Western blots.

Polyclonal Antisera

The results of microsequencing of the fish VTGs verified the strong degree of conservation at the N-terminal region of the protein (Table 2). The region of highest homology among the fishes extends between amino acid residues 7 and 20 of the striped bass VTG molecule, showing 100% identity in sequence with mummichog VTG, 87% with pinfish VTG, 93% with brown bullhead VTG, and 60% with white sturgeon VTG. This degree of homology made the N-terminus a primary candidate for generating an antipeptide antiserum for detecting VTG in a wide variety of species (23).

The antiserum generated against the consensus N-terminal VTG sequence conjugated to KLH reacted preferentially with the VTG band ($M_r \approx 170,000$) in Western blots of plasma from immature striped bass injected with E_2; this band is not visible in SDS-PAGE or Western blots of plasma from control male bass (Figure 5). The fainter immunoreactive band ($M_r \approx 36,000$) visible on the Western blot only in the lane for male plasma appears to have a lower affinity to the antiserum, fading more rapidly as the antiserum is diluted out. We are in the process of purifying and identifying this protein.

We previously reported on selective induction of vitellogenesis in tumor-bearing (hepatocellular- and cholangio-carcinomas) male and female brown bullhead collected from the Black River near Lorraine, Ohio (23). The collection site receives effluent from a steel plant and
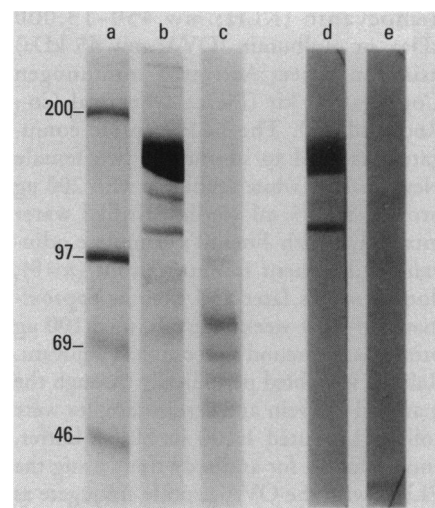
Figure 5. SDS-PAGE and corresponding Western blots of plasma from striped bass done using the anti-VTG peptide antiserum. *Lane a,* mw markers; *lane b,* plasma from an immature striped bass injected with E_2; *lane c,* Plasma from a male striped bass; *lane d,* Western blot of b; *lane e,* Western blot of c. Numbers with horizontal bars indicate the mass of the mw markers.

historically received effluent rich in polynuclear aromatic hydrocarbons (PAHs) from an associated coking facility. VTG is apparent in serum samples from the tumor-bearing adult males and females after SDS-PAGE, but not in serum from adult females collected from a pristine control creek at the same time and outside of the normal vitellogenic season for this species (Figure 6). Corresponding Western blots done using the anti-VTG peptide antiserum revealed strong immunoreactivity to the VTG band ($M_r \sim 170,000$) in the lane for plasma from a tumor-bearing female and also to a lower M_r band that is presumably a breakdown product of VTG (Figure 7). This immunostaining was not evident in the lane for plasma from a control male.

We are currently examining the extent of cross-reactivity of the anti-VTG peptide

Table 2. N-terminal amino acid sequences for several fish vitellogenins.

Source		Sequence	Homology, %
Striped bass:		HNVNFAPEFAAGKTYVYKYEAL	(100)
Mummichog:	MKAVVLALTLASYA	-GONFAPEFAAGKTYVYKYEAL	(100)
Pinfish:		YQVNLAPEFAAGRTHVYKYEAF	(87)
Bullhead:		HQINLVPEFAAGKTFVYKYE	(93)
Yellow perch:		QVNFAPEFA	
Medaka:		QLVFAPEFA	
Sturgeon:		QQTKYEPSFSGSKTYQYKYEG	(60)

Underlining indicates regions of similarity with the striped bass. Numbers in parentheses are the percent similarity with the striped bass sequence between positions 7 and 20. The sequence MKAVVLALTLASYA for mummichog is the putative leader sequence (23).
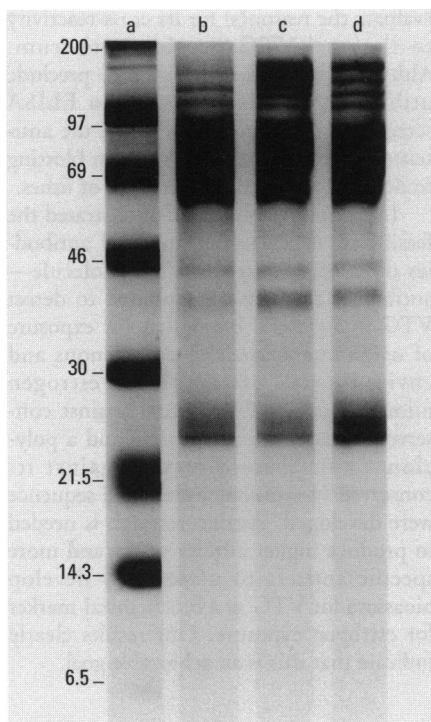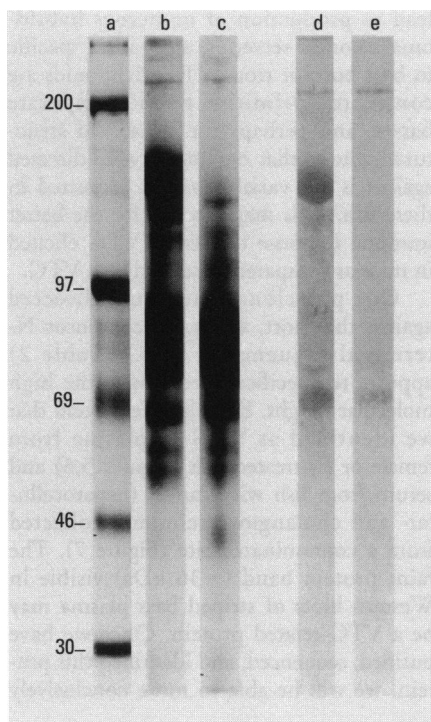
Figure 6. SDS-PAGE of mw marker proteins or serum from brown bullhead. *Lane a*, mw markers; *lane b*, serum from a female collected from a pristine control creek; *lane c*, serum from a female with hepatocellular- and cholangio-carcinoma collected from the polluted Black River; *lane d*, serum from a male with cholangio-carcinoma collected from the Black River. Numbers with horizontal bars indicate the mass of the mw markers.

Figure 7. SDS-PAGE (*lanes a–c*) and corresponding Western blots (*lanes d* and *e*) of mw marker proteins or serum from brown bullhead using the anti-VTG peptide antiserum. *Lane a*, mw markers; *lane b*, serum from a female with hepatocellular- and cholangio-carcinoma collected from the polluted Black River; *lane c*, serum from a male collected from a pristine control creek; *lane d*, Western blot of *b*; *lane e*, Western blot of *c*. Numbers with horizontal bars indicate the mass of the mw markers.

antiserum with plasma from other fish species. Initial studies indicate that the antiserum may have wide cross-reactivity with VTGs from several teleost fish including medaka ($M_r \simeq$ 193,000 and 130,000) and yellow perch ($M_r \simeq$ 120,000 and 104,000). The fish VTGs tested so far all share a high degree of similarity at their N-termini (Table 2); however, a preliminary test with rainbow trout serum gave negative results. Further work is in progress with this species and others that fill the phylogenetic gap between rainbow trout and striped bass.

Discussion

There is a demonstrable need for a biochemical marker that can be used to identify estrogenic compounds before their release into the environment (*36*). The results of this study generally corroborate prior reports on VTG as a biomarker for exposure to estrogens (*6–9*), and they demonstrate the feasibility of two approaches to developing antibodies with

the property of recognizing vertebrate VTG without regard to species of origin. We developed both monoclonal antibodies and a polyclonal antiserum possessing this property. Such antibodies have the potential to form the basis of a universal VTG immunoassay able to detect estrogen exposure in any oviparous vertebrate.

Our ELISA results for the mAbs (R1 and 2D8) show much higher absorbance values for purified VTG or plasma from E_2-treated fish or snakes than for plasma from untreated immature animals or males (Figures 1–3), but they do not provide conclusive evidence that the mAbs specifically react to VTG in the plasma. However, the results of Western blotting clearly demonstrate that the 2D8 mAb binds preferentially to VTG in plasma of rainbow trout and striped bass (Figure 4) as well as to high MW E_2-induced proteins, presumably VTG, in plasma of bullfrogs, black rat snakes, and chickens (*35*). Combined with the ELISA results, these observations indicate that the 2D8 mAb

specifically reacts to purified VTG and possibly other estrogen-inducible proteins in representatives of at least four vertebrate classes. Because the monoclonal cell line producing 2D8 was initially selected on the basis of its ability to recognize VTG from both trout and bass, species from lineages that have evolved independently for approximately 200 million years, we consider it likely that 2D8 is targeted at a conserved epitope on the VTG molecule. Whether this epitope is completely unique to VTG remains to be rigorously verified.

All of the mAbs that we developed were found to be IgM class antibodies. Such antibodies, while having strong epitope specificity, have a tendency toward high levels of nonspecific binding in immunoassays because of their large mass. This is likely to be a factor in the relatively high background seen in our ELISA (Figure 2). We plan to cleave the pentameric IgM into its monomeric Fab fragments in an attempt to reduce the high background. In our final series of immunizations, we modified our protocol to increase the probability of generating high affinity–high titer IgG mAbs but we were unable to accomplish this goal. The last five monoclonal cultures, derived from the final fusion, all produced IgM class mAbs.

IgM induction is part of the initial immune response following exposure to a novel antigen and, upon subsequent exposure to the same antigen, there is typically a class shift towards synthesis of IgGs (*26*). We have developed the concept that our inability to induce antibody class shift and generate an IgG mAb may be a function of the degree or nature of antigenicity of conserved epitopes on the VTG molecule. Because there are extensive gene sequences that are well conserved between VTG and some mammalian serum proteins (*16,17*), some of which are also estrogen inducible (*37*), it is possible that there is no antibody class shift due to lack of strong antigenicity of the conserved VTG epitopes. They may be too close to "self" to generate an IgG response. There are natural autoantibodies present in most healthy individuals; in mice, the vast majority of these are IgM class (*38*).

It should also be noted that VTG is a large and complex phospholipoglycoprotein, one that in our hands has proved to be highly antigenic (*24,25*). There is no reason to presume that dominant epitopes on the protein are conserved. Polyclonal antisera raised in rabbits against trout or bass VTG show very high levels of immunoreactivity to their homologous

VTG but are also weakly reactive to heterologous VTG from the other species (*35*), lending credence to the concept that conserved epitopes contribute little to the overall immune response to VTG. During development of the mouse immune response, many VTG epitopes may be antigenic enough to induce IgG synthesis, whereas the conserved epitopes we select for may not exhibit this degree of antigenicity. We are currently exploring this possibility using denatured and delipidated or deglycosylated VTG as the antigen for generating universal mAbs. Alternatively, we will attempt to suppress the immune response of mice to species-specific immunodominant VTG epitopes by treatment with cyclophosphamide (*39*).

It is interesting to note that although both trout and bass VTGs were used as antigens, we were only able to obtain mAbs with the desired specific cross-reactivity from mice immunized with trout VTG. The bass VTG was however able to generate an immune response in mice, which

lead to production of numerous hybridoma colonies secreting antibodies specific to bass but not trout VTG. Salmonids are considered primitive to the temperate basses, and perhaps the conserved structural epitope that our mAbs were directed against is less variable or less protected in these fish. This may account for the better immune response that trout VTG elicited in mice as compared to striped bass VTG.

Our polyclonal antiserum directed against the short, synthetic, consensus N-terminal sequence of VTG (Table 2) appears to specifically recognize the high molecular weight, E_2-inducible protein that we identified as VTG in plasma from female or E_2-treated fish (Figures 5,6) and serum from fish with cancer (hepatocellular- and cholangio-carcinoma) collected from a contaminated site (Figure 7). The faint protein band (~36 kDa) visible in Western blots of striped bass plasma may be a VTG-related protein. Once we have purified, sequenced, and identified this protein, we will be able to more conclusively

evaluate the reason(s) for its cross-reactivity to the anti-VTG peptide antiserum. Although the cross-reactivity may preclude utilization of this antiserum in ELISA screens for VTG in some species, the antiserum is clearly useful for Western blotting to detect VTG in diverse lineages of fishes.

In summary, we have demonstrated the feasibility of generating universal antibodies directed against the VTG molecule—antibodies that can be exploited to detect VTG as a general biomarker for exposure of oviparous animals to endogenous and environmental estrogens and estrogen mimics. Both mAbs directed against conserved epitopes on the protein and a polyclonal antiserum directed against its conserved N-terminal amino acid sequence were developed. Further research is needed to produce higher affinity mAbs and more specific antisera to more easily develop bioassays for VTG as a biochemical marker for estrogen exposure. Our results clearly indicate that this is an achievable goal.

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