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Original Contributions

Molecular and Immunologic Characterization of Gynogenetic Channel Catfish (*Ictalurus punctatus*)

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Abstract: Second-generation gynogenetic channel catfish were characterized by molecular and immunologic assays to determine if they were isogenic at major histocompatibility complex loci. Southern blot analyses, using channel catfish MHC class II *B* and class I *A* gene probes, revealed identical banding patterns among second-generation gynogenetic fish. In contrast, banding patterns from outbred fish differed not only from gynogenetic animals, but also among themselves. Nucleotide sequence analysis of the MHC class II β_1 domain, which encompasses the peptide binding region, from four randomly selected gynogenetic fish showed a single DNA sequence. In contrast, analysis of the same region from three outbred fish showed sequences that differed not only among themselves, but also from those of gynogenetic animals. In cytotoxic assays, peripheral blood leukocytes from outbred fish lysed both gynogenetic and allogeneic targets, whereas those from gynogenetic fish lysed only allogeneic targets. Taken together, these results suggest that this group of second-generation gynogenetic channel catfish is isogenic at MHC loci and may provide an excellent system with which to study cell-mediated immunity in teleosts.

Key words: gynogenetic fish, *Ictalurus punctatus,* MHC, cytotoxic cells, isogenic lines

Introduction

Among teleosts, the immune system of the channel catfish is one of the best characterized. Channel catfish exhibit humoral and innate immune responses involving cellular and molecular components analogous to those found in higher vertebrates (Clem et al., 1996; Miller et al., 1998). For example, channel catfish produce vigorous in vitro antibody responses against both T-independent (TI) and T-dependent (TD) antigens (Miller et al., 1985). As in mammalian systems, in vitro peripheral blood leukocyte (PBL) proliferation and antibody generation in response to TD antigens require autologous macrophages, surface-immunoglobulin-negative lymphocytes, and B cells, suggesting the involvement of both major histocompatibility complex and T cell receptor (TCR) molecules in antigen

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processing and presentation (Vallejo et al., 1991a, 1991b). The recent isolation of genes encoding MHC class I (Antao et al., 1999), class II β (Godwin et al., 1997), β_2-microglobulin (Criscitiello et al., 1998), and TCR (Wilson et al., 1998) supports the view that channel catfish possess T helper cells and cytotoxic T lymphocytes (CTLs).

Studies of cell-mediated cytotoxicity have been facilitated by the generation and characterization of cloned channel catfish B cell (Miller et al., 1994b) and T cell lines (Clem et al., 1996; Wilson et al., 1998). Using lymphoid cell lines as targets, channel catfish PBLs were shown to contain a population of NK-like effector cells (Yoshida et al., 1995; Hogan et al., 1996; Stuge et al., 1997) that differed from previously described nonspecific cytotoxic cells (Evans and Jaso-Friedman, 1992; Stuge et al., 1995). Freshly isolated PBLs from unimmunized fish exhibited high levels of cytolytic activity toward both allogeneic (Yoshida et al., 1995) and virus-infected autologous cells (Hogan et al., 1996). However, PBLs did not lyse autologous target cells, suggesting that channel catfish cytotoxic effectors are able to discriminate between "self" and "non-self." Despite compelling evidence for NK-like cytotoxic effectors and allospecific cytotoxic T cells (Stuge, 1998), xenoantigen-specific, MHC-restricted CTLs have yet to be identified in channel catfish.

Unequivocal identification of xenoantigen-specific, MHC-restricted CTLs requires MHC-matched effectors and target cells. Syngeneic mouse systems have been developed by generating inbred mouse strains and producing cell lines from those mice (Berke, 1993), whereas autologous human target cells have been generated by Epstein-Barr-virus–mediated transformation of B cells from individual donors (Seeley and Golub, 1978; Khanna et al., 1992). Although both approaches are straightforward, the first is not practical in channel catfish because of their long generation time (approx. 3–5 years), and the latter is not possible owing to the absence of any known transforming virus. Although leukocyte cell lines can be established from individual channel catfish (an autologous system), there are constraints to extensive use of this system. For example, death of the autologous donor fish would necessitate the generation of new cell lines from living animals, a task that is feasible, but impractical because of the time required. In the current study, we report the production and characterization of a family of second-generation gynogenetic channel catfish that are matched (i.e., isogenic) at MHC loci. The availability of isogenic fish and cell lines should prove useful in demonstrating authentic CTLs in catfish.

Materials and Methods

Animals

Gynogenetic fish were produced by fertilizing eggs from four Marion strain channel catfish (*Ictalurus punctatus*) (Dunham and Smitherman, 1984) with UV-irradiated sperm from blue catfish (*I. furcatus*). Retention of the second meiotic polar body (i.e., diploidization) was achieved by pressure treatment (8000 PSI for 3 minutes) at 5 minutes after fertilization (Liu et al., 1992; Goudie et al., 1995). First-generation gynogenetic channel catfish were pooled, and at sexual maturity 25 to 30 females were randomly selected as egg donors for the production of second-generation gynogenetic fish. Nine females produced surviving offspring, and at approximately 1 year of age, fish within each of the nine families were marked by barbel clip, sampled for microsatellite polymorphisms, and transferred to a common pond. At approximately 2 years of age, fish were subjected to molecular and immunologic characterization. Outbred channel catfish were obtained from ConAgra (Isola, Miss.) as well as from research and feral sources.

Cell Lines

Long-term lymphoid cell lines derived from gynogenetic fish were developed by mitogen stimulation of PBLs (Lin et al., 1992; Miller et al., 1994a). The cloned channel catfish B cell line (1G8) was generated by simulating surface-immunoglobulin-positive cells obtained from an outbred fish with lipopolysaccharide (LPS) (Miller et al., 1994b). Cloned T cell lines G14A and G14B were derived from a gynogenetic fish following LPS stimulation. All cell lines were grown in AL medium (Luft et al., 1991) containing 5% pooled channel catfish serum (AL5) (Miller et al., 1994a).

Microsatellite Analyses

A barbel sample of 5 to 10 mm was collected from individual fish and stored at −20°C. Four to eight samples from each family (only two fish were present in the family designated H12) were alkaline-denatured and used as template in a microsatellite polymerase chain reaction (PCR) assay (Waldbieser and Bosworth, 1997). Seven fluorescent primer sets were chosen to amplify microsatellite loci that displayed high levels of heterozygosity in outbred populations of channel catfish (Waldbieser and Bosworth, 1997). The 15-μl PCR reactions contained 1.5 μl of denatured barbel DNA

Table 1. Primers and Templates Used to Generate Southern Blot Probes

Probe	Sense primer	Antisense primer	Fragment size (bp)
MHC class Iα*	5′-GTTCCTCCTACAGCGTCA-3′	5′-AGGCAGCACCATCTCCTT-3′	273
MHC class IIβ†	5′-AGATTTAACAAAGAC-3′	5′-GTAGTTCATGGGTTT-3′	283

*The amplified region corresponds to the putative α3 domain of MHC class Iα (*Icpu-UBA**02). (Antao et al., 1999).
†The indicated primer set flanks the conserved β_2 domain of *Icpu-DAB**01. (Godwin et al., 1997).

template, 1 mM $MgCl_2$, 67 μM of each dNTP, 0.4 μM of each primer, and 0.5 U of *Taq* polymerase (Boehringer-Mannheim, Indianapolis, Ind.). After denaturation for 3 minutes at 95°C, microsatellite DNA was amplified by two initial cycles (95°C for 1 minute/55°C for 30 seconds) and 23 additional cycles (95°C for 30 seconds/55°C for 30 seconds/72°C for 4 minutes) in a PTC-100 thermal cycler (MJ Research, Watertown, Mass.). The resulting PCR products (1–2 μl) were analyzed by electrophoresis on 6% DNA sequencing gels (ALFexpress, Pharmacia Biotech, Piscataway, N.J.). Allele sizes were determined using Allele Links software (Pharmacia).

Southern Blot Analyses

Genomic DNA was isolated from 50 μl of pelleted erythrocytes from outbred and gynogenetic channel catfish (S.A. Miller et al., 1988). DNA samples (6 μg) were digested to completion with *Eco*RI, *Pst*I, *Dra*I, or *Pvu*II, electrophoresed on 1.2% agarose gels, and transferred to nylon membranes (Hybond N+, Amersham). Probes, generated by PCR from plasmids containing the appropriate genes (Table 1), were random-prime labeled with [^{32}P]dCTP using a commercial kit (Megaprime DNA labeling kit, Amersham). Blots were hybridized with the indicated radiolabeled probe overnight (Maniatis et al., 1989), washed at high stringency (0.2× SSC, 65°C), and exposed to x-ray film (Biomax MR, Kodak) for 6 to 24 hours at −80°C.

Sequencing of the MHC Class IIβ Peptide Binding Region

Messenger or total RNA was extracted from PBLs using either a commercial kit (Fastrack 2.0, Invitrogen) or the method of Chomcyznski and Sacchi (1987). After reverse transcription of approximately 1 μg of RNA with an oligo(dT) primer, the peptide binding region (PBR) (nucleotides 1–276) and a portion of the β_2 domain were amplified by PCR using sense (5′-ATGTCCAAGCTGCTGAAGAT-3′) and antisense (5′-AGTGTGGCCGGGTGAGTG-3′) primers. The resulting 392-bp PCR products were cloned into pCR2.1 (Original TA Cloning kit, Invitrogen), and both strands were sequenced (Sanger et al., 1977) using the above primers or vector-specific primers (5′-TAATACGACTCAC-TATAGGG-3′ and 5′-CAGGAAACAGCTATGAC-3′). DNA sequences were edited, assembled, and aligned using the Editseq, Seqman, and Megalign programs within DNASTAR (Madison, Wis.).

Cytotoxic Assays

Channel catfish were anesthetized using MS-222 (tricaine methane sulfonate, Crescent Research Chemicals) and bled from the caudal sinus into heparinized Vacutainer tubes (Becton-Dickinson). Diluted blood was layered over 51% Percoll and centrifuged at 400 *g* for 25 minutes. PBLs were isolated from the interface as described by Miller et al. (1994a) and Miller and McKinney (1994), and used as effectors as described below.

Cytotoxic assays were performed as previously described (Hogan et al., 1996). Briefly, target cells (2×10^6) were labeled with 100 μCi $Na_2[^{51}Cr]O_4$ (100–500 mCi/mg Cr, Amersham) for 2 hours at 28°C. Following labeling, target cells were washed three times in catfish RPMI-1640 (Miller et al., 1994b) and resuspended in AL5. PBL effectors were added in triplicate to wells of a 96-well round-bottom plate at effector:target (E:T) ratios ranging from 40:1 to 5:1. Labeled target cells (5×10^4 per well) were added and the plate was centrifuged at 200 *g* for 1 minute. Following a 4-hour incubation at 28°C, 100 μl of supernatant was harvested and assayed for ^{51}Cr release by gamma counting (Stuge et al., 1995; Yoshida et al., 1995). Percentage of specific lysis was calculated using the following equation:

$$\% \text{ Specific lysis} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100$$

Reproducibility among triplicate samples was high as indicated by SD < 7%. Spontaneous release values were obtained by incubation of labeled target cells in the presence of AL5 alone. Maximum release values reflect the addition of 100 μl of 4% Triton X-100.

Results

Microsatellite Analyses

To provide an MHC-matched system for identifying antigen-specific, MHC-restricted CTLs and examining other aspects of immune restriction in the channel catfish, second-generation gynogenetic fish were generated using standard procedures (Liu et al., 1992; Goudie et al., 1995). Because meiotic recombination (i.e., crossing over) occurs at readily detectable frequencies, membership within a family of second-generation gynogenetic fish is no guarantee of homozygosity at a particular locus (Estoup et al., 1993; Goudie et al., 1995). Therefore, to determine the heterozygosity of second-generation gynogens, microsatellite analyses were performed. Seven microsatellite loci, each of which demonstrated a minimum of 12 alleles following examination of 44 (locus IpCG-0014) to 818 (loci IpCG-0001 and IpCG-0008) outbred fish, were selected for analysis of heterozygosity (Waldbieser and Bosworth, 1997).

Excluding the two fish in family H12, the remaining eight families possessed the same one or two alleles at each locus (Table 2). Gynogens possessed alleles that were rare in outbred populations, such as the 254-bp allele of IpCG-0001 and the 189-bp allele of IpCG-0019, which were seen in only 0.1% and 0.5% of outbred fish, respectively. The restricted repertoire of microsatellite loci present in second-generation gynogenetic fish indicates that eight of nine gynogenetic families were highly related and clearly distinguishes them from outbred fish. Furthermore, the heterogeneity seen with six of the seven microsatellite probes was most likely due to meiotic recombination and reflects the distance of these markers from the centromere.

Since the intent in developing second-generation gynogenetic channel catfish was to develop an MHC-matched system for examining immune restriction, studies were undertaken to determine whether or not second-generation gynogens were homozygous at MHC loci. If MHC loci are located in telomeric positions, the high frequency of crossing over suggested by Table 2 indicates that it may not be possible to produce homozygous gynogenetic progeny by methods involving retention of the second polar body (Allendorf et al., 1988). However, if channel catfish MHC genes are located near the centromere as seen in some fish species (Johnson et al., 1996; Bingulac-Popovic et al., 1997), it should be feasible to generate gynogenetic fish, the majority of which should be homozygous at MHC loci. To determine if second-generation gynogens were isogenic at MHC loci, three complementary assays (Southern blotting using probes specific for channel catfish MHC class I*A* and class II*B;* nucleotide sequence analysis of the MHC class IIβ PBR; and cytotoxic assays using targets and effectors from outbred and gynogenetic fish) were employed.

Southern Blot Analyses

Genomic DNA was isolated from both outbred and gynogenetic fish and subjected to Southern blot analysis using probes specific for MHC class I*A* and class II*B*. Blots hybridized with MHC class I*A* and class II*B* probes showed multiple bands and marked restriction fragment length polymorphisms (RFLP). As shown in a representative experiment, genomic DNA from five gynogenetic fish displayed identical RFLP patterns with the class I*A* probe (Figure 1). However, genomic DNA from three outbred fish showed MHC class I*A* banding patterns that differed not only among themselves, but also from the pattern seen with gynogenetic animals. A clearer picture of this phenomenon is seen in Figure 2 where a probe for MHC class II*B* was used to analyze genomic DNA from five gynogenetic and one outbred fish. As seen with the class I probe, Southern blot analysis showed identical RFLPs with each gynogenetic animal, whereas a representative outbred fish showed a pattern markedly different from that of gynogenetic fish. Overall, RFLP analysis of 20 gynogenetic fish showed identical class II*B* profiles. In contrast, RFLP analysis of DNA from 10 outbred fish showed profiles that differed not only among themselves, but also from those of gynogenetic animals. This latter finding is consistent with results of an earlier study in which unique RFLPs were observed among five outbred fish (Godwin et al., 1997). Thus, the presence of identical RFLP profiles among all gynogenetic fish examined suggests that they are isogenic at the MHC loci tested.

Sequence Analysis of the MHC Class IIβ Peptide Binding Region

To strengthen the argument that these gynogenetic fish are isogenic at MHC loci, the MHC class IIβ_1 domain was

Table 2. Microsatellite Locus Genotypes of Gynogenetic Families

	Microsatellite loci						
Family	IpCG0001	IpCG0004	IpCG0008	IpCG0011	IpCG0012	IpCG0014	IpCG0019
F_5	215/254 (6)	132/132 (2)	134/134 (2)	104/107 (7)	185/185 (1)	195/195 (4)	200/200 (8)
	254/254 (2)	132/144 (5)	134/158 (6)	107/107 (1)	185/189 (3)		
		144/144 (1)					
F_6	215/215 (1)	144/144 (8)	134/134 (2)	104/107 (7)	185/185 (4)	195/195 (4)	189/189 (8)
	215/254 (6)		134/158 (6)	107/107 (1)			
	254/254 (1)						
F_7	254/254 (8)	132/132 (2)	134/134 (2)	104/104 (1)	185/185 (4)	195/195 (4)	200/200 (8)
		132/144 (4)	134/158 (5)	104/107 (7)			
		144/144 (2)	158/158 (1)				
H_7	215/254 (8)	144/144 (8)	134/134 (8)	107/107 (8)	185/185 (1)	195/195 (4)	189/189 (1)
					185/189 (2)		189/200 (6)
					189/189 (1)		200/200 (1)
H_8	215/254 (8)	132/132 (1)	134/134 (7)	104/104 (2)	185/185 (2)	195/195 (4)	200/200 (8)
		132/144 (5)		104/107 (5)	189/189 (2)		
		144/144 (2)		107/107 (1)			
H_9	215/215 (8)	132/132 (8)	134/158 (8)	104/107 (7)	185/185 (1)	195/195 (4)	189/189 (2)
				107/107 (1)	185/189 (3)		189/200 (4)
							200/200 (2)
H_{10}	215/254 (7)	132/132 (2)	134/158 (7)	107/107 (8)	185/189 (2)	195/195 (4)	189/200 (8)
	254/254 (1)	132/144 (5)			189/189 (2)		
		144/144 (1)					
H_{11}	215/215 (1)	132/144 (7)	134/158 (6)	104/104 (2)	189/189 (4)	195/195 (4)	189/189 (8)
	215/254 (4)	144/144 (1)	158/158 (1)	104/107 (6)			
	254/254 (3)						
H_{12}	221/254 (2)	132/132 (1)	146/166 (2)	107/107 (1)	221/221 (1)	171/171 (1)	183/189 (1)
		132/144 (1)			221/225 (1)	195/195 (1)	

Except for family H_{12}, for which only two fish were present, four to eight fish from each of nine second-generation gynogenetic families were examined for microsatellite polymorphisms by PCR analysis. The microsatellite alleles found in each family are indicated by their size (in base pairs) and whether they are homozygous or heterozygous. For example, within family F_6 at the *IpCG-0001* locus three genotypes were detected: 215/215 (homozygous), 215/254 (heterozygous), and 254/254 (homozygous). Within each family the number of offspring displaying a particular genotype is indicated within parentheses. For outbred fish, additional allelic data (e.g., the number of fish tested, the frequency distribution of alleles) can found by accessing the USDA ARS Jamie Whitten Delta States Research Center Web site (http://nola.srrc.usda.gov:80/usdadsrc/home.htm).

sequenced. The β_1 domain, which encompasses the PBR, was selected for analysis because this region contains most of the variability found within the class IIβ molecule (Janeway and Travers, 1997). Using PBL-derived complementary DNA clones from outbred and gynogenetic fish, the β_1 domains were amplified by PCR, and the resulting fragments were cloned into pCR2.1. Three randomly selected clones were sequenced from each of three outbred fish (fish numbers 24, 41, and 47) along with 12 randomly selected clones from four gynogenetic fish. As shown in Figure 3, clones isolated from the outbred fish yielded four unique nucleotide sequences (clones 24, 41, 47A, and 47C). Two of the four unique PBR sequences were obtained from fish 47 (clones 47A and 47C), suggesting heterozygosity at the class II*B* locus. Clones 24 and 47C differed by a single nucleotide at position 179. It is not clear whether this difference represents a true polymorphism or is the result of misincorporation during cDNA synthesis or PCR. In contrast, DNA sequences obtained from 11 of the 12 gynogenetic PBR clones were identical. The nucleotide sequence of the remaining clone was identical to the consensus gynogenetic sequence with the exception of a 32-bp insert

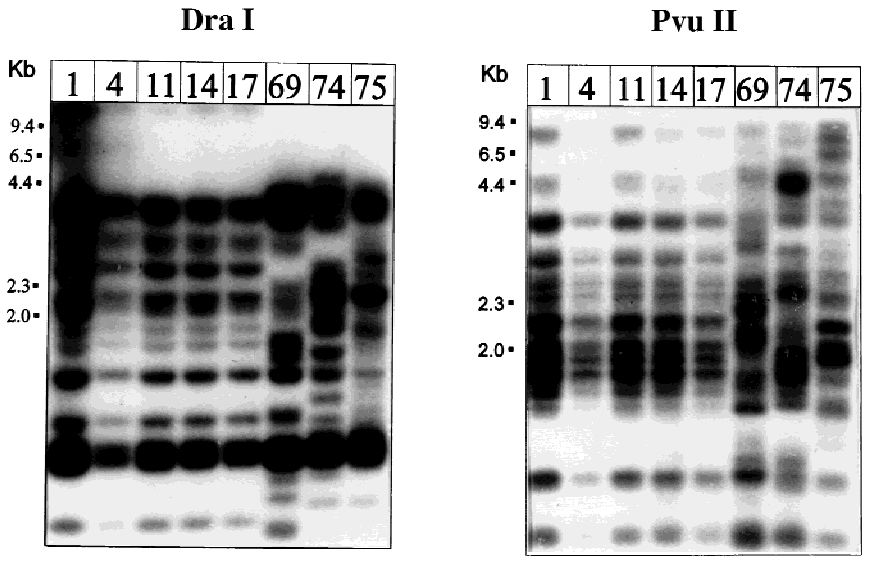

Figure 1. Southern blot analysis of genomic DNA from second-generation gynogenetic catfish: MHC class Iα probe. Genomic DNA from outbred and gynogenetic channel catfish was digested with *Dra*I and *Pvu*II and hybridized with a ^{32}P-labeled probe specific for the α3 domain of the channel catfish MHC class Iα gene. Shown are representative results from five gynogenetic fish (1, 4, 11, 14, and 17) and three outbred fish (69, 74, and 75).

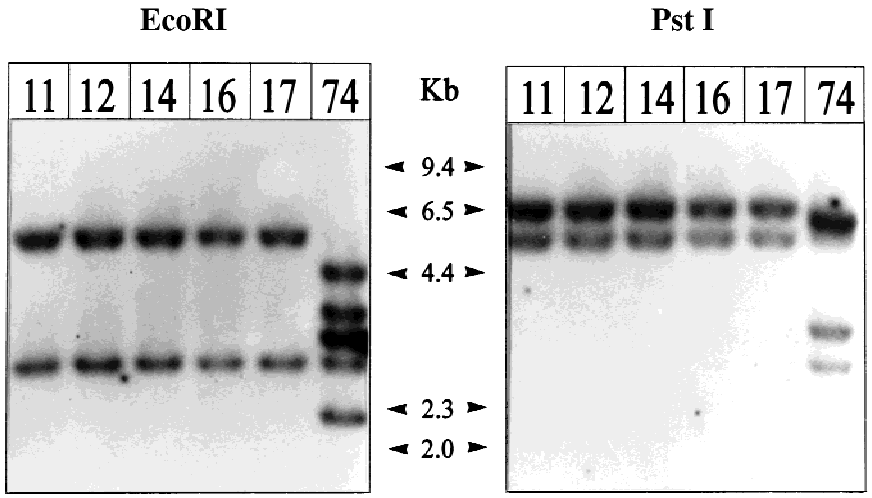

Figure 2. Southern blot analysis of genomic DNA from second-generation gynogenetic catfish: MHC class IIβ probe. Genomic DNA was hybridized with a ^{32}P-labeled probe specific for the β_2 domain of the channel catfish MHC class IIβ gene. Shown are representative results from five gynogenetic fish (11, 12, 14, 16, and 17) and a single outbred fish (74).

(5′-TGGAAAAAGGTTTTATTGTTATTTTCTCACAG-3′) at position 59 (Figure 3), which may represent an unspliced intron. Collectively, these data suggest that the gynogenetic fish tested are homozygous at the MHC class IIβ locus and provide further evidence that these fish are MHC-matched.

Cytotoxic Assays

Previous studies showed that PBLs from nonimmunized outbred fish readily lysed allogeneic target cells. However, autologous target cells were spared, indicating that channel catfish PBL effectors were able to distinguish between "self" and "nonself" (Hogan et al., 1996). To confirm that the gynogenetic fish used in this study were functionally isogenic, the ability of PBLs isolated from gynogenetic fish to kill both allogeneic and gynogenetic target cells was assessed. In a representative ^{51}Cr-release assay, PBL effectors from two separate gynogenetic fish efficiently lysed 1G8 allogeneic cells (Figures 4B and 4C). In contrast, lysis of a long-term lymphoid cell line derived from gynogenetic fish 14 was minimal. Gynogenetic targets were spared not only in an autologous system—i.e., between gynogenetic fish 14 and its cognate cell line G14A (Figure 4C), but also in an heterologous system in which PBL effectors and targets were derived from a different gynogenetic fish (Figure 4B). Furthermore, PBLs from 16 randomly selected gynogenetic fish were found to spare a second cloned T cell line (G14B) also derived from fish 14; i.e., following normalization of

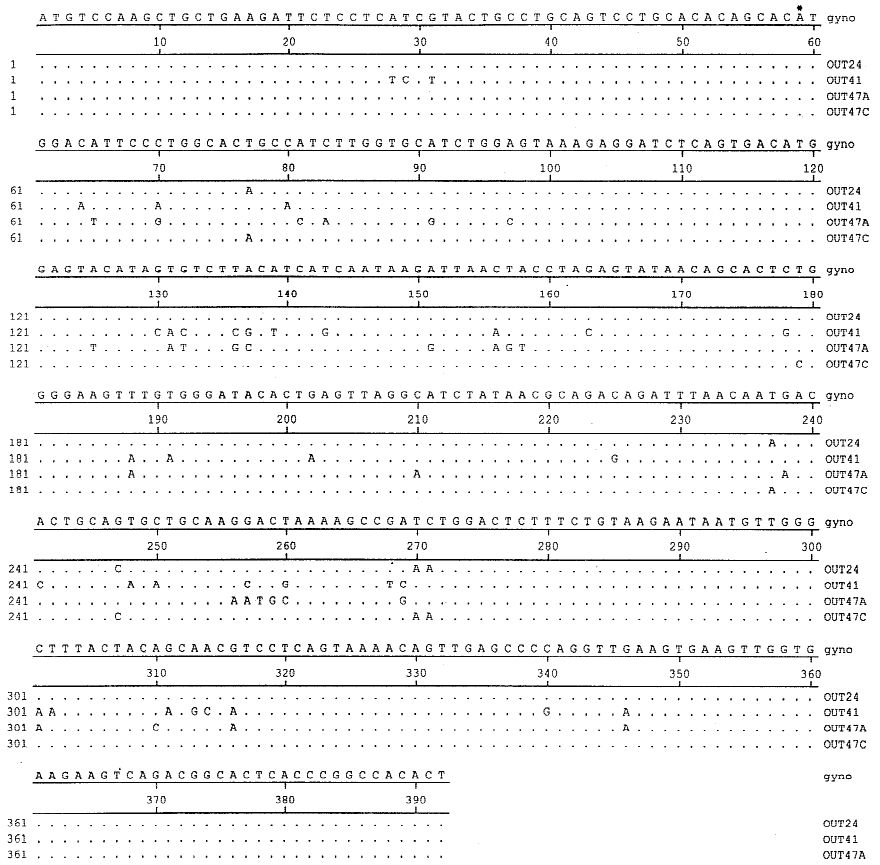

Figure 3. Nucleotide sequence analysis of MHC class IIβ peptide binding regions from gynogenetic and outbred channel catfish. A 392-bp fragment was amplified from PBL cDNA by PCR using specific oligonucleotide primers flanking the peptide binding region of the MHC class IIβ gene. Outbred class IIβ sequences (out24, out41, out47A, and out47C) were aligned by the CLUSTAL method (Megalign, DNASTAR, Madison, Wis.) and compared with the gynogenetic consensus sequence. Identity to the consensus sequence is indicated by a dot (.). The location of an intron found in a single gynogenetic clone is indicated by the asterisk (*).

1G8 lysis to 100%, lysis of G14B was 32% ± 18.4% of 1G8 levels. Because this level of ^{51}Cr release was similar to that seen in some autologous combinations, these results strengthen the suggestion that gynogenetic target cells are isogeneic at MHC loci. To ensure that the cell lines derived from gynogenetic fish were susceptible to lysis, PBLs isolated from an outbred fish were also used as effectors. PBLs from an outbred fish displayed strong cytolytic activity toward both cell lines (Figure 4A). These results indicate that gynogenetic fish possess peripheral-blood-derived cytotoxic cells, which are most likely equivalent to the NK-like cells reported in earlier studies (Yoshida et al., 1995). More importantly, these data provide evidence that this population of second-generation gynogenetic channel catfish is functionally isogenic at MHC loci (i.e., MHC-matched) because PBLs derived from any one gynogenetic fish spare (i.e., recognize as "self") target cells generated from different gynogenetic animals.

Discussion

The second-generation gynogenetic channel catfish used in this study appear to be MHC-matched as determined by Southern blotting, sequence analyses, and cytotoxic assays. In contrast, PCR analysis detected heterogeneity at six of seven microsatellite loci tested. Although heterogeneity among second-generation gynogens has been attributed to somatic mutation (Buth et al., 1995) or treatment-induced embryonic damage (Bongers et al., 1997), the variation observed in this study at six of seven microsatellite loci is most likely due to crossing over (Guyomard 1984; Allendorf et al., 1986; Estoup et al., 1993; Goudie et al., 1995). This suggestion is consistent with an earlier allozyme analysis of gynogenetic channel catfish that found gene-centromere recombination frequencies ranging from 0.36 to 1.00 (Liu et al., 1992). Thus, since meiotic crossing over occurs at frequencies proportional to the distances between the genes in question and the centromere, it is likely that the microsatellite markers used in the present study are located near the ends of chromosomes, whereas the MHC genes are most likely located nearer the centromere. Although the positions of channel catfish MHC genes have not been determined, zebrafish (*Danio rerio*) MHC class I and class II genes have been mapped to the centromeres of different chromosomes (Johnson et al., 1996; Bingulac-Popovic et al., 1997). Thus, positional effects could explain the heterozygosity seen in

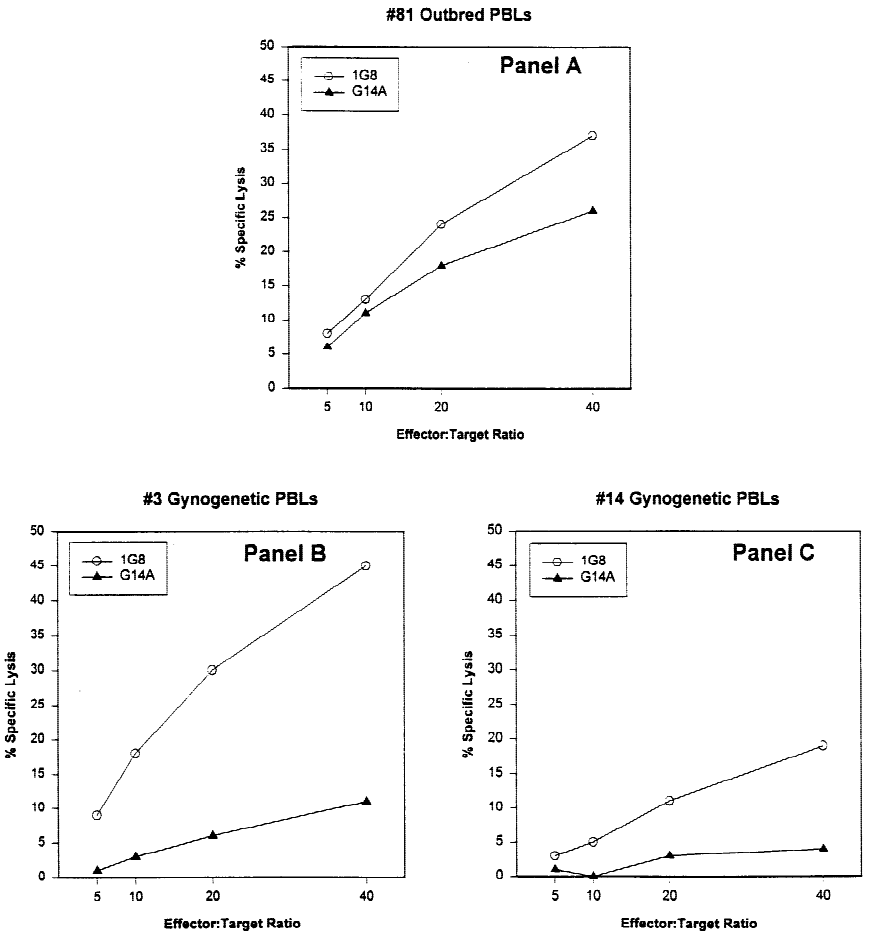

Figure 4. Gynogenetic PBL effectors spare cell lines derived from gynogenetic fish but efficiently lyse allogeneic targets. PBL effectors, isolated from outbred (81) and gynogenetic (3 and 14) channel catfish, were assayed for their ability to lyse ^{51}Cr-labeled allogeneic (1G8) or gynogenetic (G14A) targets. Effector:target ratios are shown on the *x* axis; percentage of specific lysis is found on the *y* axis. The results are representative of multiple experiments performed with 16 different fish.

channel catfish at microsatellite loci as well as the apparent homozygosity of MHC class I and II genes. If channel catfish MHC loci are located near the centromere, meiotic recombination involving these loci would be rare and most second-generation gynogenetic offspring from a heterozygous founding mother would be homozygous. Moreover, the absence of RFLPs at the class II*B* locus among 20 gynogenetic fish tested (Figure 2 and data not shown) strengthens the view that these gynogenetic fish are homozygous at MHC loci and that MHC genes are located near the centromere.

DNA sequence analysis of the variable MHC class IIβ_1 domain (which includes the peptide binding region) supports the suggestion that the gynogenetic fish used in this study are isogenic at the MHC class II*B* locus. However, sequence analyses of larger numbers of gynogenetic fish would be required to conclusively rule out the presence of a minority population of gynogens possessing different MHC class II*B* alleles. Although these data suggest that gynogenetic fish are isogenic at class II*B* loci, sequences obtained from outbred fish confirm the polymorphic nature of the channel catfish class II*B* gene. The data presented here (Figure 3), coupled with the seminal study of Godwin et al. (1997), identify seven unique channel catfish MHC class II*B* nucleotide sequences. These seven sequences were derived from four outbred fish (three fish used in this study and the one used to generate the cloned B cell library used by Godwin and coworkers) and four gynogenetic fish. In keeping with convention (Klein et al., 1990), the partial sequences reported here could be designated as *Mhc Icpu–DAB**03 through *Mhc Icpu–DAB**07 alleles. It is interesting to note that one of the cDNA sequences contained an unspliced intron within the β_1 domain. Although genomic sequences for channel catfish class II*B* are not available, the insert occurred near the position where an intron is found in the genomic DNA of other teleosts (i.e., *Aulonocara hansbaenschi* [Ono et al., 1993] and *Xiphophorus maculatus* [McConnell et al., 1998]). Perhaps this channel catfish cDNA represents a message that has been "caught" in the process of splicing. The proposed intron is 32 nucleotides long, and possesses conserved features at the 3′ acceptor junction, i.e., $(Py)_7$NCAG-3′). However, there appears to be

little conservation at the 5′ donor junction (c.f. consensus: 5′-GTAAGT vs. 5′-GaAAaa).

The cytotoxic assays depicted in Figure 4 provide functional validation for the molecular interpretations cited above. Although the molecular studies demonstrate that this group of second-generation gynogenetic fish is homozygous at specific class I and class II loci, cytotoxic assays provide strong evidence that cell lines prepared from gynogenetic fish are not recognized as "foreign" by PBL effectors prepared from the same or different gynogenetic fish. These results indicate that gynogens are functionally isogeneic at multiple MHC loci. In mammalian systems, MHC class I molecules play an important role in the susceptibility of target cells to NK-mediated lysis (Malnati et al., 1995; Kaufman et al., 1992). Resistance to killing is mediated by the interaction of killer inhibitory receptors (KIR) on NK cells and "self" MHC molecules on the surface of target cells (Pende et al., 1996). Whereas recognition of "self" MHC results in sparing, a lack of "self" MHC recognition results in NK-mediated lysis. The presence of viral or tumor peptides in association with "self" class I molecules, the presence of foreign MHC proteins, or the loss of cell surface class I molecules prevents the interaction of KIR and "self" MHC and leads to target cell lysis. Thus, the sparing of gynogenetic targets derived from one fish by effectors isolated from different gynogenetic fish suggests that this group of gynogens is isogenic at multiple MHC loci. Furthermore, this result strengthens the argument that MHC class I molecules are present on the surface of G14B cells. Moreover, β_2-microglobulin has been detected on the surface of lymphoid cells by flow cytometry (data not shown), a result consistent with the presence of membrane-associated MHC class I molecules (Antao et al., 1999).

The results described above demonstrate that the second-generation gynogenetic channel catfish characterized in this study are isogenic (i.e., matched) at MHC loci. Consequently, these fish should prove to be an excellent system for identifying antigen-specific, MHC-restricted CTL responses in catfish. In addition, this system will aid in the elucidation of NK-receptor molecules and facilitate the study of MHC organization in the channel catfish.

Acknowledgments

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