Development and Analysis of Various Clonal Alloantigen-Dependent Cytotoxic Cell Lines from Channel Catfish\(^1,2\)

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To determine the phenotypes of cytotoxic cells in channel catfish, clonal alloantigen-dependent leukocyte lines were established from mixed leukocyte cultures. Each clone was analyzed for expression of TCR \(\alpha\) and \(\beta\) genes by RT-PCR and for target cell specificity by \(^{51}\text{Cr}\)-release assay. Based on the above criteria, the following five different cell types were identified among the 19 clones analyzed: 1) TCR \(\alpha\beta^+\) allospecific cytotoxic cells, 2) TCR \(\alpha\beta^+\) non-specific cytotoxic cells, 3) allospecific TCR \(\alpha\beta^+\) non-cytotoxic cells, 4) TCR \(\alpha\beta^-\) non-specific cytotoxic cells, and 5) TCR \(\alpha\beta^-\) allospecific cytotoxic cells. The demonstration of cloned, TCR \(\alpha\beta^+\), allospecific cytotoxic effectors provides the strongest evidence to date for the existence of cytotoxic T cells in fish. *The Journal of Immunology*, 2000, 164: 2971–2977.

Although teleost fish possess subpopulations of lymphoid cells that mediate immune responses analogous to those seen in “higher” animals (reviewed in Ref. 1), relatively little is known about cell-mediated immunity in any fish species. For example, Ag-specific CTL homologues have yet to be unequivocally identified in any teleost. Similarly, the existence of bona fide NK cells in teleosts remains debatable. Major factors contributing to this dearth of information in teleosts have been the lack of physiologically relevant in vitro model systems and the paucity of molecular and cellular markers.

Previous work showed that several fish species possess nonspecific cytotoxic cells (NCC)\(^4\) that lyse xenogeneic targets (reviewed in Ref. 2). However, these effectors, suggested to be homologues of the NK cells of “higher” animals, have not been well characterized at the molecular level. In the best characterized of these systems, Evans and his coworkers (2) identified a 34-kDa cell-surface receptor protein, termed NCCR1, on channel catfish NCC that is thought to bind target cells. In addition, a previously generated mAb, 5C6, was shown to bind NCCR1 and to provide a facile way to identify NCC. However, mAb 5C6, which reacts with head kidney-derived catfish NCC, fails to react with catfish cytotoxic effectors derived from peripheral blood leukocytes (PBL), suggesting that NCC and PBL-derived cytotoxic effectors represent distinct populations (3). In our laboratory, several recent developments within the channel catfish model have greatly facilitated progress in characterizing PBL-derived cytotoxic cells. First, the development of clonal long-term autonomous (i.e., able to proliferate continuously in culture without the need for restimulation or exogenous growth factors) lines of B cells, T cells, and macrophages (4–7) makes available well-defined homogeneous populations of allotargets, which express both MHC class I and II genes (8, 9). Second, the finding of greatly enhanced cytotoxicity after one-way mixed leukocyte culture of catfish PBL with \(\alpha\)-irradiated allotargets provides a useful in vitro system for generating large numbers of teleost cytotoxic cells (10). Finally, as reported in this paper, it is now possible to clone mixed leukocyte culture (MLC)-derived effectors and determine their phenotype by monitoring their ability to express the TCR \(\alpha\) and \(\beta\) genes and to recognize and lyse specific allogeneic targets. As detailed below, the findings in the current study demonstrate that catfish possess at least four different types of cytotoxic cells that can be quantitatively expanded in vitro by MLC. In addition, a fifth population of noncytotoxic, allospecific TCR \(\alpha\beta^-\) cells that may correspond to mammalian Th cells was also isolated.

Materials and Methods

**Experimental animals and lymphoid cell isolation**

Channel catfish (*Ictalurus punctatus*; 1–2 kg) were obtained and maintained in individual tanks as described previously (11). Blood was drawn from the caudal vein of anesthetized (tricane methanesulfonate) fish into heparinized vacutainers, and PBL were isolated by centrifugation over Lymphoprep (Accurate Chemicals, Westbury, NY) as described previously (6).

**Cell lines and mAbs**

1G8 and 3B11 cells are cloned autonomous B cells generated from two different catfish by mitogen stimulation (5). 28S.1 (12) and 75C cells represent cloned and uncloned autonomous T cells, respectively; both express cloned mixed leukocyte response (MLR) effectors and determine their phenotype by monitoring their ability to express the TCR \(\alpha\) and \(\beta\) genes and to recognize and lyse specific allogeneic targets. As detailed below, the findings in the current study demonstrate that catfish possess at least four different types of cytotoxic cells that can be quantitatively expanded in vitro by MLC. In addition, a fifth population of noncytotoxic, allospecific TCR \(\alpha\beta^-\) cells that may correspond to mammalian Th cells was also isolated.

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\(^2\) The sequences presented in this article have been submitted to GenBank under accession numbers AF178012–AF178020 for TS.32.15, TS.32.49, TS.32.16, TS.32.44a, TS.32.1a, TS.32.5a, TS.32.34a, TS.32.17a, and TS.32.43a, respectively, and AF178021–AF178029 for TS.32.15b, TS.32.49b, TS.32.12b, TS.32.44b, TS.32.1b, TS.32.5b, TS.32.34b, TS.32.17b, and TS.32.43b, respectively.

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\(^4\) Abbreviations used in this paper: NCC, nonspecific cytotoxic cells; RACE, rapid amplification of cDNA ends; PBL, peripheral blood leukocyte; MLC, mixed leukocyte culture.
Channel catfish #32 was hyperimmunized i.p. with 3B11 cells, a clonal allogeneic B cell line (2–4 × 10^7 cells in 2 ml PBS). The fish was boosted after 14 days and then weekly for an additional 6 wk. Two weeks after the last boost, 10–15 ml blood was collected using Vacutainer blood collection tubes (Becton Dickinson, Franklin Lakes, NJ). The PBL were used to develop the alloreactive lymphocyte clones described below.

**MLC-derived catfish lymphoid cell lines**

MLC-generated cytotoxic effector cells were obtained as described previously (10). Briefly, 5 × 10^6 catfish PBL from a 3B11-immunized fish (fish #32) or nonimmunized fish (fish #10 and #75) were incubated with 2 × 10^6 irradiated 3B11 cells in 1 ml AL-5 medium/well using 24-well tissue culture plates (Corning Glass, Corning, NY) and incubated at 27°C in a humidified atmosphere containing 5% CO2/95% air. The cultures were passaged when cell density increased and culture medium became acidified. Typically, 1 ml AL-5 medium/well was added after day 5 of culture, and on day 6 the pooled contents of two wells were transferred to a 25-cm² tissue culture flask (Corning) with an additional 4 ml of AL-5 medium. On day 8, 8 ml AL-5 medium was added, and on day 10 the contents (16 ml) of the 25-cm² flask were transferred to a 75-cm² T-flask (Corning) along with an additional 16 ml of medium. The cells were harvested on or after day 12 and cloned by limiting dilution (see below) or continuously cultured by weekly restimulation with irradiated 3B11 cells. In the later case, 10⁶ MLC cells were cultured with 2 × 10⁶ irradiated 3B11 cells in 1 ml complete medium containing 10% conditioned medium from 75°C T cells (as a presumed source of growth factors). After 2 days, an additional 1 ml of 10% conditioned medium was added to the wells, and after 4 days, the pooled contents of two wells were transferred to a 25-cm² flask together with 4 ml of 10% conditioned medium.

**Cloning and propagation of MLC cells**

Cloning of alloantigen-responsive cytotoxic cells was performed by limiting dilution in 10% conditioned medium using freshly isolated PBL- or MLC-activated leukocytes from both 3B11 immune and nonimmune fish. Briefly, 50-μl suspensions of PBL (20 cells/ml) or MLC-activated cells (6 cells/ml) were transferred to wells in 96-well round-bottom tissue culture plates together with 50 μl irradiated (4000–5000 rad) catfish allogeneic B cells (3B11) suspended at 2 × 10⁵ cells/ml in conditioned medium. The cells were incubated at 27°C in 5% CO2/95% air. After 8–15 days in culture, proliferating responder cells were restimulated. Briefly, half of the culture supernatant in each well containing proliferating cells was removed and the cells were resuspended and completely transferred to a flat-bottom 96-well plate. Irradiated 3B11 cells (3 × 10⁶ cells/ml) were suspended in 10% conditioned medium, and 100-μl aliquots were transferred to each well containing responder cells. The clones were cultured for 6 days and then transferred to the wells of 24-well tissue culture plates together with 2 × 10⁶ irradiated 3B11 cells in 1.5 ml 10% conditioned medium and cultured 6 more days. From this point on, the cloned cells were passaged in wells of 24-well tissue culture plates at 6-day intervals by transferring 5 × 10⁵ responder cells and 2 × 10⁶ irradiated 3B11 cells in a final volume of 1 ml 10% conditioned medium per well. Optimal expansion of cloned cells during a 6-day culture period was achieved by adding 1 ml 10% conditioned medium to each well after 4 days. Alternatively, the pooled contents of three wells were transferred to 25-cm² tissue culture flasks together with 3 ml 10% conditioned medium on day 4 and cultured in flasks for 2 more days. The specificity of alloantigen stimulation was assayed by culturing 10⁶ cells from the various clonal MLC-derived lymphocyte lines with 2 × 10⁶ irradiated 3B11 cells (as specific Ag) or a cell line (as nonspecific Ag) in 200 μl of AL-5 medium with or without 10% conditioned medium in 96-well round-bottom tissue culture plates. Triplicate cultures were incubated at 27°C in a humidified atmosphere containing 5% CO2 and 95% air for 4 days. The cultures were pulsed with 0.5 μCi [methyl-³²]H-thymidine (Amer sham, Arlington Heights, IL) 18 h before harvesting by use of an automated cell harvester (Micronate 196 harvester, Packard, Meriden, CT). Incorporation of radiolabeled thymidine was measured using a direct beta-counter (Matrix 96, Packard).

**Cytotoxic assays**

³¹Cr-release assays were performed as previously described (13). Briefly, effector cells in 100 μl AL medium were mixed with 5 × 10⁵ [³¹Cr]-labeled target cells in 100 μl AL medium in round-bottom 96-well tissue culture plates (Corning). The plates were centrifuged for 1 min at 200 × g to induce contact between effector and target cells and were incubated for 4 h at 27°C. The cells were resuspended by pipetting, and the plates were centrifuged again for 3 min at 550 × g. One hundred microliters of cell-free supernatant was removed from each well and cpm were determined in a COUBA II auto gamma-counter (Packard). E:T ratios were as indicated in the text and figure legends. All experiments were done in triplicate. Percent specific release was calculated using the following formula: % specific release = [cpm (experimental) – cpm (minimum release)] / [cpm (maximum release) – cpm (minimum release)]. Maximum release wells received 100 μl 2% Nonidet P-40 (Sigma, St. Louis, MO) instead of effector cells to lyse all target cells. Minimum release wells received 100 μl AL instead of effector cells.

**Results**

Clonal alloantigen-dependent cytotoxic cell lines generated from channel catfish PBL

Previous studies demonstrated that high levels of nonspecific cytotoxic activity directed against allogeneic target cells were generated in one-way MLC using catfish PBL as responders and irradiated allogeneic cells as stimulators (10). Subsequently, cytotoxic cultures were found to continuously proliferate after weekly restimulation with alloantigen(s) in the presence of conditioned medium. Each stimulation was characterized by a short proliferative response resulting in a 3- to 4-fold expansion of cell numbers. Conditioned medium was not required for cell expansion but was important for survival, i.e., cells that were no longer proliferating remained viable for several days in various conditioned media, whereas cells suspended in nonconditioned medium died quickly. Because the phenotype(s) of the MLC-expanded catfish cytotoxic cells was not known, cells responsible for killing allogeneic targets were cloned and subsequently characterized using molecular probes for the catfish TCR α and β genes. PBL from two nonimmune and one 3B11-immunized channel catfish were cloned by limiting dilution either with or without prior stimulation and expansion in MLC. The number of wells containing proliferating cells (cloning efficiency) was estimated by microscopy. Expansion in MLC before cloning dramatically increased the cloning...
efficiency from both sources. For example, only 0.7% of PBL from nonimmune fish #75 could be cloned directly, whereas 47% of the MLC-expanded cells yielded clones. Prior immunization with allograft appeared to increase the cloning efficiency as PBL from a 3B11-immunized fish (#32) yielded significantly higher numbers of clones after both primary and repeat stimulation, i.e., 6.3% and 83%, respectively. These results showed the feasibility of cloning catfish cytotoxic cells and demonstrated that both prior immunization and MLC expansion lead to markedly enhanced cloning efficiency.

To determine their phenotype, the resulting clones were cultured for several weeks after which they were screened for expression of TCR α and β, catfish Igμ, and actin. Based on reactivity patterns, five groups of clones were identified. One group (I) consisting of 10 clones, each derived from 3B11-immune fish #32, Table 1. Characterization of cloned allograft-dependent cells derived from immune (#32) and nonimmune (#10 and #75) fish

| Group | MLC Clone | TCR αβ | 3B11 | Ig8 |
|-------|-----------|--------|------|-----|
| I     | TS.32.1   | +      |      |     |
|       | TS.32.5   | +      |      |     |
|       | TS.32.12  | +      |      |     |
|       | TS.32.13  | +      |      |     |
|       | TS.32.15  | +      |      |     |
|       | TS.32.34  | +      |      |     |
|       | TS.32.39  | +      |      |     |
|       | TS.32.42  | +      |      |     |
|       | TS.32.44  | +      |      |     |
|       | TS.32.49  | +      |      |     |
| II    | TS.32.17  | +      |      |     |
|       | TS.32.32  | +      |      |     |
|       | TS.32.43  | +      |      |     |
| III   | TS.32.4   | +      |      |     |
| IV    | TS.75.2   | -      |      |     |
|       | TS.75.3   | -      |      |     |
| V     | TS.10.1   | -      |      |     |
|       | TS.10.3   | -      |      |     |
|       | TS.75.1   | -      |      |     |

* Cloned cells were used as effectors in 4-h 51Cr-release assays against homologous 3B11 or heterologous Ig8 target cells at 1:1 ratios. Percent specific 51Cr release is indicated by “+” and “−” signs. + + + + +, 86–92%; + +++, 52–74%; + ++, 26–38%; + +, 4–24%; +, 2–2%.

FIGURE 1. Cytotoxic responses of four representative clonal catfish cell lines developed by allograft stimulation. TS.32.5, TS.32.32, TS.75.2, and TS.10.1 were cloned from immune (TS.32.5 and TS.32.32) and nonimmune (TS.75.2 and TS.10.1) fish as detailed in the text. Aliquots of cloned cells were used as effectors in 4-h 51Cr-release assays against various allogeneic or autologous target cells or for RT-PCR with CF-TCR α and β, CF-Igμ, and actin primers. +, Expression of genes.
contained cells that were TCR αβ+ (Table I). This group (represented by clone TS.32.5 in Fig. 1) lysed 3B11 cells but did not lyse any of three other allogeneic targets, each derived from individual fish different from the one that gave rise to the 3B11 cell line. A second group (II) contained three clones that were also TCR αβ+ but did not display strict specificity in target cell lysis. Clone TS.32.32, which is representative of group II, lysed 3B11 as well as 1G8 target cells with high efficiency (Table I & Fig. 1). However, this clone failed to lyse two other allogeneic targets (Fig. 1). The other two clones in group II were also capable of lysing several different allogeneic targets but not others (data not shown).

Two other groups of clones (IV and V), isolated from both immune and nonimmune fish, were TCR αβ2 but differed in cytotoxic activity. The three clones in group IV lacked allospecificity, i.e., they lysed 1G8 target cells as well as 3B11 cells (Table I). A representative member of this group, TS.75.2, lysed three allogeneic target cells including 3B11 cells with no apparent specificity (Fig. 1). However, TS.75.2 did not lyse autologous target cells.

FIGURE 2 Alignment of the inferred amino acid sequences of the TCR α (A) and TCR β (B) series 32 clones. The clone names are indicated at the left margin. Identity with 32.15; - gaps introduced to maximize the alignments; Å conserved position of charged amino acids in the TCR α; and , positions of charged amino acids in the TCR β. Conserved residues for both TCR α and β are numbered (19), and the V, CDR3, and FR4 regions are marked by "." The TCR α sequences of 32.5, 32.34, and 32.17 are not full-length and were amplified using a forward leader primer and a reverse Cα primer. The TCR α and β sequences of 32.44 obtained by 5'-RACE were shorter than expected, which may be due to the quality of the RNA isolated from this cell line. Only a single β transcript was amplified from each of the clones. However, an additional aberrant αVJC rearrangement was amplified for 32.5 and clones 32.1, 32.34, 32.17, and 32.43; all had short transcripts (beginning in FR2) identical with the Vα rearrangement found in clone 32.15. No full-length transcripts of the 32.15Vα sequence could be amplified from these clones, even by using specific 32.15Vα primers. Sequence data were not obtained for TCR αβ1 clones 32.13, 32.39, and 32.42; however, RT-PCR analysis yielded the correct size α and β fragments.

The other two clones in group II were also capable of lysing several different allogeneic targets but not others (data not shown). Two other groups of clones (IV and V), isolated from both immune and nonimmune fish, were TCR αβ+ but differed in cytotoxic activity. The three clones in group IV lacked allospecificity, i.e., they lysed 1G8 target cells as well as 3B11 cells (Table I). A representative member of this group, TS.75.2, lysed three allogeneic target cells including 3B11 cells with no apparent specificity (Fig. 1). However, TS.75.2 did not lyse autologous target cells.
Three clones were isolated that reacted with allogeneic target cells but not with autologous cells. These clones were designated TS.32.15 and TS.32.49, and they proliferated specifically to stimulation with irradiated 3B11 cells. TS.32.44, another clone isolated from the same V2a family, exhibited identical VJ rearrangements and two others, TS.32.5 and TS.32.12, had rearranged identical V genes in the second family (12). Two clones, TS.32.12 and TS.32.44, exhibited identical VJ rearrangements and two others, TS.32.15 and TS.32.49, rearranged identical V to different J in the same family (2). Each Vβ rearrangement was unique, with clones TS.32.5, TS.32.12, and TS.32.44 expressing different members from the same Vβ family (2). These findings indicate that each of these cell lines are clonal and likely derived from independent precursors.

Channel catfish alloantigen-dependent TCR αβ+ cytotoxic cells proliferate in response to stimulation with specific alloantigen

Irradiated stimulator cells were crucial for propagation of both TCR αβ+ and TCR αβ− cells during the process of cloning. To test whether or not the various cytotoxic clones required specific allostimulation for in vitro expansion (or if any allogeneic stimulator would suffice) an allospecific TCR αβ+ clone (TS.32.5) and an allospecific TCR αβ− clone (TS.10.1) were stimulated with specific alloantigen (3B11 cells) or with a different alloantigen (1G8 cells) in the presence or absence of conditioned medium. As shown in Fig. 3, TS.32.5 responded only to 3B11 cells, whereas clone TS.10.1 proliferated in response to both 3B11 and 1G8 cells. Furthermore, TS.32.5 cells proliferated equally well after stimulation with 3B11 cells whether or not conditioned medium was included in the culture. In contrast, a mixture of conditioned medium and irradiated allogeneic cells appeared to synergistically enhance proliferation of TS.10.1 cells (Fig. 3).

Discussion

Both in vivo and in vitro studies indicate that teleosts possess allospecific cell-mediated immunity similar to that seen in higher vertebrates. For example, primary and secondary rejection of allografted skin/scales in vivo has been demonstrated in representatives of nearly all major groups of fish (reviewed in Ref. 20). Moreover, leukocytes isolated from fish with allogeneic erythrocytes lysed homologous erythrocytes, demonstrating allospecific cytotoxic responses in an in vitro system (21). In another study, trinitrophenyl-modified Con A blasts from carp were lysed by autologous but not allogeneic immune leukocyte effectors, suggesting genetically restricted target cell recognition (22). Although the above studies are indicative of CTL-like activity, defined populations of teleost cytotoxic cells previously had not been isolated. Consequently, it had not been possible to determine the function and phenotype of putative subpopulations of fish cytotoxic effector cells. In the work reported here, four types of cytotoxic cells were cloned from channel catfish PBL. This was accomplished using a culture technique similar to that employed for cloning mammalian alloreactive CTL and NK cells, i.e., periodic stimulation with allogeneic cells in the presence of conditioned medium (23). Crucial for propagation of clonal catfish cytotoxic cells was the availability of allogeneic long-term leukocyte lines as continuous sources of defined MLC stimulator cells (5, 6, 12). Four different groups of cloned catfish cytotoxic cells were identified based on allospecificity and expression of TCR αβ genes. One group of effectors (group IV) was nonspecific, TCR αβ−, and was considered to be equivalent to mammalian NK cells, whereas two other groups of effectors (groups I and V) demonstrated allospecific cytotoxic activity. Clones in group I expressed messages for both TCR α and β and may be considered bona fide CTL. The second group of allospecific effector clones (group V) lacked TCR αβ expression. Therefore, it is not clear whether these cells are CTL or a second type of NK-like cell. Although mammalian NK cells typically lyse target cells in a nonspecific fashion, it has been demonstrated that human NK cells can cause specific lysis of allogeneic PHA-induced lymphoblasts after stimulation in vitro with homologous lymphoblasts (24). By analogy, a similar mechanism for target cell recognition may exist for catfish allospecific NK-like lysis. It should be noted that although the TCR αβ− alloreactive cytotoxic effector cells described here are termed NK-like, the possibility of TCR γδ expression in one or both of these groups of clones was not ruled out. Putative channel catfish TCR γδ genes have not yet been identified, although TCR γδ (as well as TCR γδ) genes have been isolated in cartilaginous fish. Thus, equivalent genes are possibly present in teleosts because it seems likely that TCR γδ genes originated in an ancestor common to all jawed vertebrates (25). Catfish cytolytic cells that were TCR αβ− but nonspecific in target cell recognition (group II) were difficult to categorize because they did not match the phenotype and function of known mammalian cytotoxic cells. It is possible that these

![Figure 3](image-url)
clones may recognize a target structure that is similar on the two target cells. Although possibly not applicable to the clones in question, it is interesting to note that mammalian CTL clones may convert from allospecific to non-specific target cell recognition after prolonged culture or after culture in high concentrations of IL-2 (26, 27). It is also possible that these particular fish cells may represent a form of NK-like T cells (28). An additional TCR αβ+ clone (group III) proliferated specifically in response to irradiated 3B11 cells but did not lyse 3B11 cells or other allogeneic targets. This clone may be a candidate for a catfish homologue of mammalian T helper cells.

Although most of the cytotoxic cells derived in this study were cloned after stimulation in MLR, some were cloned directly from freshly isolated PBL. It is noteworthy that the cloning efficiency of PBL from the nonimmune catfish tested ranged from 0.5 to 1.0%, indicating that the numbers of NK-like cytotoxic effectors and/or precursor cells in PBL were relatively low. This is consistent with previous observations of spontaneous cytotoxic responses in channel catfish, namely that E:T ratios ranging from 15:1 to 40:1 were usually required for 50% lysis of allogeneic target cells by freshly isolated PBL in 4-h 51Cr-release assays (3, 12, 29). These observations imply that the spontaneous effector cell population(s) involved in lysis of any given allogeneic target likely ranges from 1 to 4% of the total PBL in the catfish employed in these studies.

This study did not formally demonstrate that the cells designated as catfish CTL employed TCR molecules to recognize target cells, nor were MHC molecules proven to be the structures recognized on target cells (30). However, this seems likely considering the strict specificity in target cell lysis displayed by the catfish CTL clones combined with the fact that these effector cells express TCR αβ genes and the allogeneic target cells express both class I and II MHC genes (Refs. 8 and 9 and our unpublished data). Preliminary observations that blocking of target cell killing by some but not all alloantisera suggest that catfish CTL clones most likely recognize an alloantigen(s) on target cells (data not shown); whether this alloantigen is MHC class I, class II, or another molecule is not clear. Immunoprecipitation studies with such alloantisera have been equivocal and have not yet resolved this issue (31). Consequently, identification of the molecules on target and effector cells involved in target cell recognition by catfish CTL clones awaits the development of immune reagents specific for catfish TCR and MHC molecules.

The target cell recognition mechanism(s) employed by catfish NK-like effector clones was not determined in this study. However, one of the NK-like clones did not lyse autologous target cells but efficiently lysed three different allogeneic targets. It is possible that catfish NK-like cells express killer inhibitory receptors analogous in function to those found on human and rodent NK cells (32); engagement of self-MHC molecules on autologous/syngenic target cells by killer inhibitory receptors on mammalian NK cells protects these cells from lysis by effectors (33–35). The availability of autologous and allogeneic clones of effector and target cells from catfish provides a promising model with which to study target cell recognition by fish NK-like cytotoxic cells at both functional and molecular levels.

Channel catfish alloreactive cytotoxic cells were shown to be distinct from NCC by several criteria. First, an NCC-specific mAb, 5C6, failed to block lysis of allogeneic target cells by PBL effectors (3). Second, channel catfish PBL stimulated in primary MLC lysed xenogeneic target cells poorly at E:T ratios that gave maximum lysis of allogeneic target cells (10). Finally, all of the NK-like as well as CTL clones tested did not react with mAb 5C6 (data not shown). Taken together, these results indicate that the various cytotoxic cells described herein are distinct from NCC.

The results described above give the strongest evidence to date that channel catfish (and possibly other fish species) contain a variety of CTL and NK-like effector cells with different target cell preferences. The ability to clone various types of catfish cytotoxic cells along with the continued development of molecular probes and immunological reagents will likely facilitate study of both CTL and NK-like cells in fish as it has in mammals.

References

1. Clem, L. W., N. W. Miller, and J. E. Bly. 1991. Evolution of lymphocyte subpopulations, their interactions and temperature sensitivity. In The Phylogeny of Immune Functions. N. Cohen and G. W. Warn, eds. CRC, Boca Raton, FL, p. 191.
2. Evans, D. L., and L. Jaso-Friedmann. 1992. Non-specific cytotoxic cells as effectors of immunity in fish. Annu. Rev. Fish Dis. 2:169.
3. Stuge, T. B., N. W. Miller, and L. W. Clem. 1995. Channel catfish cytotoxic effectors from peripheral blood and pronephros are different. Fish Shellfish Immunol. 5:469.
4. Lin, G. L., C. P. Ellsasser, L. W. Clem, and N. W. Miller. 1992. Phorbol ester calcium ionophore activate fish leucocytes and induce long term cultures. Dev. Comp. Immunol. 16:153.
5. Miller, N. W., M. A. Ryczyw, M. R. Wilson, G. W. Warn, J. P. Naftel, and L. W. Clem. 1994. Development and characterization of channel catfish long term B cell lines. J. Immunol. 152:2180.
6. Miller, N. W., V. G. Chinchar, and L. W. Clem. 1994. Development of leucocyte lines from the channel catfish (Ictalurus punctatus). J. Tissue Cult. Methods 16:117.
7. Miller, N. W., M. R. Wilson, E. Bengten, T. B. Stuge, G. W. Warn, and L. W. Clem. 1998. Functional and molecular characterization of teleost leucocytes. Immunol. Rev. 166:187.
8. Antao, A., V. G. Chinchar, T. J. McConnell, N. W. Miller, L. W. Clem, and M. R. Wilson. 1999. MHC class I genes of the channel catfish: sequence analysis and expression. Immunogenetics 49:303.
9. Godwin, U. B., A. Antao, M. R. Wilson, V. G. Chinchar, N. W. Miller, L. W. Clem, and T. J. McConnell. 1997. MHC class II β genes in the channel catfish (Ictalurus punctatus). Dev. Comp. Immunol. 21:13.
10. Stuge, T. B., S. H. Yoshida, V. G. Chinchar, and N. W. Miller. 1997. Cytotoxic activity generated from channel catfish peripheral blood leucocytes in mixed leucocyte cultures. Cell. Immunol. 177:154.
11. van Ginkel, F. W., N. W. Miller, C. J. Lobb, and L. W. Clem. 1992. Characterization of anti-hapten antibodies generated in vitro by channel catfish peripheral blood lymphocytes. Dev. Comp. Immunol. 16:159.
12. Wilson, M. R., H. Zhou, E. Bengten, L. W. Clem, T. B. Stuge, G. W. Warn, and N. W. Miller. 1998. T-cell receptors in channel catfish: structure and expression of TCR α and β genes. Mol. Immunol. 35:545.
13. Yoshida, S. H., T. B. Stuge, N. W. Miller, and L. W. Clem. 1995. Phylogeny of lymphocyte heterogeneities: cytotoxic activity of channel catfish peripheral blood leukocytes directed against allogeneic targets. Dev. Comp. Immunol. 19:71.
14. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162:156.
15. Warn, G. W., N. W. Miller, L. W. Clem, and M. R. Wilson. 1998. Alternate splicing pathways of the immunoglobulin heavy chain transcript of a teleost fish, Ictalurus punctatus. Immunogenetics 53:253.
16. Luft, J. C., M. R. Wilson, E. Bengten, L. W. Miller, and L. W. Clem. 1996. Identification and characterization of a heat shock protein 70 family member in channel catfish (Ictalurus punctatus). Comp. Biochem. Physiol. 113B:169.
17. Frohman, M. A., M. K. Dush, and G. R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85:9588.
18. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463.
19. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and G. Foeller. 1991. Sequences of Proteins of Immunological Interest. Natl. Inst. of Health, Bethesda.
20. Manning, M. J., and T. Nakanishi. 1996. The specific immune system: cellular defenses. In The Fish Immune System: Organism, Pathogen, and Environment. G. Iwama and T. Nakamishi, eds. Academic, San Diego, p. 159.
21. Fischer, U., M. Ootoke, and T. Nakamishi. 1998. In vitro cell-mediated cytotoxicity against allogeneic erythrocytes in gibel carp crucian carp and goldfish using a non-radioactive assay. Dev. Comp. Immunol. 22:195.
22. Verhaae, V. M., S. Sage, and P. Deshaies. 1990. Cytotoxicity of carp (Cyprinus carpio) leucocytes induced against TNP-modified autologous spleen cells and influence of aclimatisation temperature. Dev. Comp. Immunol. 14:475.
23. Paul, W. E., B. Sredni, and R. H. Schwartz. 1981. Long-term growth and cloning of non-transformed lymphocytes. Nature 294:697.
24. Ciccone, C., O. Viale, D. Pende, M. Malnati, R. Biasongi, G. Melisio, E. O. Long, and L. Moretta. 1988. Specific lysis of allogeneic cells after activation of CD3+ lymphocytes in mixed lymphocyte culture. J. Exp. Med. 168:2403.
25. Rast, J. P., M. K. Anderson, S. J. Strong, C. Laer, R. T. Litman, and G. W. Litman. 1997. α, β, γ and δ T cell antigen receptor genes arose early in vertebrate phylogeny. Immunity 6:1.
26. Wilson, A., and K. Shortman. 1985. Degradation of specificity in cytolytic T lymphocyte clones: two broad specificity, H-2-independent recognition systems, one natural killer-like, develop during culture, in addition to the clonally distributed antigen-specific receptor. *Eur. J. Immunol.* 15:899.

27. Brooks, C. G., C. L. Urdal, and C. S. Henney. 1983. Lymphokine-driven “differentiation” of cytotoxic T cell clones into cells with NK-like specificity: correlations with display of membrane macromolecules. *Immunol. Rev.* 72:43.

28. Hammond, K. J. L., S. B. Pelikan, N. Y. Crowe, E. Randle-Barrett, T. Nakayama, M. Taniguchi, M. J. Smyth, I. R. van Driel, R. Scollay, A. G. Baxter, and D. I. Godfrey. 1999. NKT cells are phenotypically and functionally diverse. *Eur. J. Immunol.* 29:3768.

29. Hogan, R. J., T. B. Stuge, L. W. Clem, N. W. Miller, and V. G. Chinchar. 1996. Anti-viral cytotoxic cells in the channel catfish (*Ictalurus punctatus*). *Dev. Comp. Immunol.* 20:115.

30. Kappler, J., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2 restricted, interleukin-2-producing T cell hybridomas: lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198.

31. Vallejo, A. N., N. W. Miller, and L. W. Clem. 1992. Antigen processing and presentation in teleost immune responses. *Annu. Rev. Fish Dis.* 2:73.

32. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol.* 16:359.

33. Karlhofer, F. M., R. K. Rabaudo, and W. M. Yokoyama. 1992. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 358:66.

34. Moretta, A., M. Vitale, C. Bottino, A. M. Orengo, L. Morelli, R. Augugliaro, M. Barbaresi, E. Ciccone, and L. Moretta. 1993. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer cells: anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J. Exp. Med.* 178:597.

35. Naper, C., J. C. Ryan, M. C. Nakamura, D. Lambracht, B. Rolstad, and J. T. Vaage. 1998. Identification of an inhibitory MHC receptor on alloreactive rat natural killer cells. *J. Immunol.* 160:219.