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ARLEE LINE OF RAINBOW TROUT (*Oncorhynchus mykiss*) EXHIBITS A LOW LEVEL OF NONSPECIFIC CYTOTOXIC CELL ACTIVITY

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☐**Abstract—Nonspecific cytotoxic cell (NCC) activity was assessed in the peripheral blood of four isogenic lines of rainbow trout (*Oncorhynchus mykiss*) which were derived by the chromosome set manipulation technique of androgenesis. In these fish, whose isogenicity was previously confirmed by multilocus DNA fingerprint analysis, NCC activity was studied by the release of ^{51}Cr from YAC-1 targets. Two groups of trout (the homozygous Arlee 12 line and the heterozygous hybrid of the Arlee 63 and Arlee 12 lines) had significantly lower levels of NCC activity in peripheral blood than either outbred rainbow trout or other lines with Hot Creek or hybrid Arlee × Hot Creek ancestry. The low NCC activity in the Arlee line appears to be inherited as a recessive trait. Peripheral blood cells of the trout mediated lectin dependent cellular cytotoxicity (LDCC) with the addition of phytohemagglutinin to co-cultures of effector cells and YAC-1 cells. The low NCC activity in the peripheral blood of these fish is not due to a condition analogous to the NCC-deficient Chediak-Higashi syndrome of man or the beige mutation of mice.**

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Nomenclature

NCC	Nonspecific cytotoxic cell
PBL	Peripheral blood leukocytes
PCV	Packed cell volume
PHA	Phytohemagglutinin
CM	Complete medium

Introduction

In teleost fish, cells of the anterior kidney, spleen and peripheral blood can induce rapid unrestricted lysis of human, murine and teleost targets (1–3). Cells mediating this phenomenon are known as nonspecific cytotoxic cells (NCC). Because target cell lysis requires no allogeneic priming, it has been postulated that NCC may be the progenitors of mammalian natural killer (NK) cells (4). NK cells of mammals are of particular interest because it is believed that they are important in defending the host against certain viral infections, lysing tumor cells and regulating hemopoiesis (5).

The most extensive studies of NCC have been performed on catfish (*Ictalurus punctatus*). In catfish, NCC are nylon wool non-adherent, exhibit lysis only when they contact the target, and show high lytic activity against ^{51}Cr-labeled human lymphoblastoid targets (1,4). Catfish NCC can lyse the fish parasite, *Teterahymena pyriformis* (6). Most recently, the mechanism of action of catfish NCC has become of much broader interest with the discovery that NCC and mammalian NK cells share a vimentin-like membrane determinant recognized by a monoclonal antibody (7–9). The target cell antigen to which the NCC receptor of catfish binds has been identified by an anti-idiotype monoclonal antibody which reacts with target cell lysates on Western blots (10). In contrast to mammalian NK cells, which are large granular lymphocytes, NCC from both rainbow trout and channel catfish are small agranular lymphocytes (4,11). NCC of rainbow trout kill target cells by both necrotic and apoptic mechanisms (11).

We have recently produced isogenic lines of rainbow trout by androgenesis (12) with the purpose of utilizing them as aquatic animal models for human and fish disease research. Androgenetic trout, having total paternal inheritance, are produced by fertilization of irradiated eggs (13). Subsequently, diploidy is restored by applying a heat or pressure shock to prevent the first cleavage (14). Likewise, gynogenesis, another method for the production of homozygous individuals, is performed by fertilizing normal eggs with sperm which has been inactivated by radiation, usually ultraviolet light. Subsequently, a shock is applied to the eggs to prevent the first cleavage (15). Induced gynogenesis or androgenesis applied to the gametes of the homozygous parent produces isogenic progeny (16). Since inbred fish have low viability and fertility (12,17), it is sometimes desirable to produce isogenic F1 hybrids between inbred lines (18).

In order to fully utilize these isogenic lines of trout as models for disease research, they must be immunologically characterized. In this preliminary communication, we examined the NCC activity, lectin dependent cellular cytotoxicity, and the leucocytes of the peripheral blood of several interrelated isogenic lines of rainbow trout of known heritage.

Materials and Methods

Production of Isogenic Lines of Fish

The isogenic lines of rainbow trout (*Oncorhynchus mykiss*) utilized in this study were developed by Gary Thorgaard and co-workers at Washington State University and were produced by chromosome set manipulation techniques in several steps. The isogenicity of the lines has been confirmed by DNA fingerprinting (19). Homozygous diploid individuals were first produced by androgenesis (13,14). Isogenic heterozygous hybrids were produced by mating homozygous females with homozygous males. For example, Arlee 63 (a homozygous androgenetic female) was crossed to a homozygous androgenetic male (Hot Creek 71) to produce the isogenic hybrid line, Arlee 63 × Hot Creek 71. The ancestries of the lines used in this study were as follows (20): Arlee was derived from a strain cultured by the Montana Department of Fish, Wildlife and Parks at Arlee, Montana. Hot Creek was derived from an inbred line cultured at the Hot Creek Hatchery operated by the California Department of Fish and Game. Outbred trout used in this study were obtained from Clear Springs Foods, Buhl, Idaho. Table 1 decribes the isogenic lines used in these experiments.

Table 1. Description of Isogenic Lines of Rainbow Trout Used in This Study.

- Arlee 12: A diploid isogenic line whose members are homozygous androgenetic males (YY).
- Arlee 63: A diploid homozygous, androgenetic female (XX). Arlee 12 and Arlee 63 were derived from separate sperm cells of the same normal male (XY) parent; therefore, these two lines (Arlee 12 and Arlee 63) are not genetically identical to each other. The Arlee 63 female was not available for testing.
- Hot Creek 71: A diploid isogenic line which is homozygous, androgenetic and male (YY). This line is genetically dissimilar to the Arlee families.
- Arlee 63 × Hot Creek 71: A diploid isogenic hybrid line whose members are all heterozygous, naturally produced males (XY). The parents are non-related, homozygous individuals.
- Arlee 63 × Arlee 12: A diploid isogenic hybrid line which was naturally produced. These fish are all XY males. This line is heterozygous but the two parents are closely related to each other.

Isolation of Peripheral Blood Leukocytes

Leukocytes were isolated from peripheral blood using a modified protocol of Greenlee and Ristow (21). Fish were bled at 2-month intervals to ensure that they did not become anemic. Four Arlee 12, six Hot Creek 71, seven Arlee 63 × Arlee 12 and five Arlee 63 × Hot Creek 71 rainbow trout were bled at the beginning of the experiment. After 10 months, their numbers were reduced to two Arlee 12, six Hot Creek 71, five Arlee 63 × Arlee 12, and four Arlee 63 × Hot Creek 71.

Fish were anesthetized with 2-phenoxyethanol and 1.5 mL of blood was drawn by cardiac puncture into 3 mL heparinized syringes (100 U heparin). Blood was immediately diluted 1:5 into RPMI 1640 and placed over a bed of ice. The mixture was underlaid with 6 mL Histopaque 1.077 (at 20°C) (Sigma #1077-1) and centrifuged at 1500 × *g* at 15°C for 40 min. Peripheral blood leukocytes (PBL) sedimenting at the Histopaque-media interface were harvested, resuspended, washed three times in 15 mL of RPMI 1640 and resuspended in 1–2 mL complete medium (RPMI 1640 with 10% fetal bovine serum plus 100 U/mL of penicillin and 100 μg of streptomycin per mL) (CM) and the cell number adjusted to 1×10^7 cells/mL.

Assay for NCC Activity

The ^{51}Cr release assay was performed as previously described by Greenlee et al. (11). YAC-1 or P815 targets (2×10^7) in log phase growth were labeled in 1 mL CM containing 200 μCi sodium chromate (New England Nuclear NEZ 030S) for 1 h at 37°C in a humidified incubator in an atmosphere of 7% CO_2. Cells were washed three times in RPMI 1640 containing 2.5% fetal bovine serum. Ten thousand YAC-1 target cells in 100 μL of CM were dispensed into the wells of a 96 well microtiter plate. Effector PBL isolated from each fish were dispensed into the wells in triplicate at an effector: target ratios of 100:1, 50:1 and 25:1 in initial experiments. Thereafter, only the 100:1 target:effector ratio was performed. To measure spontaneous release, 100 μL of complete medium was added to the labeled targets. To measure total release, 100 μL of 2% sodium dodecyl sulfate was added. Wells were harvested by withdrawing 100 μL of cell-free supernatant from the wells after 4 or 20 h incubation at 20°C. Samples were counted for 5 min on a gamma spectrophotometer. Percent specific chromium release was calculated by the following formula:

$$\frac{\text{CPM(experimental)} - \text{CPM(spontaneous)}}{\text{CPM(total)} - \text{CPM (spontaneous)}} \times 100,$$

where CPM = counts per min, spontaneous release equals radioactivity in wells receiving target cells plus 100 μL complete medium and total release equals radioactivity in wells receiving target cells plus 100 μL of 2% sodium dodecyl sulfate. Seasonal effects on NCC activity of the PBL were measured over the period of 10 months.

Lectin Dependent Cellular Cytotoxicity (LDCC) Assays

Phytohemagglutinin (PHA, Sigma-9132) was added at 5 μg per mL to each of the triplicate cocultures of effector plus target ^{51}Cr labeled YAC-1 or P815 cells. After an incubation period of 20 h, lectin dependent cellular cytotoxicity (LDCC) was assayed and calculated in the same manner as NCC activity.

Microscopic Examination of PBL

Blood smears and cytospin preparations made on clean glass slides were stained with modified Wright's stain and examined under oil by light microscopy. Because NCC values for trout of the Arlee heritage tended to be low, particular attention was directed toward the size and structure of the cytoplasmic granules of the leukocytes in order to discover whether these fish possessed a condition analogous to the Chediak-Higashi Syndrome (22,23). The packed cell volumes (PCV) of the individual fish were also assessed by microhematocrits over the 10 month period of the study because it was observed that there were differences between the groups; in particular, the Hot Creek trout appeared to be anemic.

Statistics

Data were subjected to a least squares analysis of variance (ANOVA) using the General Linear Models (GLM) procedure of SAS (24). Separate analyses were performed for data collected at each bimonthly time point. For NCC and PCV data, ANOVA were performed for a randomized block design with main effect of family and block effect of day of assay being cross-classified. For PHA-stimulated LDCC data, ANOVA for a randomized block design was performed with main effects of family and PHA treatment, fish nested within family by day group and block effect of day of assay. Effects of family, PHA treatment and day of assay were cross-classified. All tests of hypotheses were performed using the appropriate error terms, according to the expectations of the means squares (25). Least squares means and associated standard errors were obtained using the least-square means statement of the GLM procedure.

Results

NCC activity was measured in the lines of rainbow trout described in Table 1 over a period of 10 months (Table 2). Figure 1 records our initial observation of low NCC in trout of Arlee heritage at 20 h and at three effector:target ratios: 100:1, 50:1 and 25:1. During the ensuing 10-month period, trout possessing purebred Arlee heritage had significantly lower NCC activity in the peripheral blood than either the outbred trout (Clear Springs) or those with purebred Hot Creek or hybrid Arlee × Hot Creek heritage (Table 2). The Arlee 12 and Arlee 63 × Arlee 12 isogenic lines, representing pure Arlee heritage, maintained an NCC activity level of less than 10% specific chromium release throughout the 10-month period at an effector:target ratio of 100:1. NCC activity in the Arlee 63 × Hot Creek 71 isogenic hybrid remained the same or less than the outbred controls, but was always at a level significantly greater than the two

Table 2. NCC in Isogenic Lines of Rainbow Trout Over 10 Months*

Isogenic Family	N†	% Specific ^{51}Cr Release After 20 h ± SELSM‡				
		May	July	Sept	Dec	Feb
Arlee 12	4–2	2.8^{b} ± 5.9	2.1^{c} ± 2.3	3.9^{b} ± 2.8	0.8^{b} ± 2.8	0.6^{c} ± 2.0
Hot Creek 71	6–2	32.4^{a} ± 4.8	16.8^{b} ± 2.4	39.5^{c} ± 3.0	40.7^{c} ± 2.8	ND
Arlee 63 × Arlee 12	7–5	5.8^{b} ± 4.4	5.6^{c} ± 1.9	8.9^{b} ± 2.0	0.4^{b} ± 2.0	0.6^{c} ± 1.8
Arlee 63 × Hot Creek 71	5–4	33.9^{a} ± 5.2	21.2^{b} ± 2.4	16.9^{d} ± 2.4	19.2^{a} ± 2.4	9.4^{b} ± 1.6
Outbred	16–12	30.3^{a} ± 2.5	33.0^{a} ± 1.3	29.0^{a} ± 1.4	18.0^{a} ± 1.5	20.8^{a} ± 1.3

* Values with different superscripts are compared vertically within each month and are significantly different at $p \leq 0.05$.
† Shows variable numbers of trout included in assays. In the isogenic animals, numbers decreased over time.
‡ Effector:target ratio 100:1, 20 h incubation with YAC-1 targets at 20°C.
SELSM = standard error least squares means. ND = not done.

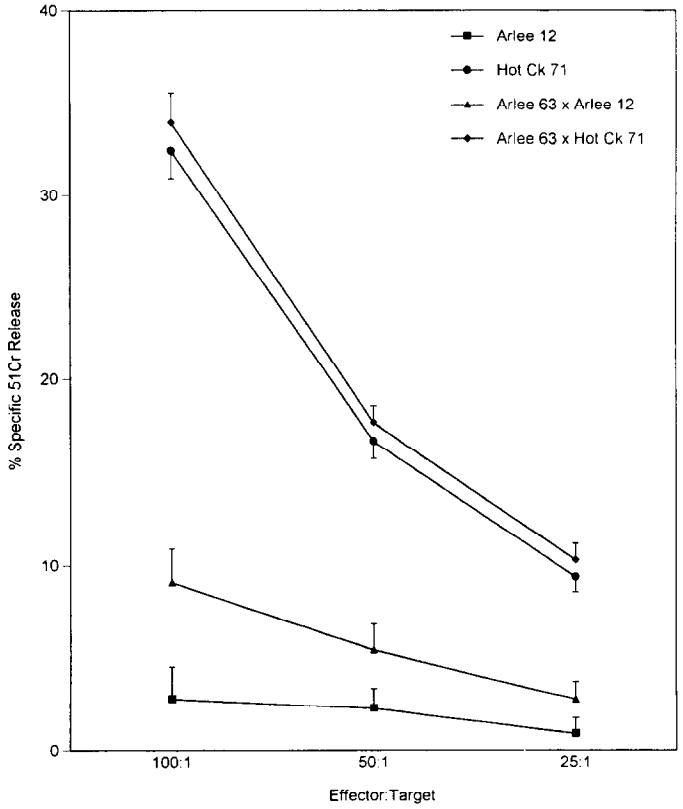

Figure 1. NCC activity in isogenic lines of rainbow trout at 20 h against YAC-1 targets. 1×10^4 targets were incubated with the indicated effector ratios for 20 h at 20°C.

purebred Arlee lines with the exception of February. The Hot Creek 71 homozygous line was normal to significantly high throughout the period compared to the controls, with the exception of July when their NCC activity was significantly less than during the remainder of the 10-month period. In spite of this low activity, the mean Hot Creek NCC activities were still significantly greater than those of the two Arlee lines. It should be noted that the homozygous Hot Creek 71 trout are not a vigorous line and that only two fish survived the experimental period. Overall, when compared with the outbred group, all isogenic Arlee lines possessed low NCC activity whereas Hot Creek and Arlee × Hot Creek hybrid lines were normal and low-normal, respectively. These data suggest that very low NCC is inherited as a recessive trait.

That peripheral blood leukocytes from trout of Arlee heritage can perform lectin-mediated cellular cytotoxicity is illustrated in Figure 2. Figure 2 demonstrate, with each isogenic line or with outbred trout, that killing is significantly increased against YAC-1 with the addition of PHA over 20 h of incubation. Likewise, we also observed increased killing against P815 cells with the addition of PHA (data not shown).

Because the Hot Creek 71 trout appeared to be anemic, microhematocrits were performed on all trout blood samples over a period of 10 months (summarized in Table 3) and revealed a trend. In general, the packed cell volumes of Arlee 12 and Arlee 63 × Arlee 12 were significantly greater than the controls throughout much of the year. Hot Creek 71 individuals remained lower than outbred control fish. Packed cell volumes of the Arlee 63 × Hot Creek 71 line were low to normal.

Although the Arlee trout maintained a low peripheral blood NCC over the 10-month period, they did not have a dilution in coat color nor did their blood smears or cytospins indicate the presence of abnormal granulocyte morphology. No differences were detectable by light microscopy in the size and structure of the cytoplasmic granules in the leukocytes of Arlee and outbred trout. No other abnormalities were noted in the smears.

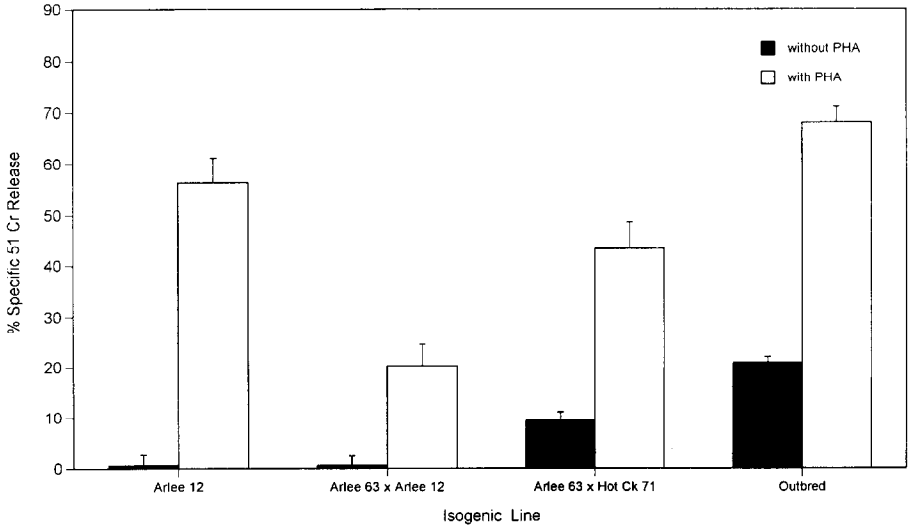

Figure 2. Effect of PHA on NCC activity against YAC-1 targets in isogenic lines of rainbow trout. Effector to target ratio 100:1 against YAC-1 targets. Incubation period was 20 h at 20°C. Spontaneous release (targets + PHA) was not greater than 12% of the total release. PHA concentration was 5 μg/ml. All groups of trout exhibited significant differences in killing YAC-1 targets when co-cultured with and without PHA.

Table 3. Packed Cell Volumes in Isogenic Families of Rainbow Trout Over 10 months

Isogenic Family	N	Packed Cell Volume (%) ± SEM				
		May	July	Sept	Dec	Feb*
Arlee 12	4–2	66^{b} ± 4	54^{b} ± 1	59^{b} ± 6	60^{a} ± 6	57 ± 4
Hot Creek 71	6–2	28^{c} ± 6	39^{c} ± 5	33^{c} ± 9	18^{b} ± 1	ND
Arlee 63 × Arlee 12	7–5	52^{d} ± 6	54^{c} ± 3	55^{ab} ± 8	66^{d} ± 3	62 ± 4
Arlee 63 × Hot Creek 71	5–4	49^{d} ± 2	42^{c} ± 2	52^{ab} ± 6	47^{c} ± 3	52 ± 4
Outbred	12–16	41^{a} ± 1	48^{a} ± 2	49^{a} ± 2	57^{a} ± 2	57 ± 3

Values with different superscripts are compared within each month and are significantly different at $p \leqslant 0.05$.
* Mean values are not significant from controls at $p \leqslant 0.05$.

Discussion

This preliminary communication begins the examination of isogenic lines of rainbow trout as aquatic animal models for biomedical research, particularly as immunological models. Although they were not available in large numbers, these trout, originally developed by chromosome set manipulation techniques, will be useful as trout of known genotype for salmonid disease research and as aquatic animal models. Just as with strains of inbred mice now widely used by the biomedical community (26), these fish undoubtedly possess a defined major histocompatibility complex (MHC) (27); and experiments performed with a particular line should be repeatable over time.

Based upon the findings in this study, the low NCC activity in the Arlee line of rainbow trout appears to be inherited as a recessive trait. The beige trait in mice is a model of the Chediak-Higashi syndrome of humans and other mammalian species, and in the various models of this autosomal recessive disease there is a decreased level of natural killer (NK) cell activity. Also associated with the syndrome, however, is an increased size of cytoplasmic granules, compared to the granules found in the leukocytes of normal controls of the same species (22,23). The absence of such an enlargement of cytoplasmic leukocytic granules in the Arlee line indicates that the low level of nonspecific activity in this line has a different basis than the low NK cell activity of the various models of Chediak-Higashi syndrome in mammals.

Hot Creek 71 trout display normal NCC activity, but have very low packed cell volumes. They were consistently anemic throughout the course of the study and suffered the highest mortality. In the future, it may be fruitful to study the pathways of hemopoiesis in these fish.

Effectors from all of the isogenic lines are capable of performing LDCC. It may be speculated that the lectin is able to bring the effectors and targets together; and that once the cells contact, the apparatus is in place to effect killing. Interestingly, in our experiments, the addition of PHA also improved killing of the target cell, P815. Our results,

however, do not preclude the recruitment of cell types other than NCC to effect killing by the LDCC mechanism. For example, Phillips and Lanier (28) showed by flow microfluorimetry with monoclonal antibodies, that in human LDCC, a $CD3^+$ T lymphocyte predominates. Several pieces of evidence point to a T cell function in rainbow trout. They possess the ability to reject allografts (29), and respond to some of the traditional mammalian T cell mitogens. A gene has been sequenced which codes for the putative β chain of the T cell receptor of trout (30); and in addition, a gene which is similar to the murine MHC II β chain has also been sequenced from trout spleen (31), illustrating that trout do exhibit some of the genes for important T cell associated molecules. Since there are no monoclonal antibodies at present for an analogue of the CD3 marker for trout, we cannot yet perform the analogous experiment with the isogenic fish to determine the cell mediating LDCC.

Propagation of syngeneic lines of animals has been of enormous benefit to biomedical sciences. Elimination of genetic dissimilarity within a group of experimental animals provides investigators with the ability to perform replicate studies without confounding variability. Studies using inbred mice have been an excellent example of the value of inbred animals to basic research (26,32). Inbred lines of mice, crosses between lines, and new inbred lines generated from crosses have proven valuable in understanding the genetic basis of important physiological differences and in identifying single genes with major effects.

In contrast to mammals, which require 10–20 generations of inbreeding to produce inbred lines, fish are amenable to several methods of chromosome set manipulation, including gynogenesis and androgenesis, techniques which can screen out lethal genes in the first generation (16). Genetically identical progeny can be produced from homozygous diploid individuals. In addition, the hybrid between two homozygous lines can be used to generate a number of new heterozygous isogenic lines of progeny, each of which can be used to found a recombinant inbred line (33). It is anticipated that fish derived by these techniques will become useful aquatic animal models for biomedical research.

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