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Major histocompatibility complex class I involvement in the rejection of allogeneic erythrocytes in rainbow trout (*Oncorhynchus mykiss*)

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Abstract
Major histocompatibility complex genes are thought to be involved in allogeneic graft rejection but not many reports are available on their functional analysis in fish. Analysis of available sequences of MHC genes suggests functions in antigen presentation similar to those found in higher vertebrates. In mammals, the MHC class I and class II molecules are major determinants of allogeneic graft rejection due to their polymorphism in conjunction with their antigen presenting function. In fish, MHC class II molecules are found to be involved in rejection of allogeneic scale grafts. The present study was designed to investigate the involvement of MHC class I molecules in allograft rejection. Erythrocytes were collected from donors of rainbow trout expressed different MHC class I alleles, stained with two dyes, mixed and grafted to the recipients that were of the same sibling group as the donors. The grafts were rejected by allogeneic recipients and the MHC class I linkage group was the major determinant for the rejection.

Key words: MHC class I, Rainbow trout, Allogeneic graft, Erythrocyte, Fluorescent dye

Introduction

The major histocompatibility complex (MHC) contains a tightly linked cluster of genes, which encode cell membrane glycoproteins, the MHC class I and MHC class II molecules. They appear to be intimately involved in a variety of immunological processes including the restriction of antigen recognition by lymphocytes, the acquisition of the T cell repertoire, and the co-operative interaction among subsets of mononuclear leucocytes. Endogenous antigens are thought to be degraded into peptide fragments that bind to MHC class I molecules within the endoplasmic reticulum, while exogenous antigens are processed into peptide fragments within endosomal compartments and bind to MHC class II molecules (Germain 1999).

Allogeneic MHC molecules can induce both antibodies as well as cytotoxic T -cells directed against the graft (Auchincloss *et al.* 1999). It is generally accepted that mammalian T cells recognize a complex of MHC and endogenous peptide ligands via

TCR (Wang *et al.* 1998). The interaction of TCR with a complex of foreign MHC molecules and foreign peptides results in acute graft rejection. Chronic rejection occurs when the MHCs of the graft donor and the graft recipient are identical but foreign peptides are complexed with the MHC of the grafted cells (Janeway and Travers 1995).

MHC class I and II genes have been identified in many fish (McConnel *et al.* 1998, Hashimoto *et al.* 1999) and are supported to be involved in allograft rejection. Though allograft rejection was studied in many fish species (reviews: Manning and Nakanishi 1996, Nakanishi *et al.* 2002), very rare works with the identification of MHC gene function in allorejection were published. So far only one study in the Gila top minnow (*Poeciliopsis o. occidentalis*) was reported where MHC class II allele-matched scale grafts were better accepted by the recipient than non-matched allografts (Cardwell *et al.* 2001). Functional analysis of MHC class I is also scarce and inconclusive (reviewed in Nakanishi *et al.* 2002).

Only one classical MHC class I locus in rainbow trout, (*Oncorhynchus mykiss) Onmy-UBA*, with very low homology between alleles was identified so far (Hansen *et al.* 1996, Shum *et al.* 2001, Aoyagi *et al.* 2002, Xia *et al.* 2002). Different lineages are distinguished for the different domains, and they co-exist in various combinations at the *Onmy-UBA* locus (Aoyagi *et al.* 2002). In rainbow trout, the lineages Sal-MHCIa*A to – K were distinguished (Hansen *et al.* 1999, Shum *et al.* 2001, Aoyagi *et al.* 2002, Xia *et al.* 2002).

Fish erythrocytes are nucleated as like other lower vertebrates such as birds and amphibians (Delany *et al.* 1987, Flajnik and Du Pasquier 1988) and have been shown to express MHC class I molecules on their surface. The purpose of the present study was to investigate the involvement of MHC class I locus in allogeneic graft rejection by excluding involvement of MHC class II. Since erythrocytes do express only MHC class I and do not express MHC class II, erythrocytes were collected from the unsensitized MHC class I characterized rainbow trout and grafted it to the allogeneic DD and DF siblings to determine the rejection of grafted erythrocytes *in vivo.* The involvement of MHC class I to allogeneic erythrocyte graft rejection was clearly demonstrated.

Materials and methods

Experimental fish

Rainbow trout (Donaldson strain) stock were maintained in a flowing water system at the National Research Institute of Aquaculture (NRIA), Nikko branch, Japan and used in the present study. The fish were first investigated for determining the Sal-MHCIa lineages using the RT-PCR system described by Xia *et al.* (2002). After characterization, the broodstock, which expressed sequences belonging to the lineages Sal-MHCIa*D and F were selected. Sequence analyses (data not shown) demonstrated that all the brood fish express both the sequences *Onmy-UBA* *701 and *4901, which are classified into the Sal-MHCIa lineages D and F, respectively (Xia *et al.* 2002). Eggs from a *701/*4901 female were collected and fertilized with the sperm of a *701/*4901 male,

resulting in sibling offspring. During the eyed-egg stage, the siblings were transported to the Tamaki branch of the NRIA and kept in 30l tanks supplied with aerated running spring water at 15 °C. The fish were fed twice a day *ad libitum* with commercial trout dry pellets. When the fish became approximately 40-60 g, they were used in the experiment. For identifying the fish, they were tagged at dorsal fin with a plastic anchor tag using a Tagging Gun (103-XL, Bano'k, Tokyo, Japan).

*Detection of Onmy-UBA*701 and *4901 expression*

The adipose fin samples of the rainbow trout were collected and total RNA was isolated using TRIzol Reagent (Gibco BRL, Life Technologies, Grand Island, U.S.A.) following the manufacturer's recommendations. For RT-PCR amplification the 'RT-PCR high-PLUS' kit (Toyobo, Osaka, Japan) was used. The RT-PCR reaction mixtures were formulated following the manufacturer's suggestions, with 2.5 mM $Mn(OAc)_2$, 1 μM of each primer and 0.5 μg total RNA.

The *Onmy-UBA**701 and *Onmy-UBA* *4901 fragments were amplified following the methods of Xia *et al.* (2002) and Sarder *et al.* (2003). The primers used for amplification of the *Onmy-UBA**701 fragment are specific for sequences belonging to lineage Sal-MHCIa*D and can not amplify *Onmy-UBA**4901. Similarly, the primer set used for amplification of *Onmy-UBA* *4901 fragment were specific for sequences belonging to lineage Sal-MHCIa*F and can not amplify *Onmy-UBA* *701. The conditions for both RT-PCR amplifications were: First 60 °C for 30 min, then 94 °C for 2 min, then 35 cycles (94 °C for 1 min, 60 °C for 1.5 min) and finally 60 °C for 7 min. All parents and siblings used in this study were analyzed with both RT-PCRs.

The two parents of the siblings were analyzed with an RT-PCR system for amplification of 'full-length' *Onmy-UBA* fragments encoding the whole protein. The primers *pG-LPf*, 5'-GTATTATCTTGCTGGTGCTGGGAA (forward), binding to the leader peptide region, in conjunction with primer *pG-3'UTRr*, 5'-TTATGTTCTTGAGAAGTTCCTCTTC (reverse), binding to the 3'UTR, can amplify most *Onmy-UBA* alleles discovered thus far (Xia *et al.* 2002). The RT-PCR mixtures were set up as described above, and the amplification schedule was: First 60 °C for 98 min, then 94 °C for 2 min, then 35 cycles of (94 °C for 1min, 55 °C for 5 min), and finally 55 °C for 7 min.

Sequence analysis

Full-length *Onmy-UBA* fragments were cloned into the vector pGEM-T Easy (Promega Corporation, Wisconsin, U.S.A.). The nucleotide sequences were determined by the dideoxychain termination method using a 'CEQ Dye Termination Cycle Sequencing Kit' (Beckman Coulter Inc., California, U.S.A.) and suitable primers. Sequence analysis was conducted with an automated sequencer (CEQ 2000 DNA analysis system, Beckman Coulter.Inc.). For each fish at least three *Onmy-UBA**701 and three *Onmy-UBA**4901 clones were analyzed to exclude PCR errors. Comparison of deduced amino acid sequences was performed using the program 'Multiple alignment' of

GENETYX version 10.1 (Software Development Co. Ltd, Tokyo, Japan) computer software.

Staining of erythrocyte grafts

Fish erythrocyte grafts were stained with PKH67-GL (Green Fluorescent-cell Linker Kit, Sigma, Saint Louis, U.S.A.) and PKH26-GL (Red Fluorescent-cell Linker Kit, Sigma) by following manufacturer's instructions with some modifications. Blood (100 µl) was drawn from the caudal vessel of anaesthetized fish into a syringe containing an equal volume of MEM/FBS (Eagle's minimal essential medium plus 10% foetal bovine serum, pH adjusted to 7.2) with 2% heparin solution. The blood was centrifuged at 250 g for 5 min at 4 °C for crude separation of leukocytes (buffy coat) from erythrocytes. The erythrocyte cells were drawn from the bottom of the pellet, and washed once with MEM (MEM without FBS, pH adjusted to 6.95). The erythrocytes were resuspended in 1 ml of diluent C (supplied with the Sigma staining kits) and then 1 ml of freshly prepared 0.8 µM PKH67-GL (in diluent C) or PKH26-GL was added. Five min after incubation, an equal volume of MEM/FBS was added to stop the staining reaction. The cells were then washed twice with MEM/FBS and finally resuspended in MEM/FBS. Staining of cells was verified by fluorescence microscopy. At the end of staining, the green- and red-stained erythrocytes were mixed together in an approximately equal ratio with a final concentration of approximately 10^7 total erythrocytes/ml. Leukocyte contamination in the erythrocyte grafts was less than 1%.

Determination of survival of the erythrocyte graft

Approximately 400 µl of the green- and red-stained erythrocyte mixture was injected into the caudal vessel of anaesthetized recipients weighing 40-60 g and maintained them in tanks with regular feeding. For determining survival of the grafts at different time points after grafting, 100 µl of blood was collected from the caudal vessel of anaesthetized recipients. These blood samples were examined by fluorescence microscopy.

Results

*Expression of Omny-UBA *701 and *4901 lineages*

The siblings used in the experiments were produced from the same parents who expressed only sequences from the lineages of Sal-MHCla*D (701) and *F (4901). Because the parents were 701/4901, the allelic segregation in the siblings were 701/701(DD), 701/4901(DF) or 4901/4901(FF) in a Mendelian fashion (Fig. 1). This was confirmed by use of RT-PCR amplification reactions specific for sequences of lineages Sal-MHCla*D and *F. Nucleotide sequence analysis for full length *Onmy-UBA* fragments of the parents of the siblings demonstrated that there was low homology between the *Onmy-UBA**701 and *Onmy-UBA**4901lineages (data not shown).

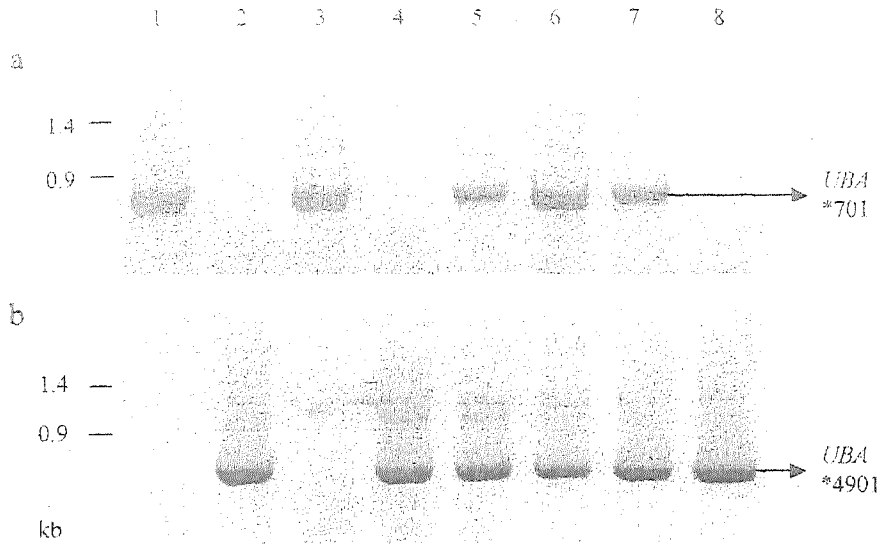

Fig. 1. RT-PCR amplification reactions specific for sequences of lineages Sal-MHCIa*D (a) and F (b). Eight individuals (1-8) of the sibling group were analyzed. The allelic sequences *UBA**701 and *4901 had been determined for the sibling parents. The data (a plus b) indicate that fish 1 and 3 express *UBA**701, fish 2, 4 and 8 express *UBA**4901, and fish 5, 6 and 7 express both alleles.

Survival of autologous grafts

In order to assess the potential toxic effects of the two stains on erythrocyte grafts, an experiment was carried out using four fish not belonging to the same sibling group. Erythrocytes were collected from the four fish separately and stained with either the red or green dye. The red and green stained cells were mixed and re-injected into the same donor fish. To determine the survivability of the stained erythrocytes, blood samples were collected from fish at days 21, 28 and 42 after grafting and checked under fluorescence microscope. Analysis showed that the red and green stained erythrocytes equally survived (Fig. 2a) and the intensity of the stain was quite strong even after 42 days of grafting.

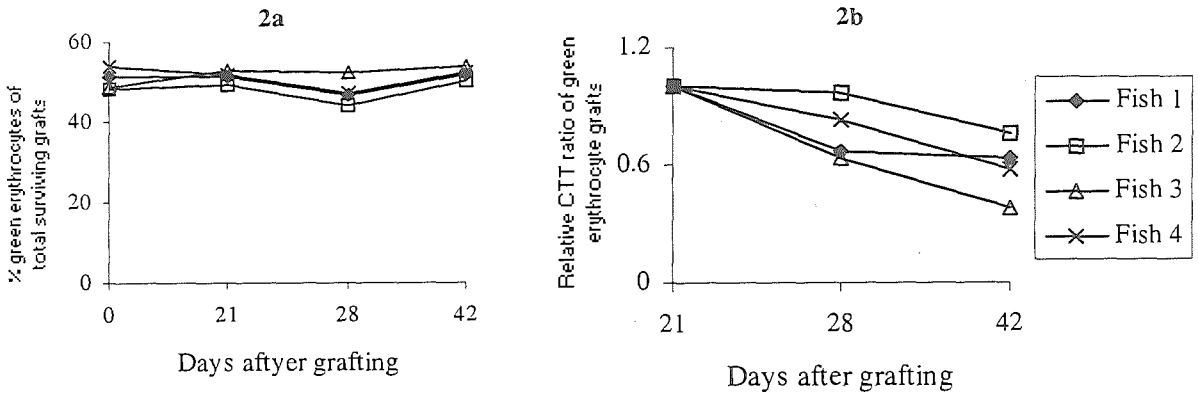

Fig. 2. The graphs (2a) show similar survival of green compared to red autologous erythrocyte grafts. The different symbols indicate four different individuals. The injected graft mixtures contained 51, 48, 49 and 54% green erythrocytes respectively. The graphs (2b) compare the survival of green grafts to the total erythrocytes in the host with the CTT (compared to total) ratio at day 21 arbitrarily assigned a value of 1.

Survival of allogeneic erythrocytes in vivo

Two experiments were carried out to compare the survival of *Onmy-UBA* matching and mismatching allogeneic erythrocytes. In the first experiment, erythrocytes collected from a DD and an FF individual were differently stained and injected to the recipients DD3, DD6, DD8, DD9, DF11, DF19, DF21 and DF23. Similarly, in the second experiment erythrocytes from a DD and an FF were stained and grafted to the recipients DD10, DD14, DD22, DD24, DF1, DF4, DF13 and DF15. In both the experiments donors did not include in the recipient groups. It is necessary to mention that there was no many FF fish other than donors, that's why DF siblings were chosen as recipients instead of FF. To observe the survivability of the grafts the fish blood were sampled at days 7, 14, 21, 28 and 35 after grafting in the first experiment. While fish blood were sampled at 7, 14, 28 and 35 days after grafting in the second experiment. The survival results of the matched and mismatched grafts of the first and second experiments are presented in Fig. 3a and Fig. 4a, respectively. In Fig. 3a, mismatched grafts showed significantly ($p<0.05$) lower survival in all DD recipients. The survival of FF grafts in all DF recipients were significantly ($p<0.05$) lower than those of DD grafts. Although, the experiment continued for 35 days all the DD and the DF23 recipients died before ending the experiment. In Fig. 4a, mismatched grafts had lower survival in all DD recipients except DD22, which died just after first sampling (7 days). All the DF recipients except DF4 showed equal survival for both matched and mismatched grafts. The DF4 recipient demonstrated only 7% survival of mismatched grafts. The survival of the MHC class I-matched grafts was significantly ($p<0.05$) higher than that of the MHC class I-mismatched grafts in all DD recipients in both experiments at all the sampling points.

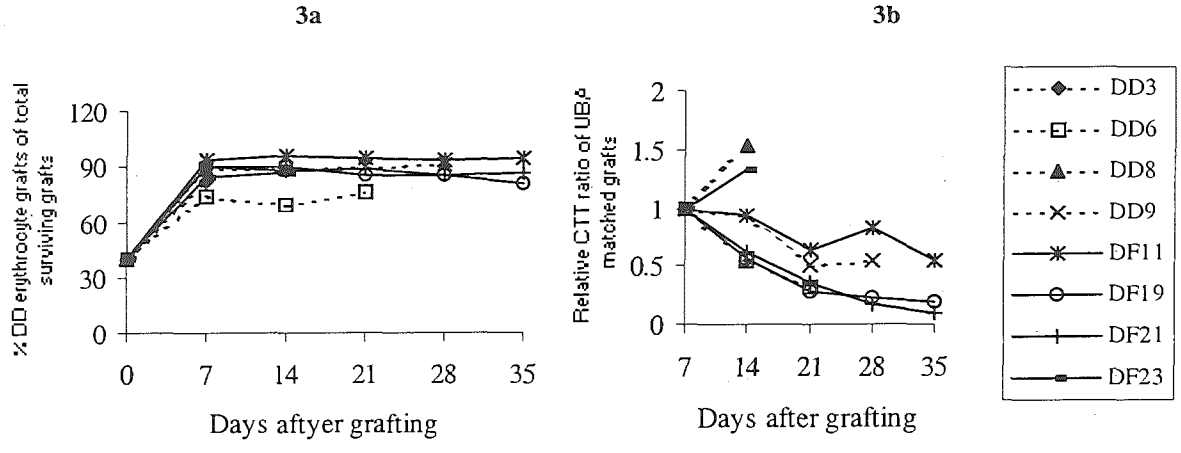

Fig. 3. MHC class I matching is important for the survival of erythrocyte grafts. The injected graft mixture contained 40% DD erythrocytes. Survival of erythrocyte grafts was determined at days 7, 14, 21, 28 and 35 after grafting. Graph indicators at the right apply to both (3a) and (3b) graphs and refer to the recipients. The (3a) graphs indicate the survival of the DD grafts as a percentage of the total surviving grafts (red plus green). The (3b) graphs compare the survival of the MHC class I matched grafts to the total erythrocytes in the host with the CTT (compared to total) ratio at day 7 arbitrarily set to value 1.

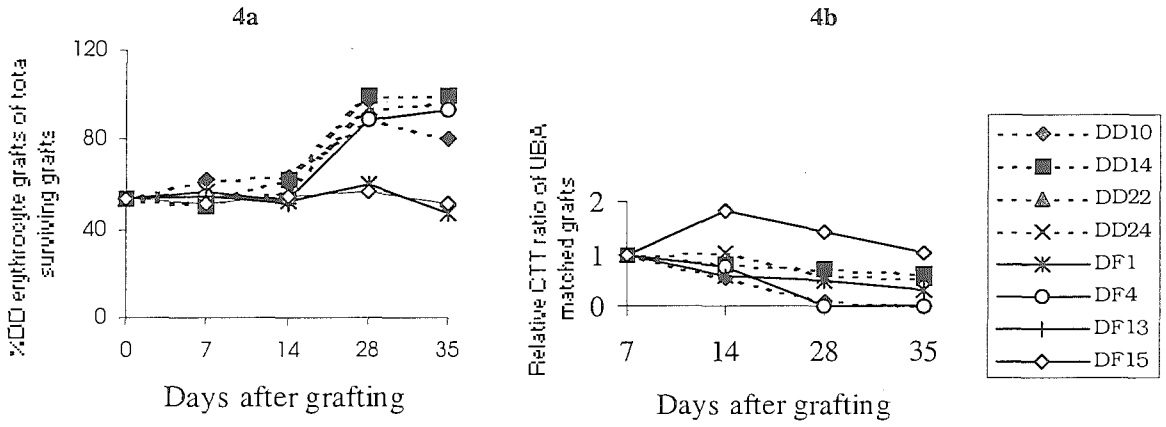

Fig. 4. Survival of MHC class I matched grafts *in vivo.* The injected graft mixture contained 54% DD erythrocytes. Survival of erythrocyte grafts was determined at days 7, 14, 28 and 35 after grafting. Graph indicators at the right apply to both (4a) and (4b) graphs and refer to the recipients. The (4a) graphs indicate the survival of the DD grafts as a percentage of the total surviving grafts (red plus green). The (4b) graphs compare the survival of the MHC class I matched grafts to the total erythrocytes in the host with the CTT (compared to total) ratio at day 7 arbitrarily set to value 1.

Discussion

Data presented in this paper describes the involvement of classical MHC class I locus in the rejection of allogeneic erythrocyte grafts in rainbow trout. In the allogeneic experiments, all DD recipients except DD3 (experiment 1) and DD22 (experiment 2) (died after 7 days of grafting) demonstrated better survival of MHC class I- matched grafts compared to those of MHC class I-mismatched grafts (Fig. 3a and 4a). In the first allogeneic experiment, all the DF recipients rejected the FF allogratfs significantly faster than those of DD allografts. In the second allogeneic experiment, three out of four DF recipients showed equal survival of both allogeneic DD and FF grafts, but the DF4 recipient showed very fast rejection of FF allografts compared to the DD grafts. The overall analysis of results showed that the MHC- mismatched grafts were rejected by all eight DD recipients, five out of eight DF sibling recipients rejected FF grafts significantly faster than those of DD grafts, which was a clear indication of the involvement of MHC class I- linkage groups in the rejection of allogeneic erythrocytes.

The equal survival of FF grafts in DF recipients or faster rejection of FF grafts by DF recipients in allogeneic experiments predicts some assumptions other than the MHC class I linkage group involvement in the allorejection. The first assumption might be the toxic effects of certain stains on erythrocytes that caused high mortality of FF grafts in some DF recipients but it is not convincingly acceptable. Because, the autologous grafts stained with the red and green stains as allogeneic graft staining demonstrated equal survival of both red and green stained grafts (Fig. 2a). The another assumption could be the involvement of phenotypic differences in the immune system of the recipients. Individual phenotypical differences are commonly observed in the fish found immune system (Yoshinaga *et al.* 1994, Alcorn *et al.* 2002).

When compared the survivability of stained grafts with the total harvested erythrocytes in the allogeneic experiments (Fig. 3b and 4b), it was observed that most of the recipient fishes showed similar pattern of survivability as found in the autologous grafts (Fig. 2b), only DD10 and DF4 had profound reduction of stained grafts compared to the total cells, which could be resulted from the loss of big amount of blood from the body due to sampling error. It is wise to mention here that based on the experimental procedures of the two allogeneic experiments, another few more experiments involving DD and FF recipients were conducted and similar rejection responses were observed (Sarder *et al.* 2003).

Only one classical MHC class I sequence is expressed per haploid rainbow trout genome (Shum *et al.* 2001, Aoyagi *et al.* 2002, Xia *et al.* 2002). The homology between many Onmy-UBA allomorphs (proteins encoded by alleles) is very low. For example, the amino acid identity between the α1 and α2 domains of *Onmy-UBA**701 and *4901 is only 42% and 56% respectively (data not shown).

The low homology between the *Onmy-UBA**701 and *4901 allomorphs may have influenced the nature of the cytotoxic response to the erythrocyte allografts. There are two logical effector cell candidates, cytotoxic T -cells and natural killer (NK) cells. These cell types share many characteristics and can under certain conditions even kill the same targets (Yokoyama 1999). In mammals, cell-mediated killing of allografts is primarily performed by cytotoxic T -cells, but dependent on the type of graft tissue NK cells can play a limited role (Manilay and Sykes 1998, Auchincloss *et al.* 1999). NK cells play a more important role in xenograft rejection (Manilay and Sykes 1998, Auchincloss *et al.* 1999). While both cell types have MHC class I binding receptors, MHC binding activates T cells but inhibits NK cells. In fish no cytotoxic T cells or NK cells have been clearly distinguished, but indications for identification of both cell types exist. Genes for the probable T -cell markers TCR (Hawke *et al.* 1999) and CD8 (Hansen and Strassburger 2000) genes, and a possible NK marker in channel catfish (a ligand for MAb CC41, Shen *et al.* 2002) have been identified.

Some *in vitro* cell-mediated cytotoxicity assays for rainbow trout have shown a need for sensitization to detect allograft killing (Fischer *et al.* 2003), reminiscent of T cell activity, while other studies showed spontaneous killing of allogeneic targets by leukocyte populations from some rainbow trout individuals, reminiscent of NK activity (Yoshinaga *et al.* 1994). Cells similar to cytotoxic T cells and cells similar to NK cells

seem to exist in fish and they are capable of killing allogeneic targets. If antibody independent cytotoxicity has played an important role in the erythrocyte graft rejections observed, this was probably mediated by cytotoxic T cells and not NK cells. If antibodies were involved in the graft rejection, NK cells may have killed the allografts by means of antibody-dependent cell-mediated cytotoxicity (ADCC, Yokoyama 1999).

From the above discussion it is concluded that the *Onmy-UBA* classical MHC I lineages are thought to be determinant of rejection of allografts and the present study demonstrated MHC class I involvement in the rejection of allogeneic erythrocytes. However, more studies need to identify the kinds of immune response involving allograft rejection and also to determine some other possible factors, such as blood groups and influence of low homology between MHC class I lineages.

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