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Kinetics of lymphocyte subpopulations in allogeneic grafted scales of ginbuna crucian carp

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ABSTRACT

In mammals the rejection of allografts is primarily accomplished by cell-mediated immunity including T cells. Recently, considerable studies reveal the existence of helper and cytotoxic T cell subsets in fish. Here we investigate the kinetics of CD4$^+$ and CD8α^+ T cells along with sIgM$^+$ cells and phagocytic cells in an allogeneic scale graft model using ginbuna crucian carp for understanding the mechanisms of cell-mediated immune response. The results showed that CD4$^+$ T cells first infiltrated into allogeneic scales followed by CD8α^+ and sIgM$^+$ cells, and finally phagocytic cells appeared in the graft. Furthermore, most of the CD8α^+ T cells appeared on the border of the allografted scales at the time of rejection. These results suggest that T cells play crucial roles and work together with other cell types for completion of allograft rejection.

1. Introduction

In mammals the rejection of allografts is primarily mediated by cell-mediated immunity (Schlitt et al., 1993) and the response is divided into three stages (reviewed by Le Moine et al., 2002). In the first stage, recipient naive T cells recognize alloantigen directly through donor dendritic cells (DCs; Braun et al., 1993; Talmage et al., 1976) or indirectly through recipient DCs (Braun et al., 2001). In the second stage, alloreactive effector T cells become activated and the subpopulation expands. In the final stage, effector T cells accumulate at the site of the target allograft and attack it.

In fish, previous findings indicate that alloantigen specific rejection is mediated by T cells as in mammals. McKinney et al. (1981) reported that in the holostean fish, *Lepisosteus platyrhincus*, the majority of infiltrated cells in acute allograft rejection were mononuclear leukocytes. Abelli et al. (1999) showed that lymphocyte subpopulations were involved in allogeneic muscle graft rejection in sea bass, *Dicentrarchus labrax* (L.), using a monoclonal antibody (mAb) recognizing T cells. However, the subset of T cells involved in allograft rejection remains unknown.

In the 2000s, several studies suggested that alloantigen specific cytotoxicity is mediated by CD8$^+$ T cells. Stuge and his coworkers revealed that alloantigen specific cytotoxic cell clones expressed TCR α and β mRNA (Stuge et al., 2000). Fischer et al. (2003) reported that a T cell fraction enriched by magnetic depletion of B cells and phagocytic cells expressed CD8 mRNA and showed cell-mediated cytotoxicity (CMC) against allogeneic cells.

Recently, we have succeeded in producing monoclonal antibodies (mAbs) against CD8α and CD4 in ginbuna crucian carp, *Carassius auratus langsdorfii* (Toda et al., 2009, 2011b). With the aid of these mAbs, we found that CD8α^+ T cells are the primary cell types showing alloantigen specific cytotoxicity ex vivo (Toda et al., 2009, 2011a) and demonstrated in vitro proliferation of CD4$^+$ T cells by allogeneic combination in mixed leukocyte cultures and antigen-specific proliferation of CD4$^+$ T cells following in vitro and in vivo sensitization with Ovalbumin (OVA) (Toda et al., 2011a). In addition, we investigated the kinetics of CD4$^+$ and CD8α^+ T cell subsets in graft-versus-host reaction (GVHR) along with the pathological changes associated with graft-versus-host-disease (GVHD) and found that donor derived CD4$^+$ and CD8α^+ T cells both play essential roles in the induction of acute GVHR/D in teleosts as in mammals (Shibasaki et al., 2010).

In the present study, we aimed to reveal the mechanisms of cell-mediated immune response *in vivo* in allograft rejection model using

Abbreviations: CMC, cell-mediated cytotoxicity; CTL, cytotoxic T cell; DC, dendritic cell; sIgM, surface IgM.

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ginbuna crucian carp. We investigated the kinetics of CD4$^+$ and CD8α^+ T cell subsets along with sIgM$^+$ and macrophage/granulocyte by flow cytometry and immunohistological study during the scale allograft rejection.

2. Materials and methods

2.1. Fish

Triploid clonal ginbuna crucian carp (*Carassius auratus langsdorfii*) from Lake Suwa (S3N) and Okushiri Island (OB1) were used as donors and recipients, respectively. Fish weighing 20–25 g (1 year old) were maintained in tanks with running water at 25 ± 1 °C and fed twice daily with commercial pellets throughout the experiments.

2.2. Monoclonal antibodies and rabbit antiserum against ginbuna crucian carp CD4 and CD8α molecules

For flow cytometry analysis, previously reported rat mAbs, 6D1 and 2C3 recognizing ginbuna crucian carp CD4 and CD8α, respectively (Shibasaki et al., 2010; Toda et al., 2009, 2011a) were used. For analyzing the tissue distribution of T cell subsets by immunohistochemistry, mAb 2C3 against ginbuna CD8α, rabbit anti-CD4 antiserum and mouse mAb GB21 recognizing macrophages/granulocytes were used.

2.3. Scale grafting

Donor S3N and recipient OB1 fish were anesthetized with 35 ppm ethyl-4-aminobenzoate (Benzocaine, Sigma, St. Louis, MO, USA). Six scales from S3N fish were transplanted above the lateral line, and six others were transplanted below the lateral line of OB1 recipients. After transplantation, scales were observed at regular intervals under a stereomicroscope (Olympus SZX12, Olympus, Tokyo, Japan) with digital camera and software (Olympus DP73). The clinical stage of graft rejection was determined as shown in Table 1 based on the frequency of expansion and breakdown of melanophores in grafted scales.

2.4. Preparation of cell suspensions from epidermis

OB1 recipient fish were anesthetized, bled from the caudal vessels, and euthanized. Grafted scales and surrounding epidermis were harvested at various time intervals after grafting and incubated with PBS containing 1 mM DTT (Wako Chemicals, Osaka, Japan) and 1 mM EDTA for 15 minutes. After incubation, tissues were rinsed with PBS(−), minced with scissors and dissociated by incubating with calcium- and magnesium-free Hank's Balanced Salt Solution (CMF-HBSS) containing 0.1 mg/ml collagenase (Wako Chemicals), 0.1 mg/ml DNase (Sigma) and 5% FBS for 90 min with shaking. After digestion, the tissue was disaggregated by pressing through a 150-gauge mesh stainless steel sieve into OPTI-MEM (Gibco, UK) supplemented with 0.5% FBS (OPTI-MEM-0.5). The cells were washed once with OPTI-MEM-0.5, layered onto a Percoll density gradient of 1.08 g/ml and centrifuged at 450 × *g* for 30 min at 4 °C. The cells at the interface were collected and washed three times with the medium.

Table 1
Criteria for clinical stages of allograft rejection.

Stage	Expansion of melanophore	Breakdown of melanophore
0	–	–
1	+	–
2	+	0–20%
3	+	20–80%
4	+	80–100%
5	Disappearance of melanophore (completion of rejection)	

Clinical stages of allograft rejection were defined as indicated. Stage was determined by randomly assessing five scales of each recipient fish using a stereomicroscope.

2.5. Flow cytometry

Cells from recipient epidermis were resuspended in PBS containing 0.5% FBS at a concentration of 1×10^7 cells/ml and incubated with 1:10^4 diluted rat anti-ginbuna CD4 or CD8α mAb (mouse ascites) for 45 min at 4 °C. The cells were then washed three times with buffer, resuspended and incubated for 30 min at 4 °C with 1 ml of a 1:500 dilution of Alexa 488 donkey anti-rat IgG antibody (Life Technologies). The cells were washed an additional three times and then suspended in 0.5 ml of PBS with 2.5 μg/ml propidium iodide (Life Technologies) and 1×10^5/ml of CaliBRITE beads (Becton Dickinson). Lymphocytes were gated on FS & SS dot plot and stained lymphocytes excluding dead cells were analyzed with a FACS Canto (Becton Dickinson). Data collections were stopped when the bead count reached 2.5×10^3. The absolute cell number was calculated as follows: (number of cells counted in lymphocyte or mAb positive fraction) × 20 [×separation number (if the sample was separated for immuno staining)].

2.6. Immunohistological analysis

Skin with associated muscle was dissected from recipients and fixed in 4% paraformaldehyde (Wako Chemicals, Osaka, Japan) for 24 h at 4 °C. After being washed in phosphate-buffered saline (PBS), each tissue was infiltrated with 30% sucrose–PBS for 24 hours at 4 °C, embedded in Tissue-Tek OCT compound (Sakura Finetek), and sectioned at 8 μm with a cryostat (Leica Microsystems, Tokyo Japan) using the Kawamoto film method.

For double-labeling, frozen sections were incubated with a mixture of anti-CD8α mAb and anti-CD4 anti sera, washed, and then stained with secondary antibodies (Alexa 488 donkey-anti-rat-IgG and Alexa 594 goat-anti-rabbit IgG (Life Technologies)). For staining of macrophages/granulocytes, anti- macrophage/granulocyte mAb, GB20, was used as first antibody. After incubation with the antibody, macrophages/granulocytes were stained with Alexa 594 goat-anti-mouse IgG (Life Technologies) as secondary antibody. Negative controls were processed without a first antibody. Sections were then washed three times and nuclei were stained with DAPI (Sigma). Finally the sections were mounted with ProLong Gold anti-fade mounting medium (Life Technologies). Sections were examined by fluorescence microscopy (Olympus IX71) with a digital camera and software (Olympus DP73).

2.7. RNA extraction and cDNA synthesis for expression analysis

Total RNA was extracted from grafted scales and surrounding epidermis of recipient fish using ReliaPrep RNA Tissue Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's protocols and guidelines. cDNA was synthesized from total RNA from each sample using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocols and guidelines.

2.8. Expression analysis of immune-related genes by real-time RT-PCR

For real-time PCR, each target was amplified on the same plate with the housekeeping gene, ef1a (GenBank accession number: AB491676) as reference, using a Thermal Cycler Dice Real Time System (TaKaRa Bio), and relative mRNA quantities were