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Direct antibacterial activity of CD8$^+$/CD4$^+$ T-cells in ginbuna crucian carp, *Carassius auratus langsdorfii*

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ABSTRACT

Cytotoxic T cells (CTLs) constitute an important component of the specific effector mechanism in killing against microbial-infected or transformed cells. In addition to these activities, recent studies in mammals have suggested that CTLs can exhibit direct antimicrobial activity. Therefore, the present investigation was conducted to find out the microbicidal activity of CD8α^+ T cells of ginbuna crucian carp, *Carassius auratus langsdorfii*. The CD8α^+ T cells from immunised ginbuna exhibited the antibacterial activity against both facultative intracellular bacteria and extracellular bacteria. The maximum reduction of viable count of pathogens was recorded with effector (sensitized) cells and target (bacteria) ratio of 10:1 co-incubated for a period of 1–2 h at 26 °C when effector cells were derived from ginbuna 7 days after one booster dose at 15th day of primary sensitization/immunisation. Sensitized CD8α^+ T cells are found to kill 92.1 and 98.9% of *Lactococcus garvieae* and *Edwardsiella tarda*, respectively. No significant difference in the bacterial killing activity could be recorded against facultative intracellular bacteria and extracellular bacteria. The specificity study indicated the non-specific killing of bacteria. CD8α^+ T cells from *E. tarda* immunised ginbuna exhibited 40% of non-specific killing activity against *L. garvieae* and those from *L. garvieae* immunised ginbuna showed 42.7% of non-specific killing activity against *E. tarda*. Furthermore, CD4$^+$ T cells also killed 88% and 95.7% of *L. garvieae* and *E. tarda*, respectively. In addition to T cell subsets, surface IgM$^+$ cells also killed both types of pathogens. Therefore, the present study demonstrated the direct antibacterial activity of CD8α^+, CD4$^+$ T-cells and surface IgM$^+$ cells in fish.

1. Introduction

Cytotoxic lymphocytes which are composed of natural killer (NK) cells and CD8$^+$ cytotoxic T lymphocytes (CTLs) [1–3], are the principal contributors to immune protection from microbial infections and cell transformation. CTLs constitute an important component of adaptive immune response for specific effector mechanism in controlling tumours and viral or bacterial infections. Killing mechanism of CTLs against intracellular pathogens involves the MHC-restricted and antigen-specific recognition and binding of infected host cells [4,5]. Upon recognition by CTLs, killing of the target cell is induced through pathways initiated by death receptors (e.g. Fas) or through granule exocytosis [6,7]. CD8α^+ T cells recognize antigens processed and presented by antigen in the context of MHC Class I antigens. Recently, cytotoxic nature of CD4$^+$ T cells against alloantigens, viral-infected cells in various mammalian system has been also reported [8,9]. Although these activities are well documented, the mechanisms of microbicidal activity of CD4$^+$ T cells have been under investigated.

Recent studies have suggested that CTLs have direct antimicrobial activity and can kill different types of pathogens belonging to bacteria, parasites, and fungi [10]. In contrast to killing of tumour and microbial infected cells, direct killing of extracellular pathogens by CTLs is an apparent MHC-independent event since microorganisms do not express MHC [10]. Recognition and killing mechanisms in direct microbicidal activity of CTLs are largely unknown even in mammals, although specific recognition of antigen via MHC and killing mechanism of infected host cells by CTLs are well-known. Therefore, direct antimicrobial activity of CD4$^+$ and CD8$^+$ T cells could be vital in promoting the antimicrobial cell-mediated immune response.

The presence of alloantigen or virus-specific cytotoxic T cells has been reported in channel catfish [11], ginbuna [12]. Recently, alloantigen specific killing by CD8α^+ T cells [13] along with perforin-dependent cytotoxic mechanism [14] and helper function of CD4$^+$ T cells of ginbuna crucian carps have been also reported [15].

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However, direct microbicidal activity of fish T cell subsets has not been reported yet. Furthermore, recent evidences indicate the phagocytic and microbicidal nature of fish B cells [16–18]. Therefore, we are aiming to evaluate the direct microbicidal activity of T cell subsets (CD8α^+ and CD4$^+$ cells) and other cell types such as surface IgM$^+$ cells and adherent cells of ginbuna.

In the present study we found significant bacterial killing activity by sensitized T cell subsets (CD8α^+ and CD4$^+$ cells) and surface IgM$^+$ cells from immunised ginbuna against target bacterial cells. This is the first report to demonstrate the conservation of direct microbicidal activity of lymphocytes throughout vertebrates and these findings may shed light on the understanding of protection mechanisms against pathogens from phylogenetical point of view.

2. Materials and methods

2.1. Fish

Ginbuna crucian carp, *Carassius auratus langsdorfii* (OB1 clone, collected from the Okushiri island) of 15–20 g was studied in the present investigation. Ginbuna were maintained at 25 (±1 °C) in 60 l glass tanks with running water and were fed twice with commercial pellets.

2.2. Bacteria

One extracellular pathogen *viz.*, *Lactococcus garvieae* and one facultative intracellular pathogen namely *Edwardsiella tarda* was used in the present study. *E. tarda* was kindly supplied by Dr. Mano, Marine Biotechnology Lab., College of Bioresource Sciences, Nihon University, Japan and *L. garvieae*, was obtained from Kyoritu Seiyaku Co. Ltd., Japan.

2.3. Monoclonal antibodies

Monoclonal antibodies (MAbs) against ginbuna CD8α and CD4 were produced as per the method reported by Akashi et al. [19] and the characteristics of the MAbs have been described in our previous papers [13,15]. A MAb against ginbuna IgM was produced in mice by injecting purified ginbuna IgM according to the standard protocol and has been used to separate sIgM$^+$ and sIgM$^-$ cells [12,20].

2.4. Preparation of bacterial antigen

E. tarda and *L. garvieae* were separately grown at 26 °C in brain heart infusion (BHI) broth (Fluka, Japan) for 24 h, and then cells were harvested by centrifuging the broth at 10,000 × *g* for 10 min at 4 °C. The bacterial antigen was prepared by inactivating the live bacterial suspension in phosphate buffered saline (PBS, pH 7.2) with 1% formalin overnight at 4 °C. After overnight inactivation, bacterial cells were centrifuged in a similar manner as described above and finally the pellets containing inactivated cells were suspended in PBS (pH 7.2) after three times washing in PBS (pH 7.2).

2.5. Immunisation of effector donors

Two sets of immunisation schedule (single and with one booster dose) was evaluated to find out the best immunisation schedule for optimum sensitization of effector donors. Ginbuna were intraperitoneally immunised with inactivated bacterial antigens (*E. tarda* and *L. garvieae* @ 10^8 CFU/fish). In the single set of immunisation schedule, fish were immunised with one dose of antigen followed by sampling at 1, 3 and 7th day of post injection. Similarly, with one booster dose immunisation schedule, fish were given booster dose after 7 and 15th day of primary injection followed by sampling at 3 and 7th day of post immunisation. As per the immunisation schedule, trunk kidney from 3 immunised ginbuna on due sampling time was aseptically removed to prepare the effector cells.

2.6. Preparation of effector cells

Leucocyte suspension from the trunk kidney of the immunised ginbuna was prepared by aseptically disaggregating the tissue through sterilized 150-gauge mesh stainless steel sieve in OPTI-MEM (Gibco) supplemented with 0.5% heat-inactivated foetal bovine serum (FBS). The leucocytes suspension was then layered over the Percoll density gradient (1.08 g/ml) at equal proportion followed by centrifugation at 450 × *g* for 30 min at 4 °C. Lymphocytes rich fraction was finally, collected from the interface of cell suspension and percoll. Different cell fractions (CD8α^+/CD4$^+$/sIgM$^+$ cells) from the lymphocytes rich fraction were then separated by using specific mAb raised against respective types of cells followed by magnetic activated cell sorting (MACS; Mini Macs, Miltenyi Biotec) according to the method described by Toda el al. [13]. In brief, 1.0×10^7 cells/ml of trunk kidney cells were incubated with $1{:}10^4$ diluted rat anti-ginbuna CD8α MAb (mouse ascites) for 45 min on ice. The cells were then washed three times with the medium, adjusted to 1×10^8 cells/ml, incubated for 15 min at 4 °C with 1 ml of a 1:5 dilution of magnetic bead-conjugated goat anti-rat Ig antibody (Miltenyi Biotec GmbH, Germany), and washed three additional times. CD8α positive and negative cells were separated with MACS by applying the cell suspension to a plastic column equipped with an external magnet. The CD8α^+ cells were retained in the column, while the CD8α^- cells were not. CD8α^- fraction cells were further separated into CD4 positive and negative fractions using rat anti-ginbuna CD4 MAb (mouse ascites) according to the method described above. Furthermore, CD8α and CD4 double negative cells were separated into sIgM positive and negative fractions using mouse anti-ginbuna IgM MAb (mouse ascites).

2.7. Viability and purity of individual cell fractions

The viability of MACS sorted cell fractions was confirmed by trypan blue dye exclusion. The purity of individual cell fractions was checked by flow cytometry by incubating a portion of CD8α^+ and/or CD4$^+$ cell fraction with FITC conjugated goat anti-rat IgG + M + A antibody (Rockland) with anti-ginbuna CD8 and/or CD4 monoclonal antibodies. Similarly, a portion of sIgM$^+$ cells was incubated with FITC conjugated goat anti-mouse IgG + M antibody (KPL) with an anti-ginbuna IgM monoclonal antibody.

2.8. Preparation of glass adherent leucocytes cells

The glass adherent leucocytes were prepared by spreading 1 ml of trunk kidney leucocyte suspension (1.0×10^7 cells/ml) into sterilized petri-dishes and then the plates were incubated at 30 °C for 4 h in a humidified 5% CO_2 incubator followed by three washes in OPTI-MEM supplemented with 0.5% heat-inactivated FBS to remove the majority of non-adherent cells. The cells were then removed from the petri-dishes by trypsinisation and washed three times with the medium and then the viability and types of glass adherent cells were observed under microscope. Antibacterial activity of adherent cells was also determined in a similar manner done for other effector cells as described elsewhere in the text.

2.9. Antibacterial activity of different effector cells

Colony-forming unit (CFU) assay was conducted to find out the *in vitro* antibacterial activity of different types of effector cells