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Lack of a contact requirement for direct antibacterial activity of lymphocyte subpopulations in ginbuna crucian carp

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ABSTRACT

Cytotoxic T lymphocytes (CTL) recognize and kill cells infected with viruses, intracellular bacteria and tumors with MHC restriction and antigen specificity. In addition to these activities, recent studies in mammals have suggested that CTL can exhibit direct microbicidal activity. In our previous study we documented direct antibacterial activity of CD4+ T cells and sIgM+ cells as well as CD8α+ T cells from immunized fish. However, we also found weak non-specific killing activity of lymphocytes against bacteria. In the present study we further analyzed the weak killing activity of lymphocytes, increasing the effector cell to target bacteria ratio from 10:1 to 10^3:1. Sensitized and non-sensitized effector lymphocytes (CD8α+, CD4+ and sIgM+) separated by MACS were incubated with target bacteria. CD8α+ T cells from *Edwardsiella tarda*-immunized ginbuna crucian carp killed 98%, 100% and 70% of *E. tarda*, *Streptococcus iniae* and *Escherichia coli*, respectively. CD8α+ T cells from non-immunized fish showed similar but slightly lower killing activity than sensitized cells. CD4+ and sIgM+ lymphocytes also showed high killing activity against *E. tarda* and *S. iniae* as found for CD8α+ T cells, although the activity was lower against *E. coli*. Supernatants from all three types of lymphocytes showed microbicidal activity, although the activity was lower than that evoked by effector lymphocytes. Furthermore, the presence of a membrane between effectors and targets did not affect the killing activity. The present results suggest that both sensitized and non-sensitized lymphocytes non-specifically killed target bacteria without the need of contact. The major difference between the present and previous experiments is the E:T ratio. We suspect that there are two different mechanisms in the direct bacterial killing by lymphocytes in ginbuna.

1. Introduction

All over the world one of the fastest rising sectors of food production is aquaculture. However, this growth is hindered by severe economic losses due to many infectious diseases [1]. Antibiotics are not the best choice in countering infectious diseases because of accumulation in the environment and the emergence of antibiotic resistant strains [2]. The best alternative to antibiotics for protection against infectious diseases is vaccination and usage of effective vaccines to control persistent and emerging diseases have significant positive impact on the reduced usage of antibiotics [3]. However, evaluation of protection for most of vaccines was based on parameters relevant to humoral immunity and not cell-mediated immunity. In spite of accumulating evidences that cell-mediated immunity play important roles in the protection against viruses and intracellular bacteria, little is known about cell-mediated immunity in fish [4].

Immunity against extracellular bacteria depends on antibodies, complement factors and phagocytic cells while protection against intracellular pathogens is highly dependent on cytotoxic T lymphocytes (CTLs) [5] and activated monocytes/macrophages stimulated with IFN-γ [6]. Effective immunity to intracellular bacterial infection often requires lysis of infected cells as well as killing of the invading pathogen [7]. CTLs are required for clearance of intracellular bacteria and recognize bacterial peptides presented by MHC molecules on the surface of infected cells. However, in mammals MHC-independent direct antimicrobial activities of T cells against different pathogens have been reported [8].

Recently, we reported the direct antibacterial activity of CD8α, CD4+ T cells and sIgM+ cells against both intracellular and

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extracellular bacteria in ginbuna crucian carp [9]. Although we demonstrated higher killing activity of immunized effector cells when compared to that of non-immunized cells at an E:T ratio of 10:1, we detected low (15–20%) but significant killing activity even in non-immunized effector cells. Furthermore, in mammals, results from *in vitro* studies indicate that contact is required between T cells and pathogens such as fungi and parasites to achieve direct killing activity [8], while the contact is not required for bacteria [10]. At present, a contact requirement between fish effector cells and target bacteria remains unknown.

In the present study we further analyzed the weak killing activities of non-immunized effector cells, increasing the E:T ratio from 10:1 to 1000:1. We also examined the requirement for contact between effector cells and target bacteria together with the specificity of the killing. We found high antibacterial activity in T cell subpopulations ($CD8\alpha^+$ and $CD4^+$) and $sIgM^+$ cells from both immunized and non-immunized ginbuna crucian carp at an E:T ratio of 1000:1, without contact between bacterial targets and effector cells. Our findings suggest that there are two different mechanisms in the direct bactericidal activity of lymphocytes, and that lymphocytes are involved in innate cellular immunity in fish.

2. Material and methods

2.1. Fish

Ginbuna crucian carp, *Carassius auratus langsdorfii* (OB1 clone, collected from Okushiri Island) weighing 25–40 g were used in this study. Fish were maintained at 25 ± 1 °C in 60 l tanks with running water and were fed once daily with commercial pellets.

2.2. Bacterial strains

Two gram-negative bacteria, *Edwardsiella tarda* FPC347 as an intracellular bacterium and *Escherichia coli* IAM1239 as an extracellular bacterium as well as *Streptococcus iniae* no. 20 as a gram-positive extracellular bacterium were used in this study. These were kindly provided by Dr. Mano in the Department of Marine Science and Resources of our university.

2.3. Monoclonal antibodies

Monoclonal antibodies (MAbs) against ginbuna CD8α and CD4 were produced in rat according to Akashi et al. [11] and the characteristics of the MAbs have been described in our previous papers [12,13]. A MAb against ginbuna IgM was produced in mice using a standard protocol and has been used to separate $sIgM^+$ and $sIgM^-$ cells [14,15].

2.4. Preparation of bacterial antigen

E. tarda and *S. iniae* were grown separately in Tryptic Soy (TS) broth (Eiken chemical Co. Ltd, Japan) at 26 °C for 24 h. *E. coli* was grown in TS broth at 37°°C for 24 h. Bacterial cells were harvested by centrifuging the broth at 1000× *g* for 10 min at room temperature. Bacterial antigen was prepared by inactivating the live cells in PBS (pH 7.3) with 1% formalin overnight at 4 °C. The bacterial cells were then collected as described above and finally suspended in PBS after three washes.

2.5. Immunization of effector donors

Ginbuna were intra-peritoneally immunized with inactivated bacterial antigen (*E. tarda* at 10^8 CFU/fish) followed by one booster dose seven days after the first immunization. Seven days after the booster injection, fish were killed and head and trunk kidney from three immunized ginbuna were aseptically removed to prepare effector lymphocytes. For the studies with neutrophils, tissues were sampled three days after the final immunization because of their short life span.

2.6. Preparation of effector cells

Head and trunk kidney tissues from both immunized and non-immunized fish were aseptically disaggregated through a sterilized 150-gauge mesh stainless steel sieve in HBSS (Nissui Pharmaceutical Co. Ltd, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.5% bovine serum albumen (BSA) (Wako Pure Chemical Industries, Ltd, Japan), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Wako Pure Chemical Industries, Ltd, Japan).

2.6.1. Preparation of lymphocyte subpopulations

The leucocyte suspension was layered over a Percoll (GH Healthcare, USA) density gradient ($r = 1.08$ g/ml) and centrifuged at 450× *g* for 30 min at 4 °C. The lymphocyte-rich fraction at the interface was collected and washed three times with HBSS. Magnetic activated cell sorting (MACS; Mini Macs, Miltenyi Biotec) was used to obtain different cell fractions ($CD8\alpha^+$/$CD4^+$/$sIgM^+$ cells) from the lymphocyte-rich fraction after incubation with specific mAb against the respective cell types according to Toda el al [12]. Briefly, 1.0×10^7 cells/ml of kidney lymphocytes in HBSS were incubated with $1{:}10^4$ diluted rat anti-ginbuna CD8α MAb (mouse ascites) for 45 min on ice. Cells were then washed three times with HBSS and adjusted to 1×10^8 cells/ml, incubated for 30 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. Each cell suspension was then applied to a plastic column equipped with an external magnet to separate the $CD8\alpha^+$ and $CD8\alpha^-$ fractions. Similarly, $CD4^+$ and $sIgM^+$ cells were separated using rat anti-ginbuna CD4 MAb and mouse anti-ginbuna IgM MAb, respectively.

2.6.2. Preparation of neutrophil cells

The kidney cell suspension was layered over two gradients ($r = 1.075$ and 1.09 g/ml) of Percoll and centrifuged at 400× *g* for 30 min at 4 °C. Cells above the 1.09 Percoll layer were then collected followed by three washes with HBSS and the cells were then counted.

2.7. Viability and purity of individual cell fractions

The viability of lymphocytes and neutrophils was assessed by Trypan blue dye exclusion while the purity of the MACS-sorted lymphocyte cell fractions was confirmed by flow cytometry. A portion of MACS-sorted $CD8\alpha^+$ and/or $CD4^+$ cell fractions was incubated with FITC-conjugated goat anti-rat IgG + M + A antibody (Rockland) following incubation with anti-ginbuna CD8α and/or CD4 MAbs. Similarly, a portion of the $sIgM^+$ cells was incubated with FITC-conjugated goat anti-mouse Ig G + M antibody with anti-ginbuna IgM MAb. In order to remove any antibiotics, effector cells were washed three times and re-suspended in antibiotic-free RPMI 1640 (Nissui pharmaceutical co. ltd, Japan) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Wako Pure Chemical Industries, Ltd, Japan).

2.8. Antibacterial activity of different effector cells

The direct killing activity of effector cells ($CD8\alpha^+$, $CD4^+$, $sIgM^+$ cells and neutrophils) was evaluated using the colony-forming unit