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Preparation method of lamprey antisera and activity assay

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ABSTRACT

Lampreys, the surviving representative of jawless vertebrates, have been a focal point in the search for the evolutionary origin of adaptive immunity. They have independently evolved the variable lymphocyte receptor (VLR)-based adaptive immune system that protects themselves from infection by a variable of microorganisms. The standard immunization schedule for Japanese lamprey (*Lampetra japonica*) was established to prepare antisera by injection of *Escherichia coli*, *Bacillus proteus*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, RRBCs, SRBCs, NB4 cells and Hela cells. In this study, we demonstrated the activities of lamprey antisera, which might be helpful to research the collaboration between VLR-based adaptive immune system and complement system in jawless vertebrates.

1. Introduction

Jawless vertebrates (with Lamprey for example) have been a focal point in the search for the evolutionary origin of adaptive immunity because of their unique position in chordate phylogeny [1]. Prior to the jawless vertebrates, the invertebrates relied on germline-encoded molecules and phagocytes for immune recognition and microbial defense, which are known as the innate immunity [2]. Then the jawed vertebrates have evolved adaptive immune system which is based on highly diverse antigen receptors. A characteristic of the system is its capacity to maintain a memory of previous pathogenic encounters. Moreover, this memory provides an accelerated response for the organism to repel a second invasion.

In 2004, Pancer et al. [3] confirmed the adaptive immune system of jawless vertebrates is based on variable lymphocyte receptors (VLRs) which are generated by tandem array of highly diverse leucine-rich-repeat (LRR) motifs as basic structural units. Therefore, lamprey could be the best model organism to investigate the adaptive immune system that is not based on immunoglobulin. Three types of VLR genes (VLRA, VLRB and VLRC) have been identified [4,5]. Only VLRB$^+$ lymphocytes could undergo lymphoblastoid transformation, proliferation, and differentiation into plasmacytes and finally secrete multivalent VLRB antibodies [6–8].

In this study, we established the immunization schedule for Japanese lamprey (*Lampetra japonica*) to prepare antisera by injection with various kinds of antigens: *Escherichia coli* (*E. coli*), *Bacillus proteus* (*B. proteus*), *Staphylococcus aureus* (*S. aureus*), *Mycobacterium smegmatis* (*M. smegmatis*), rabbit red blood cells (RRBCs), sheep red blood cells (SRBCs), NB4 cells and Hela cells. Moreover, the remarkable activities of lamprey antisera against corresponding antigens have also been testified.

2. Material and methods

2.1. Animals, bacterial strains and cells

Adult male and female Japanese lampreys were obtained from the Songhua River region of Heilongjiang province, China. These lampreys were kept in aquariums before being immunized according to the schedule. The bacteria were kindly supplied by Mingjie Xie, Department of Microbiology of Liaoning Normal University, RRBCs and SRBCs were obtained from rabbit and sheep, respectively. Hela and NB4 cells were presented by Prof. Jianing Zhang of the Institute of Dalian Medical University.

2.2. Immunization schedule with various doses of Escherichia coli

96 healthy lampreys (without trauma) were divided equally into four groups. They were separately immunized with 100 μl

Abbreviations: VLR, variable lymphocyte receptor; IgSF, immunoglobulin superfamily; LRR, diverse leucine-rich-repeat; RRBCs, rabbit red blood cells; SRBCs, sheep red blood cells; i.p., intraperitoneal injection; i.m., intramuscular injection.

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Table 1
Immunization schedule of *Escherichia coli*.

Time of immunization	Dose of *E. coli*	Way of immunization/test
1 (Day 1)	Group 1: 1×10^2 each Group 2: 1×10^4 each Group 3: 1×10^6 each Group 4: 1×10^8 each	Intraperitoneal injection
Day 5	Antiserum preparation	Test of bacteriolytic activity of antisera
2 (Day 10)	Group 1: 1×10^2 each Group 2: 1×10^4 each Group 3: 1×10^6 each Group 4: 1×10^8 each	Intraperitoneal injection
Day 15	Antiserum preparation	Test of bacteriolytic activity of antisera
3 (Day 20)	Group 1: 1×10^2 each Group 2: 1×10^4 each Group 3: 1×10^6 each Group 4: 1×10^8 each	Intraperitoneal injection
Day 25	Antiserum preparation	Test of bacteriolytic activity of antisera
4 (Day 30)	Group 1: 1×10^2 each Group 2: 1×10^4 each Group 3: 1×10^6 each Group 4: 1×10^8 each	Intraperitoneal injection
Day 35	Antiserum preparation	Test of bacteriolytic activity of antisera

0.01 M PBS containing 10^2 *E. coli*, 10^4 *E. coli*, 10^6 *E. coli* and 10^8 *E. coli* at 10 day (named Day1, Day10, Day20 and Day30) intervals by four intraperitoneal injections. The control animals were injected with normal saline. Then the bacteriolytic effect of lamprey antiserum was testified 5 days after the immunization each time. The specific details of immunization schedule with *E. coli* are in Table 1.

2.3. Immunization schedule with various kinds of antigens

Lampreys were immunized with eight kinds of antigens at 10-day intervals by four intraperitoneal injections. The antigens were 10^8 *E. coli*, 10^8 *B. proteus*, 10^8 *S. Aureus*, 10^8 *M. smegmatis*, 10^7 RRBCs, 10^7 SRBCs, 10^6 NB4 cells and 10^6 Hela cells.

2.4. Collection of lampreys antisera stimulated by various kinds of antigens

Each lamprey was sterilized with 75% alcohol first. Total blood was collected and incubated at 4 °C for 1 h before centrifugation at 7000 rpm for 15 min. The upper antiserum were extracted and preserved at −20 °C.

2.5. Agglutination assay

The antisera were heated at 56 °C for 30 min, and then serially diluted in 2-fold increments from 1/2 to 1/256 with normal saline. 50 μl of each antisera dilution was added to 50 μl antigen in 96-well flat-bottom plates and incubated at 37 °C for 1 h and 4 °C overnight before visual inspection for antigen agglutination by light microscopy.

2.6. Determination of cytolytic effects of lamprey antisera

To determine the temperature dependence of the stimulated sera, the lamprey antisera were pre-treated at various temperatures (4 °C–65 °C) for 20 min. Then the treated sera were incubated with RRBCs. Determination of the optimum sera concentration for cytotoxicity was analyzed according to the method of Liang et al. [9]. In brief, antisera were mixed with RRBCs at sera concentrations ranging from 0.5% to 40% (v/v). The optimum time research of antisera cytotoxicity was carried out through incubating respective antigens at 4 °C for different period (5 min–120 min). To determine the effects of Ca^{2+} and Mg^{2+} on cytotoxic activity, the antisera were pre-incubated with serial dilutions of EDTA, and then mixed with RRBCs and incubated at 4 °C for 30 min before evaluation of the cytotoxic activity.

2.7. Analysis of cytolytic effects of lamprey antisera

Tumor cells (Hela cells and NB4 cells), and RBCs (RRBCs and SRBCs) were suspended to 5×10^7 cells/ml in normal saline. Lamprey antisera were added to corresponding antigens and incubated at 4 °C for 20 min. The treated cells were washed and re-suspended in normal saline. The numbers of survival cells were directly counted with a hemocytometer. Normal saline was used as blank control; the sera stimulated by normal saline and naive sera were used as negative controls.

$$\text{Percentage of cell lysis}\,\% = \frac{5\times10^7 - \text{The numbe of survival cells treated by lamprey antiserum}}{5\times10^7} \times 100\% \quad (1)$$

Bacteria (*E. coli*, *B. proteus*, *S. Aureus* and *M. smegmatis*) were suspended to 5×10^7 cells/ml in normal saline. Lamprey antisera were added to corresponding antigens and incubated at 4 °C for 30 min. The treated bacteria were washed and re-suspended in normal saline. The bacteria were inoculated onto LB agar plates and incubated for 14 h at 37 °C. Colony-forming units were counted for viability and the average number of colonies was estimated from three plates. At time zero (before incubation with antiserum), the number of colony-forming units (CFU) was taken as 100%. Normal saline was used as blank control; the sera stimulated by normal saline and naive sera were used as negative controls.

$$\text{Percentage of cell lysis}\,\% = \frac{\text{The number of CFU(time zero)} - \text{The number of CFU(14hours later)}}{\text{The number of CFU(time zero)}} \times 100\% \quad (2)$$

2.8. Analysis of antigen specificity

In order to research the antigen specificity of lamprey antisera, *E. coli*, *B. proteus*, *S. Aureus*, RRBCs and Hela cells were incubated with the antiserum stimulated with *E. coli*. The cytolytic and agglutination effects of lamprey antisera were analyzed as mentioned above.

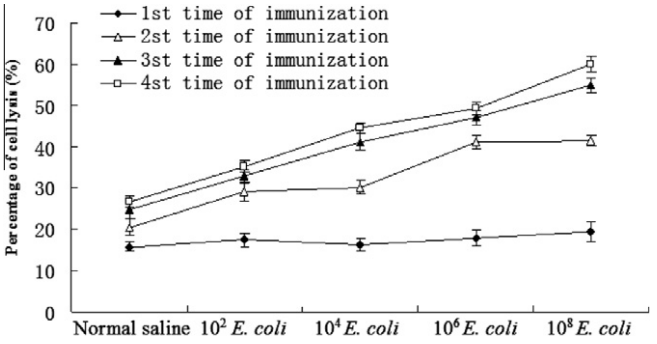

Fig. 1. Bacteriolytic activity of lamprey antisera immunized by *E. coli*. Data represent mean percentages ± SE, Error bars indicated standard error of mean (s.e.m), $n = 3$.