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IN VITRO CULTURE OF PERIPHERAL BLOOD LEUKOCYTES OF *CHANNA PUNCTATUS*

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Cytokines are the most studied immunological molecules which play a crucial role in immunomodulatory processes involving functional T leukocytes. Cytokines are small cell-signaling protein molecules that are secreted by numerous cells mostly by peripheral blood leucocytes. *In vitro* culture of leucocytes plays an important role in the study of different cytokines and its role in immune system. We developed and optimized a simple and efficient method for *in vitro* culture of peripheral blood leukocytes of snakehead fish (*Channa punctatus*), using various parameters including culture medium, different mitogens and its concentration and incubation time. In the present study, peripheral blood leukocytes (PBLs) were isolated from the blood of *C. punctatus* using percoll and ficoll gradient solutions and were cultured *in vitro* in different culture media. The PBLs were stimulated with different mitogens such as phytohaemagglutinin (PHA), concanavalin A (ConA) and phorbol myristate acetate (PMA) to optimize the growth of peripheral blood leukocytes. Growth kinetics was observed to be comparatively more in PHA induced culture media. This study established an *in vitro* culture model that allowed us to study the growth kinetics of peripheral blood leukocytes in different media in response to different mitogens and incubation period.

INTRODUCTION

The diversifications of aquaculture practices for the rapidly expanding sector have necessitated a greater understanding of disease mechanism in fish. Recent advancement in mammalian cellular immunology has provided new investigative and diagnostic procedures for accessing immune function and post infection interaction. Many of these procedures required the isolation and enrichment of leucocytes population. Various modifications of the discontinuous density gradient procedure developed by Böyum (1968) have been utilized to achieve highly pure leucocytes fraction in mammalian and avian systems. Among teleosts, single layer 1.077 gm/ml discontinuous gradient of ficoll and sodium diatrizoate (Histopaque 1.077, Sigma, St. Louis, MO, USA) has been used to separate leucocytes from blood (Faulmann *et al.*, 1983, Scott and Klesius, 1980). Multiple layers of density gradient have also been used successfully to separate leucocyte

populations from the pronephrous of rainbow trout and Atlantic salmon (Braun-Nesje *et al.*, 1981) and carp (Bayne, 1986). Waterstrat *et al.* (1988) used a discontinuous percoll gradient technique for the separation of channel catfish peripheral blood leucocytes. The discontinuous percoll gradient technique provides a straight forward procedure for obtaining enriched population of leucocytes for using assay of cellular immune function or interleukin assay. Faulmann *et al.* (1983) used a variety of mitogens to determine whether peripheral blood leucocytes (PBLs) of channel catfish undergo *in vitro* mitogenic responses. They conducted the experiments with PBLs cultured at 28 °C in RPMI-1640 medium supplemented with 10% human serum in presence of various mitogens. Each of these mitogens such as LPS, dextran sulphate, PHA and pokeweed mitogen stimulated the leucocytes to varying degrees of ^{3}H thimidine incorporation during 2-4 days. They observed the mitogenic response to LPS and ConA was stronger and more predictable than these two other mitogens. Few reports are available for lymphocyte cultures of tortoises in different culture media and its response to different mitogens (Maecha, 1998; Ulsh *et al.*, 2000; Ortiz and Rodríguez, 2003). Mitogenic response in PBL culture produced some of the interleukins and growth of the leucocytes was more within 72 h culture periods.

The present study was aimed to find out short term growth of PBLs of *Channa punctatus* in different culture media with incorporation of different mitogens like PHA, ConA and PMA. As culture of *C. punctatus* is growing day by day, it is important to standardize the *in vitro* PBL culture in order to study some of the immune related genes and provide a gateway of *in vitro* immunity study.

MATERIALS AND METHODS

Chemicals

Standard cell culture media like modified Eagle's medium/Ham's F12 (MEM-F12), RPMI 1640 and B-302, antibiotics (penicillin-streptomycin) and trypsin (from porcine pancreas), MS-222 (E10521, CAS Number: 886-86-2) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); while phosphate buffered saline (PBS), fetal bovine serum (FBS), non-essential amino acid (NEAA) and pancreatin were from Gibco Laboratories (Grand Island, NY, USA). Millipore filters were purchased from Millipore Corp. (Billerica, MA, USA). Anti-coagulating EDTA KE tubes were obtained from Sarstedt Ag. & Co (Nürecht, Germany). Ficoll Paque-Plus and Percoll were purchased from Amersham Biosciences (GE Healthcare, NJ, USA).

Experimental fish

Apparently clinically healthy snakehead fish *C. punctatus* (average weight of 200 g) were collected from the private fish ponds of Bhubaneswar, India and were acclimatized for three weeks in fiber reinforced circular tanks of 500 l capacity in the wet

laboratory of Fish Health Management Division of Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India. Fish were fed daily with a laboratory hand made diet at 3% body weight twice per day.

Isolation of peripheral blood leukocytes (PBLs)

Experimental fish were anesthetized with MS-222 (E10521, CAS Number: 886-86-2, Sigma, USA) using one ml syringes with 25 x g 5/8'' gauge needle. 2 ml of peripheral blood was taken from caudal vein of each fish using syringe with one milliliter syringes with 25 x g 5/8'' gauge needle containing heparin sulfate (100units/ml; BDH chemicals) to prevent blood coagulation. The blood was diluted with equal volume of phosphate buffered saline (PBS, pH 7.2) and allowed to stand for 5 min before being layered on the top of the percoll (Sigma, Cas Number: 65455-52-9, Product Number:F4375)/ ficoll gradient solution (Sigma, CAS Number: 26873-85-8, Product Number:P1644). The diluted blood was carefully layered on top of the gradient solutions in 15 ml sterilized centrifuge tube by using small pipette. The tubes were capped and the blood was centrifuged without brakes at 400 x g for 40 min at RT. The top layers of lysed RBC and plasma were discarded and PBL were carefully collected at the interface. PBL were washed once in 10 ml of 1x PBS and cells were pelleted by centrifuging at 400 x g for 5 min at RT. Traces of RBC remaining were further removed by adding 2 ml of sterile distilled water for 30 s to lyse the RBC. An equal volume of 2x PBS was quickly added (1:1 ratio) to equilibrate the osmotic pressure. PBLs were centrifuged at 400 x g for another 5 min and cell counts were performed using a haemocytometer to determine cell concentration. The cells were suspended in a small volume of medium containing penicillin and streptomycin and the live cells were counted by dye exclusion method using trypan blue solution (Sigma, Product Number: T8154).

Cell seeding and cell viability

The culture media were prepared as per the specifications given by the manufacturer; 10% FBS was added and filter sterilization was done by using Millipore filter. Sterility of the above prepared cell culture media were checked prior to use. A concentration of $1x10^6$ cells/ml was seeded in 25 cm^2 tissue culture flask in all the three media RPMI 1640, B-302 and Minimum Essential Media (MEM). Cultures were incubated at 28 °C in 5% CO_2 in a CO_2 incubator (Thermo stericult, Model-3307, USA) for 3 days until confluent. In 24 hour cultured flasks, the cells were stimulated with different mitogens like phytohemagglutinin-M (PHA) (Grand Island Biological Co, USA), concanavalin A (Con A, L7647, Sigma, USA) and phorbol 12-myristate 13-acetate (PMA, P8139, Sigma, USA) at a concentration of 5 µg/ml and incubated further at 28 °C and 5% CO_2. After the post stimulation incubation of 24 h, 48 h and 72 h, growth kinetics was observed by viable cells counting by dye exclusion method using trypan blue solution.

RESULTS AND DISCUSSION

PBL was separated by using percoll and ficoll gradient solutions. In each method, ten trials (10 numbers of fish) were taken and leucocytes were separated. In percoll gradient solution, the PBL count was varied from $13.6x10^6$ to $22.8x10^6$ cells/ml of the blood whereas ficoll gradient solution, the leucocyte cell density was varied from $10.2x10^6$ to $20.6x10^6$ cells/ml of blood. The detailed estimation of PBL count from snakehead fish separated by percoll gradient solution and ficoll gradient solutions are given in Fig. 1. It was observed that percoll gradient solution is more efficient than ficoll gradient solution for the separation of PBLs.

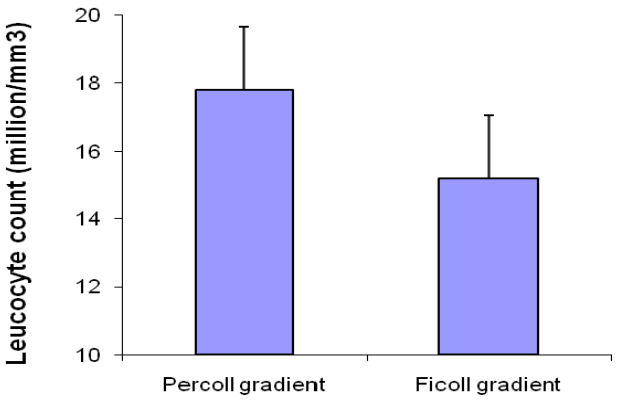

Fig. 1. Comparison of separation of leucocytes by percoll gradient and ficoll gradient solutions.

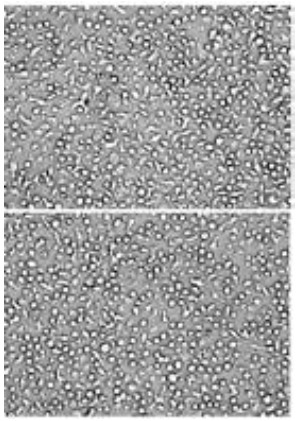

Fig. 2. Image showing culture of PBL in T-25 flask in MEM media

$3x10^6$ numbers of cells were seeded in T-25 cm^2 flask containing different media (Fig. 2). The growth kinetics was observed in each media after 24, 48 and 72 hour of culture period. It was observed that unstimulated PBLs showed highest growth in MEM media after 72 hour culture period while slowest growth was observed in B-302 media. In 24 hour culture, RPMI showed highest growth in comparison to MEM and B-302 media. The growth kinetics was significant in RPMI from 24 to 72 hour incubation whereas B-302 media did not show any significant growth from 24 to 72 hour (Fig. 3).

The cells were stimulated with different mitogens like PHA, conA and PMA. The growth kinetics of mitogen induced cell cultures were recorded after 24, 48 and 72 hours. In response to PHA stimulation PBLs showed maximum growth in RPMI media in comparison to MEM and B-302 media. Highest growth was observed after 72 hour culture period in RPMI media (Fig. 4). The mitogenic response of conA in different culture media was observed (Fig. 5) and found that the rate of growth was more in B-302 media in

comparison to other two media, which indicated that mitogenic response of conA is much more in B-302 media. MEM and RPMI media also showed significant growth from 24 to 72 hour culture period. With respect to PMA stimulation RPMI showed highest growth in 72 hour culture period followed by B-302, while MEM showed the lowest rate of growth (Fig. 6).

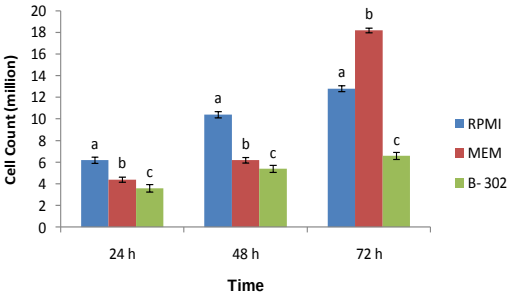

Fig. 3. Growth kinetics of PBL on RPMI, MEM and B-302 media. Data are expressed as Mean ± SE. Means bearing different superscript are significantly (P< 0.05) different from each other.

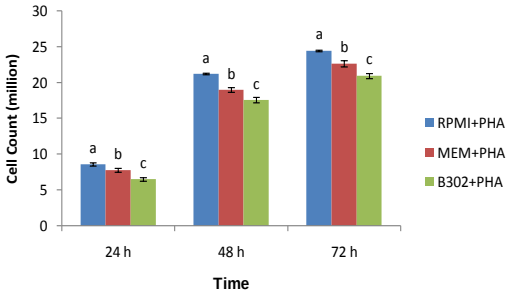

Fig. 4. Growth kinetics of PBL on RPMI, MEM and B-302 media in response to PHA. Data are expressed as Mean ± SE. Means bearing different superscript are significantly (P< 0.05) different from each other.

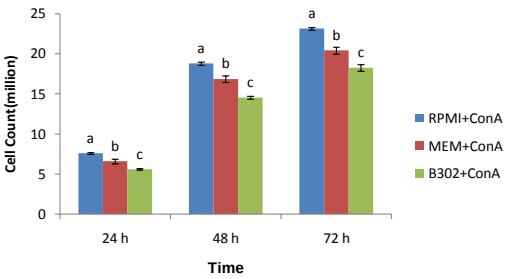

Fig. 5. Growth kinetics of PBL on RPMI, MEM and B-302 media in response to conA. Data are expressed as Mean ± SE. Means bearing different superscript are significantly (P< 0.05) different from each other.

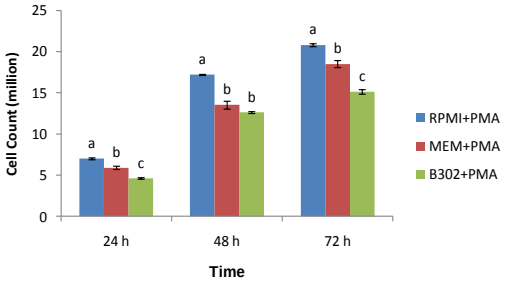

Fig. 6. Growth kinetics of PBL on RPMI, MEM and B-302 media in response to PMA. Data are expressed as Mean ± SE. Means bearing different superscript are significantly (P< 0.05) different from each other.

Unstimulated PBLs showed maximum growth in MEM after 72 hour culture period while RPMI showed maximum growth with PBLs stimulated with mitogens like PHA, ConA and PMA. There is no significant growth was observed in unstimulated cells in B-302 media from 24 to 72 hour culture period while the highest growth was observed with stimulated cells in B-302 media.

In our present set up experiment we have used 3 mitogens i.e. PHA, Con A and PMA to test degree of response and standardize the mitogens that will responsible for more classic B and T-cell mitogens. In the process of screening it was noticed that the degree of mitogenic response for lymphocyte proliferation was highest in PHA followed by conA and PMA respectively in RPMI-1640 medium. Snakehead fish PBL can be stimulated to synthesize DNA *in vitro* by compounds that are considered to be T-cell and B-cell mitogen in other vertebrate system. Their responses to PHA and Con A are quite strong and reproducible, provided suitable culture conditions are used. The dose response of snakehead PBLs to the mitogens are similar to those reported in other fish cells.

There are various cell culture media used for proliferation of fish cells developed from different organs. In the present study we used 3 types of cell culture media with equal enrichment of FCS to find out the growth kinetics of PBLs. Out of the three media, the growths kinetics of PBLs grown in RPMI 1640 was highest followed by MEM and B-302. This might be attributed due to the lack of required constituents in these two media, which did not favor the PBLs to proliferate. The need for serum supplements for *in vitro* culture of cells is a testament to our relative ignorance of the specific requirements for different cells to live and grow. *Channa* cells are not unusual in this regard and the concentration of fetal calf serum (FCS) we used was achieved through trial and error and it observed that 10% FCS showed optimum result. The relative weak responses by *Channa* PBLs to other mitogens initially tested could be due to serum supplements used.

The present work shows the existence of lymphocyte growth promoting activity in snakehead lymphocyte culture supernatant, which is released following mitogenic stimulation such as PHA, Con A and PMA and possibly is similar to IL-2 production in mammals. This study corroborated the findings of Farrar et al. (1982). Lymphocyte growth promoting activity in the piscine counter part of IL-2 is best on its ability to stimulate proliferation of activated, but not of resting carp T-like cells (Caspi & Avtalion, 1984). Indirect evidence presented by them with respect to PMA and ConA reactive lymphocytes suggests that this is indeed the case of stimulating the leucocytes for producing IL2. Results found in our experiment show that mitogen induction could help lymphocyte growth promotion as revealed by cell density enrichment in three culture media tested.

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