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PHA and Con A as Mitogenic Factors for the Cultivation of Lymphocytes of Catfishes

N. GUPTA¹ and D.K. GUPTA²

¹*Department of Animal Science
MJP Rohilkhand University
Bareilly
India*

²*Department of Zoology
Bareilly College
Bareilly
India*

Abstract

The lymphocytes of *Heteropneustes fossilis* and *Clarias batrachus* were isolated by monoculture (centrifugation of diluted blood or Plasmagel method) and Mixed lymphocyte culture (cultured directly from the spleen) and comparative efficacy of both methods were discussed. The lymphocytes were cultured in two growth media, medium A (mitogen Con A; antibiotic penicillin) and medium B (mitogen PHA; antibiotic streptomycin) in the medium RPMI 1640 with 10% FCS and HEPES buffer. The samples were assessed for lymphocyte growth every 24 h for 96 h. The results indicated that PHA is a superior mitogen and MLC is a better technique for causing blastogenesis and proliferation of lymphocytes.

Introduction

Progress in the field of fish immunology goes hand in hand with the increasing interest in fish farming. With the intensification of fish production, increasing stock density and changes in the rearing technology applied, current methods of prophylaxis and therapy are becoming less effective. Thus, fish culturists have begun to pay closer attention to new methods based on the natural ability of fish to protect themselves against pathogens. The preliminary results on immunoprophylaxis in fish culture are encouraging.

Lymphocytes have recently become the focus of interest of immunological investigations due to their protective capability. Mitogens (blastogenic factors) stimulate their proliferation (Carpenter 1975). While the number of valuable contributions on these aspects is gradually gaining momentum, (Kang & Park 1975; Anderson & Hennessen 1981; van Muiswinkel et al. 1985) sporadic investigations on lymphocyte culture are also increasing (Hadden 1978; Blaxhall 1985).

The present work describes isolation of lymphocytes from two species of catfish, *Heteropneustes fossilis* (Saccobranchidae) and *Clarias batrachus* (Clariidae) and their subsequent cultivation (monoculture and mixed lymphocyte culture) in two growth media: growth medium A with Concanavalin A (Con A); and culture medium B with Phytohaemagglutinin (PHA), as mitogens. The relative efficacy of the mitogens is also discussed.

Materials and Methods

Lymphocyte isolation

Live specimens of *H. fossilis* (size 15-20 cms) and *C. batrachus* (size 20-25 cms) were collected from ponds of Rohilkhand region, India and acclimatized to laboratory conditions for 10 days. Blood was collected aseptically from the dorsal vessel using a sterile syringe containing heparin (500 units heparin in 0.2 ml.). One ml of blood was diluted four times in Hanks balanced salt solution, mixed and the lymphocytes separated by centrifugation of diluted blood and Plasmagel method according to Blaxhall (1985). Observations were made on 10 samples for each method. All glassware used were sterilized in hot air and autoclaved prior to experimentation.

CENTRIFUGATION OF DILUTED BLOOD

The diluted blood was centrifuged at 300-350 rpm at room temperature. The supernatent, rich in leucocytes was removed at 5 minutes interval for three times.

PLASMAGEL

Plasmagel (1-2 ml) was mixed with diluted blood and centrifuged at 40-50 g for 10 min. The supernatent leucocyte rich plasma was removed and the original tube was centrifuged again.

Lymphocyte cultivation

The lymphocytes were cultured either after separation from the blood as described above or directly from the spleen (Mixed lymphocyte culture - MLC). In the latter case, the spleen was collected from sacrificed fish and minced carefully in phosphate buffer at pH 7.2. The composition of growth media (A and B) are given in Table 1. The media were membrane filtered (0.34 μ) prior to the addition of the mitogens. Lymphocytes were cultured at 20°C. Observations on blastogenesis were made every 24 h for 96 h. The lymphocytes were smeared on glass slides, fixed in methanol, stained in Giemsa, washed two to three times in acetone, cleared in xylene and mounted in canada balsam.

All investigations were conducted aseptically under a laminar flow hood.

Results and Discussion

Lymphocyte separation methods

The mean recovery of lymphocytes was 45% by centrifugation of diluted blood and was significantly higher ($P < 0.01$) than the plasmagel method (30%).

In mammalian immunology, lymphocyte separation procedures have been established and reviewed by Denman (1973) who has stressed the role of pH, osmolarity of the gradient, cell content, volume and dilution of the sample to affect the separation of cells. However, similar studies on fish blood are far from adequate.

The aim of the separation technique is to obtain maximum recovery of viable lymphocytes for subsequent use. During experimentation, the low yield of lymphocytes by Plasmagel method is probably due to the reason that this procedure yields a mixed suspension of lymphocytes, granulocytes, thrombocytes and a variable number of erythrocytes. Careful controlling of the centrifugation speed is required to avoid undue sedimentation of lymphocytes and gross contamination with erythrocytes. Erythrocyte contamination is generally 5 - 10% after careful handling. The method may be more effective using larger volume of blood.

On the other hand, the cells or their receptors are not damaged due to the low centrifugation speed and the absence of additives in the centrifugation method (Blaxhall 1985). This method proved to be simple and yielded a better recovery rate of lymphocytes than the plasmagel method (as mentioned above). Nevertheless, accurate dilution of the blood, appropriate adjustment of the speed (300 to 350 rpm) and time (5 mins interval) are required to prevent the loss of lymphocytes into the erythrocyte layer.

Lymphocyte cultivation

Results indicate that in both species of catfish, the lymphocytes in growth medium A started to develop in 24 h but the lymphocyte concentration was comparatively very low (Table 2). Apparently, Con A binds to glycoprotein containing α -mannosyl moieties. On the other hand, the growth of lymphocyte in

Table 1. Composition of growth media (A and B) for culture of lymphocytes of *Heteropneustes fossilis* and *Clarias batrachus*.

Composition	Growth Medium A	Growth Medium B
RPMI 1640	100 ml	100 ml
10% FCS	10 ml	10 ml
HEPES buffer	10 ml	10 ml
Antibiotic		
Penicillin	100 IU/ml	-
Streptomycin	-	100 μ g/ml
Mitogen		
Con A	10 mg/ml	-
PHA	-	1 mg/ml

Table 2. Cultivation of fish lymphocytes in RPMI 1640 with Con A and PHA as mitogens (pH 7.2).

Cultivation Method	Growth Medium	Lymphocyte Culture (h)					
		24	48	60	72	84	96
Monoculture							
<i>Heteropneustes fossilis</i>	A	±	+	+	+	+	+
	B	++	++	++	++	++	++
<i>Clarias batrachus</i>	A	±	+	+	+	+	+
	B	++	++	++	++	++	++
MLC	A	++	++	++	++	++	++
	B	+++	+++	+++	+++	+++	+++

The number of + signs denotes the intensity of lymphocyte culture.

medium B peaked at 24 h and persisted up to the culmination of the experiment (96 h). Phytohaemagglutinin is known to induce lymphokine production in cultures (Bellanti 1978).

The best results are obtained in MLC where maximum lymphocytes could be harvested throughout the test period in both the growth media. Mixed lymphocyte culture is an ideal *in-vitro* test for cell mediated immune reaction towards cell surface alloantigens encoded by Major histocompatibility complex (MHC) and can be used for the assay of blastogenesis. During this cultivation, genetically dissimilar lymphocytes are cultured together, blast transformation and mitosis occur and each population of lymphocyte reacts to the foreign histocompatibility antigens of the other population which may account for the enhanced division.

Mitogens are plant proteins which can be added to cultures of non-sensitized lymphocytes to initiate blast transformation (Bellanti 1978). Phytohaemagglutinin (PHA, source - kidney bean, *Phasoleus vulgaris*) and Concanavalin A (Con A, source - jackbean *Canavalia ensiformis*) have been used as mitogens to initiate lymphocyte blastogenesis during the present course of investigations. Both mitogens are known to be specific for T cell sub-set (Chakravarty 1996). The lymphocytes are stimulated to divide by membrane pertruberation induced by cross linking of surface molecules. Mitogens have the potential to bind to and activate large sub-populations of lymphocytes. Con A and PHA are regarded as non-specific stimulants which selectively stimulate the lymphocytes to synthesize DNA and divide thereby inducing blastogenesis in the lymphocyte sub-populations (Oppenheim & Rosenstreich 1976).

Many morphologic and metabolic changes accompany blast transformation. It has been proposed that in the initial stages, lectin binding to specific receptors on lymphocytes lead to changes in membrane permeability and transport of various nutrients and cations, particularly calcium (Segel 1981).

Lymphocyte stimulation has lately been defined in terms of biochemical changes accompanying the process of transformation (Wintrobe 1981). The mitogens interact with a variety of specific membrane receptors (Douglas 1971). In PHA stimulated cells, this step is followed by an increased turnover of membrane phosphatidylinositol and is followed in the next 24 hours by increased accumula-

tion of phospholipids (Fisher & Mueller 1968). An increased acetylation of histones appears to precede the increase in nuclear RNA synthesis indicating changes in the fine structure of chromatin and the ability of DNA to serve as a template for RNA synthesis (Cooper 1971). This is followed by an increased protein synthesis. Morphological changes begin within 24 hours, followed by endocytosis and redistribution of acid hydrolases within lysosomes. Within 24 to 36 hours, there is a gradual increase in the incorporation of 3H -thymidine (Bloom 1971) which has been used as an indicator for the measure of DNA synthesis and the degree of lymphocyte stimulation (Wintrobe 1981).

A number of variables may play a role in the cultivation of fish lymphocytes viz. cyclic nucleotides (Hadden 1978), atmosphere of fish lymphocytes, dose of the mitogen, serum added to the culture medium, stress, hormones, diet, age, disease and drugs (Barta 1983). Results of our investigation indicate that PHA is a superior mitogen specially in the species investigated which can trigger non-specific blastogenesis and proliferation of non-sensitized lymphocytes and MLC, a better technique for lymphocyte culture as compared to monoculture. The present work is an attempt to stimulate studies on antibody formation and lymphokines under adequate sensitization as well as the probability to utilize lymphocytes as biotechnological tools in order to determine their immunizing potency. In the long term, the relationship between lymphocyte blastogenesis and immune response due to sensitization by parasites may prove to be an exciting new area of research in order to unravel many unsolved questions on fish immunity due to an array of fish pathogens.

Conclusion

Fish lymphocyte culture techniques have not been able to give as consistent results as those with mammalian lymphocytes. However, some of the factors affecting the cultivation of fish lymphocytes have been worked upon and PHA has been found to yield a better lymphocyte culture as compared to Con A. Moreover, an attempt to cultivate genetically dissimilar lymphocytes (during MLC) gave the best results among the catfishes examined.

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