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Full Length Research Article

Proliferative Responses of Tilapia T-Like Lymphocytes to Stimulation by Concanavalin A

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ABSTRACT
Peripheral blood lymphocytes were cultured in vitro in the presence of a mitogen; Concanavalin A (Con A), this resulted into a process described as lymphocyte transformation. The morphological changes in the cells were studied using phase contrast and electron microscopes. The results presented corroborated the existence of lymphocyte heterogeneity in tilapias.

Keywords: *Mitogenic stimulation, T-like Lymphocytes, Concanavalin A.*

INTRODUCTION

Nowell (1960) found that normal human lymphocytes proliferated into blast forms in vitro in the presence of Phytohaemaglutinin (PHA), a lectin extracted from the red kidney bean *Phaseolus vulgaris*. Subsequently, proliferative and blastogenic responses of lymphocytes of man and other higher vertebrates, mice and chickens to other mitogens such as Concanavalin A (Con A), Pokeweed mitogen (PWM) and Lipopolysaccharide have been reported (Chessin *et al* 1966). These observations and the subsequent discoveries of many other forms and sources of mitogens have since been applied by numerous researchers (Jannossy & Greaves 1971;Etlinger *et al* 1976; 1978) to study the characteristics of lymphocytes, particularly in relation to the phylogenetic appearance, occurrence and differentiation of thymus-derived (T) and Bursa or bone marrow-derived (B) lymhocytes.

The presence of mitogenic responses suggestive of lymphocyte heterogeneity has been reported in fishes. PHA stimulated leucocytes from the Paddlefish, *Polyodon spathula*; the Stingray, *Dasyatis Americana* and the Sea Lamprey, *Petgromyzonus marinus* (Olson 1967). Lymphocytes of Rainbow trout, *Salmo gairdneri* (Warr & Simon 1983); Brown trout, *Salmo trutta*; Carp, *Cyprinus carpio* and Channel catfish, *Ictalurus punctatus* (Faulmann *et al* 1983; Clem *et al* 1984) have all been shown to respond to stimulation by various mitogens. Con A and PHA have been used to induce stimulation of peripheral blood leucocytes from the nurse shark, *Ginglymusoma cirratum* (Lopez *et al* 1974).

As with the response of B cells to lipopolysaccharide (LPS) and purified protein derivative (PPD), T-cells undergo division when stimulated by specific mitogen (Etlinger *et al* 1978). Stone *et al* (1995) reported that PWM stimulates both B and T lymphocytes. Response is usually accompanied by a morphological change to a blast cell. The degree of lymphocyte stimulation in culture may therefore be examined either by determining the blastogenic changes or by measuring the amount of radioactive DNA

analogue incorporated into the newly synthesized DNA.

MATERIALS AND METHODS

Fish

Adult tilapia (*Oreochromis niloticus*) weighing between 280-350g and 25-28cm total lengths were obtained from the department of Wildlife and Fisheries' fish farm located within the University of Ibadan campus. They were acclimated at 27 $\pm$ 1^{o}C in the aquarium facilities of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Ibadan, Nigeria.

Blood collection

Donor fish were gently caught in a net and transferred to the laboratory in a bucket containing about 8 litres of pond water at 27^{o}C, mixed with 0.2g benzocaine dissolved in 5ml acetone to anaesthetize the fish. 2ml of blood and lymphoid organs were obtained from 3 adult fish at each sampling period; they were sacrificed at regular intervals. Blood was collected from the vein running ventrally along the vertebral column (Vertebral vein) with a 22G hypodermic needle and was discharged into test tubes containing the sodium salt of ethylene diamine tetra-acetic acid (Na-EDTA) as anticoagulant.

The blood was immediately mixed with the anticoagulant by gently inverting and rotating the syringe 5 times and the uncoagulated blood immediately mixed with 8ml phosphate buffered saline in a sterile plastic universal bottle to aid the subsequent separation of lymphocytes over a Ficoll-paque density gradient (Pharmacia fine chemcab).

The test tubes were spinned in a BR 401 coded ultra-centrifuge at 1000 X G for 30 minutes. The separated lymphocytes were then washed three times following the same centrifugation procedure.

Setting Up the Culture System

Concanavalin A was reconstituted according to manufacturer's instruction and serially diluted to obtain the required working concentration (Fig 1).
All procedures were aseptically carried out in a laminar airflow cabinet. Approximately 1 X 10^{6} lymphocytes in 1 ml Leibovitz L-15 medium and 1ml of Con. A solution concentration. A mixture consisting of 0.2ml cell suspension. 1ml medium and 0.2ml Con A was also inoculated into each well of a four-well plate flasks and plates were incubated at 28^{o}C for a maximum of 8 days. Cultures were sacrificed at 2,4 and 8 days to monitor cell growth and at 5 days for electron microscopy. The growth response to the lowest mitogen concentration was taken as the control level with which responses to the higher concentrations were compared.

Preparation for Electron Microscopy

Lymphocyte suspensions were obtained from the blood of healthy donors using the method of Coulson and Chalmers (1964). The lymphocyte suspension was diluted with tissue culture medium Leibovitz L-15 at 28^{o}C to give a standardized concentration of lymphocytes usually 1.0 or 1.5 X 10^{6}ml. packed cells were pooled from all flasks and plates sacrificed on the 5th day and pelleted by centrifugation at 1000 r.p.m for 5 minutes in MSE minor centrifuge. The cells were similarly centrifuged after each step in their fixation and dehydration. The cells were fixed for 1 hr in 1% glutaraldehyde in 0.1N Sorenson's buffer, washed three times (total time is 30 minutes) in the same buffer, post-fixed in buffered osmium tetroxide (Millonig 1962) and embedded in MARAGLAS? Using the method of Erlandson (1964). The pellet was divided into equal parts. One part was processed for TEM and the other processed for SEM.

Transmission Electron Microscopy

Ultra thin sections were cut with glass knives in an LKB ULTROTOME III^{R} ultramicrotome and mounted on uncoated grids. The sections were double-stained on the grid with uranyl acetate and lead acetate by the methods of Chiu *et al* 1993. Stained sections were observed with a JEOL-JEM 100S TEM.

Scanning Electron Microscopy

The pellet of cells was processed after Hayat (1978) and Blaxhall (1983). The dried pellets were then mounted on stubs, gold-coated in an S150m sputter-coater and viewed in a model ISI-60 scanning electron microscope (International scientific instruments, U.K.).

RESULTS

Enhanced growth was observed in al Con A treatment cultures, particularly at high mitogen concentrations (Fig. 1). Intense cell density was observed from 48 hours of culture up to 8 days, when the culture was terminated. At the higher mitogen concentrations, cell death was also similarly rapid, leading to a change in the colour of phenol red (pH). The morphological change in

mitogen-transformed lymphocytes differs from the morphology of normal lymphocytes as seen under scanning electron microscope (Plates 1 and 2). Cells examined by showed characteristics pore formation (Plate 3) and some tendency to adhere together. Most responding lymphocytes as seen in Plate 2, had smooth surfaces with pores.

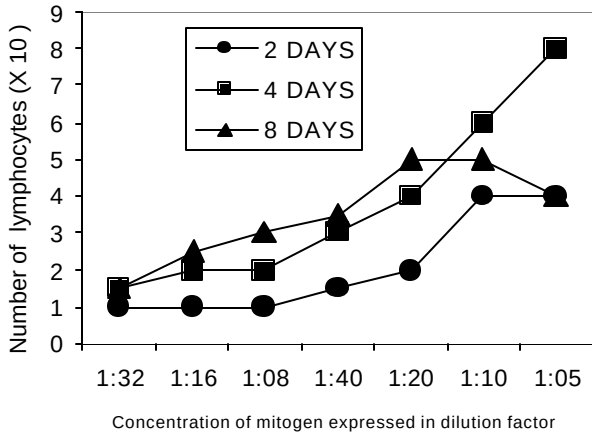

Fig. 1
Dose-response curves of Tilapia lymphocytes stimulated by Con A.

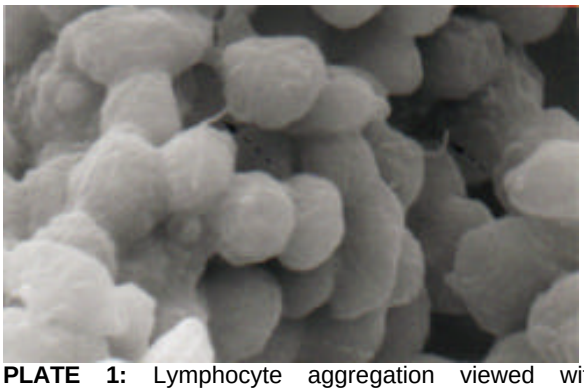

PLATE 1: Lymphocyte aggregation viewed with scanning electron microscope showing microvilli on the surface of some lymphocytes (arrows)

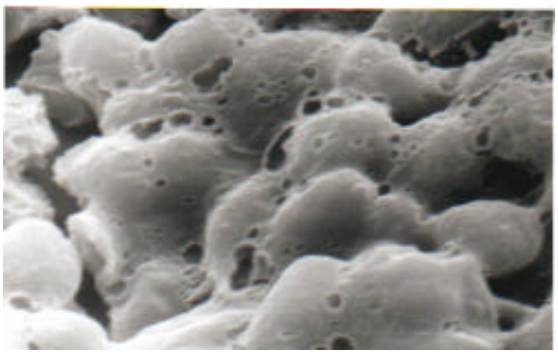

PLATE 2: Scanning electron micrograph of lilapia lymphocytes stimulated by Con A. Each cell has a smooth surface with or without pores. Cells are attached to form peripheral pores.

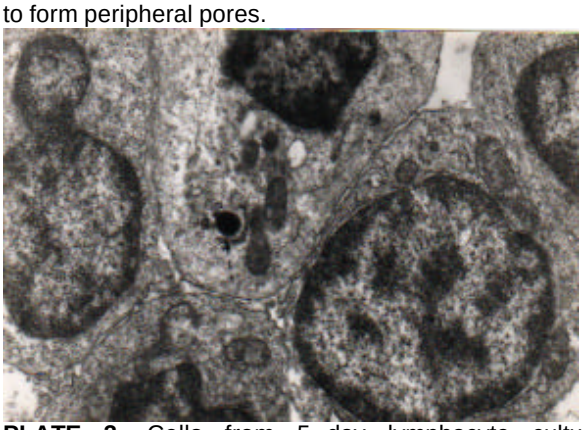

PLATE 3: Cells from 5--day lymphocyte culture stimulated by Concananalin A showing partial nuclear division of one of the cells. Mitochondria are abundant; one has become electron-- opaque (arrow).

The ultrastructure of mitogen transformed lymphocytes as revealed by transmission electron microscopy showed that the predominant cell-type after 48 hours had a rather extensive cytoplasm containing many free ribosome and mitochondria which appeared in some cells to contain electron opaque material.

The nuclear membrane many of the cells were incomplete and appeared to flake off into the cytoplasm. The blast cells after 4-5 days of culture were large, some with a dividing nucleus and had much cytoplasm rich in ribosome, mitochondria and endoplasmic reticulum in the form of small vesicles or sacs, which were either dilated or flattened. Endoplasmic reticulum were often seen in association with large bodies, usually rounded in outline and containing materials that are similar to electron-opaque dense bodies. Smaller bodies, which were less regular in outline, were occasionally observed in the 2-days cultures. Throughout the 8-days period of culture, small lymphocytes were only seen at the latter sampling periods.

DISCUSSION

The results presented indicated that Tilapia possess lymphocytes capable of responding to Con A, a mitogen considered to be stimulators of T cells in higher vertebrates. Many mitogens have been described; some are selective in their ability to stimulate T or B-lymphocytes. PHA and Con A activate T but not B cells, whereas Pokeweed mitogen (PMW) stimulates both types of cell.

Galeotii *et al* 1996, observed that the peripheral blood leucocytes of Sea bass

(*Dicentrarchus labrax*) were stimulated by PHA, Con A and Bacterial lipopolysaccharide, they however concluded that the greatest reaction was derived using PHA at a concentration of 6.25µg/ml.

Activated cells enlarge or transform into blast-like cells prior to, or in concomitant with DNA synthesis. Douglas (1972) noted that mitogens are capable of inducing a transformation rate of 80-90% of lymphocyte population into blast forms, whereas specific antigens are capable of transforming a maximum of 5-7% of a lymphocyte population into blast form.

Bagara *et al* 1995 also reported that there was a significant increase in the proportion of all T cells subsets following Con A stimulation. The morphological transformation of lymphocytes by mitogenic lectins is accompanied by extensive biological changes, the most striking of which is an increase in the synthesis of RNA and DNA (Naspitz and Richter 1968). Peripheral blood lymphocytes from Eimeria tenella infected chickens produce gamma-interferon after stimulation in vitro by mitogens (Breed *et al* 1997). Lymphocytes of murines and humans are responsive to PHA, Con A and PWM, which induce the lymphocytes to mitosis and lymphoblasts. Though blast transformation showing a similarity to the lymphoblasts of murines and humans has been qualitatively detected in a few amphibians and fishes (Manning and Turner 1976).

The present study shows that cells cultured for five days provided suitable samples for morphological assessment of proliferative activity. The majority of the cells appeared to be transformed between the 2nd and 5th days. The characteristic feature of the mitogen-transformed blast cells is the presence in the cytoplasm of large osmiophilic bodies, which the finding of this study suggests to be degenerating mitochondria. The difference in blastogenic response to a mitogen, therefore can be applied as one of the principal discriminating criteria between T and B cells (Chess and Schlossman 1980).

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