

Whole blood assay for examining lymphocyte blastogenesis of percichthyid bass (*Morone*) mrr01

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Abstract

A simple and reproducible method was developed for the measurement of blastogenesis of peripheral blood lymphocytes using whole blood of hybrid bass (striped bass [*Morone saxatilis*] female × white bass [*M. chrysops*] male) stimulated with Concanavalin A, phytohemagglutinin-P, lipopolysaccharide or pokeweed mitogen. Compared to traditional methods which use leucocyte separation procedures, whole blood culture is faster and less expensive. Only small aliquots of blood (10 μl per culture well) were needed, which would be beneficial for sampling small fish as well as for taking multiple samples from single animals. Optimal culture conditions for hybrid bass, including mitogen concentration, incubation temperature and incubation period, were determined. This is the first report to demonstrate a blastogenic response of whole blood cells in fish. © 1997 Elsevier Science B.V.

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1. Introduction

Mitogen-stimulated lymphocyte blastogenesis is widely used as an indicator of immune competence in vertebrates, including fish. Incorporation of radiolabelled nucleic acid precursors is the major method for testing the functional status of fish peripheral blood lymphocytes (PBL). Lymphocytes are usually separated from other cell types by density gradient centrifugation (Luft et al., 1991; Reitan and Thuvander, 1991). How-

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ever, this technique is labor-intensive and time-consuming (Wemme et al., 1992). A simplified alternative to density gradient centrifugation is the use of whole blood culture. In this paper, we describe the adaptation of whole blood culture to studying mitogen blastogenesis in fish, using hybrid bass (striped bass [*Morone saxatilis*] female × white bass [*M. chrysops*] male) as a model.

2. Materials and methods

2.1. Animals

Hybrid bass (about 3 years old, with an average body weight of 570 g) were spawned at the Pamlico Aquaculture Center, Aurora, NC. They were kept in tanks supplied with aerated, flow-through, fresh well water at a temperature of 20°C. Fish were fed a commercial pelleted feed twice daily, and were kept on a photoperiod of 12 h light:12 h dark.

2.2. Sampling

Fish were sedated with about 21 mg l⁻¹ of tricaine methanesulfonate. Blood samples were collected from the caudal sinus into heparinized syringes and needles (22-G). The blood was immediately diluted 1:10 with A-L medium, a chemically-defined, serum-free medium (Luft et al., 1991). All blood samples were transported to the laboratory within 30 min.

2.3. Assay procedures

Lyophilized mitogens were purchased from Sigma, St. Louis, MO. Concanavalin A (ConA, Lot No. 12H9408), phytohemagglutinin-P (PHA-P, Lot No. 122H9401), lipopolysaccharide (LPS, Lot No. 112H4011, from *E. coli* 055:B5), and pokeweed mitogen (PWN, Lot No. 81H9585) were rehydrated with A-L medium to a concentration of 200 µg ml⁻¹ (ConA, PHA-P, and PWM) or 1000 µg ml⁻¹ (LPS), and stored at -20°C.

100 µl of mitogen dilutions were placed into wells of a 96-well microtiter plate (Falcon). Then, 100 µl of cell suspension (equivalent to 10 µl of whole blood) were added to each well. Duplicate cultures were treated with each mitogen concentration.

Cultures were incubated in a 95% air:5% CO₂ atmosphere in modular incubation chambers (Billups-Rothenburg). Lymphocyte proliferation was measured by the incorporation of [methyl-³H]-thymidine (ICN, Irvine, CA, specific activity 6.7 Ci µmol⁻¹) into DNA. ³H-thymidine (0.8 µCi) in 20 µl of A-L medium was added to each well 16 h prior to harvesting the cells.

Cells were harvested with a multiple, semi-automatic cell harvester (Bellco Glass, Vineland, NJ) under 23 inch Hg vacuum, and rinsed with distilled water followed by 95% alcohol. Cell debris and macromolecules incorporating ³H-thymidine-labeled DNA were collected on fiberglass filter disks (Cambridge Technology, Cambridge, MA). Filter disks were dried and placed in liquid scintillation vials (7 ml) and 4 ml

scintillation fluid (Ecoscint O, National Diagnostics, Atlanta, GA) was added to each vial. The radioactivity was quantitated by liquid scintillation counting in a beta counter (1219 Rackbeta, LKB). Results were expressed as counts per minute (cpm) or a stimulation index (SI), which was calculated according to the following formula: $SI = \text{cpm of stimulated culture} / \text{cpm of non-stimulated control culture}$.

2.4. Statistical analysis

For comparing the mitogen-stimulated responses between different incubation periods and temperatures, multiple analysis of variance (ANOVA) plus Tukey's multiple comparisons (Neter et al., 1990) was performed with the Statistical Analysis System (SAS; SAS Institute, Cary, NC), and $p < 0.05$ was set as the limit for statistical

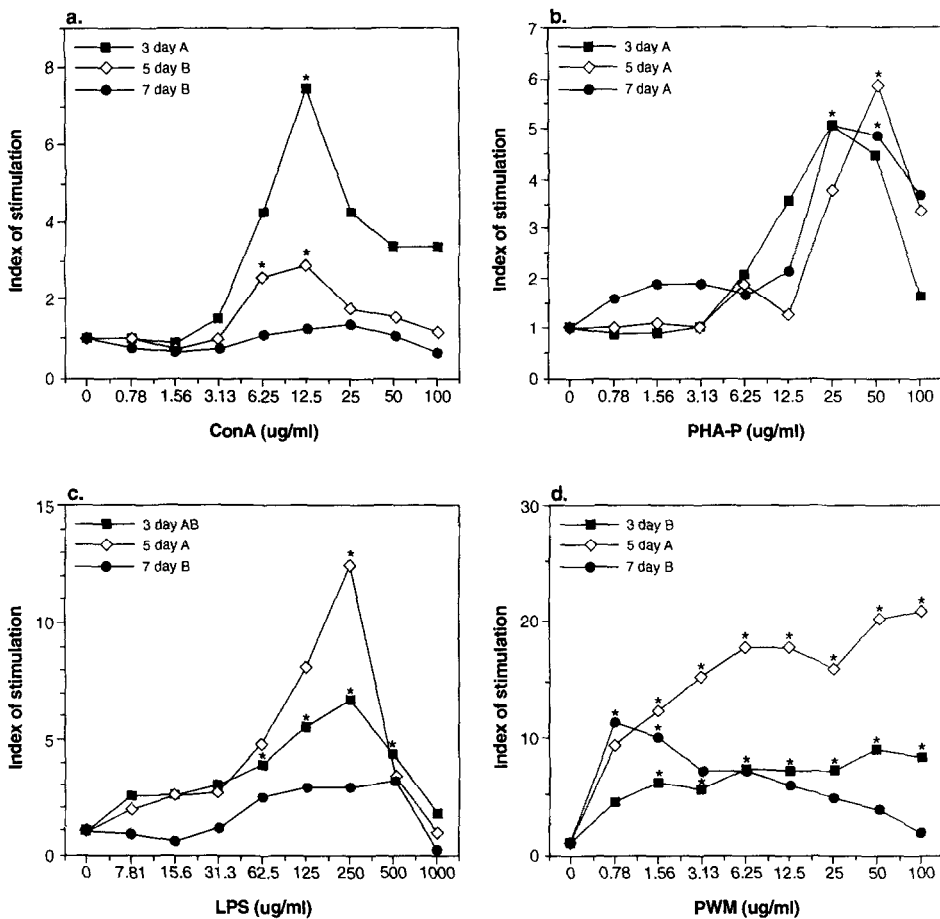


Fig. 1. SIs of whole blood samples from three hybrid bass that were stimulated with serially diluted ConA (a), PHA-P (b), LPS (c), or PWM (d) and incubated at 24°C for 3, 5, and 7 days, respectively, before ^3H -thymidine labelling. Significant ($p < 0.05$) differences among treatment times are indicated with different letters (A, B). Treatments having the same letters (e.g., 'AB' vs 'B') were not significantly different. SIs labelled with an '*' were significantly different from their negative control (no mitogen).

significance. Means, standard deviations (SD), standard errors of mean (SEM), and coefficients of variation (CV) were calculated using Microsoft Excel (Microsoft).

3. Results

3.1. Optimal conditions for mitogen stimulation using whole blood

3.1.1. Effect of incubation period

Whole blood samples from three hybrid bass were stimulated with serially diluted ConA, PHA-P, LPS, and PWM. Duplicate samples from each fish were incubated for 3,

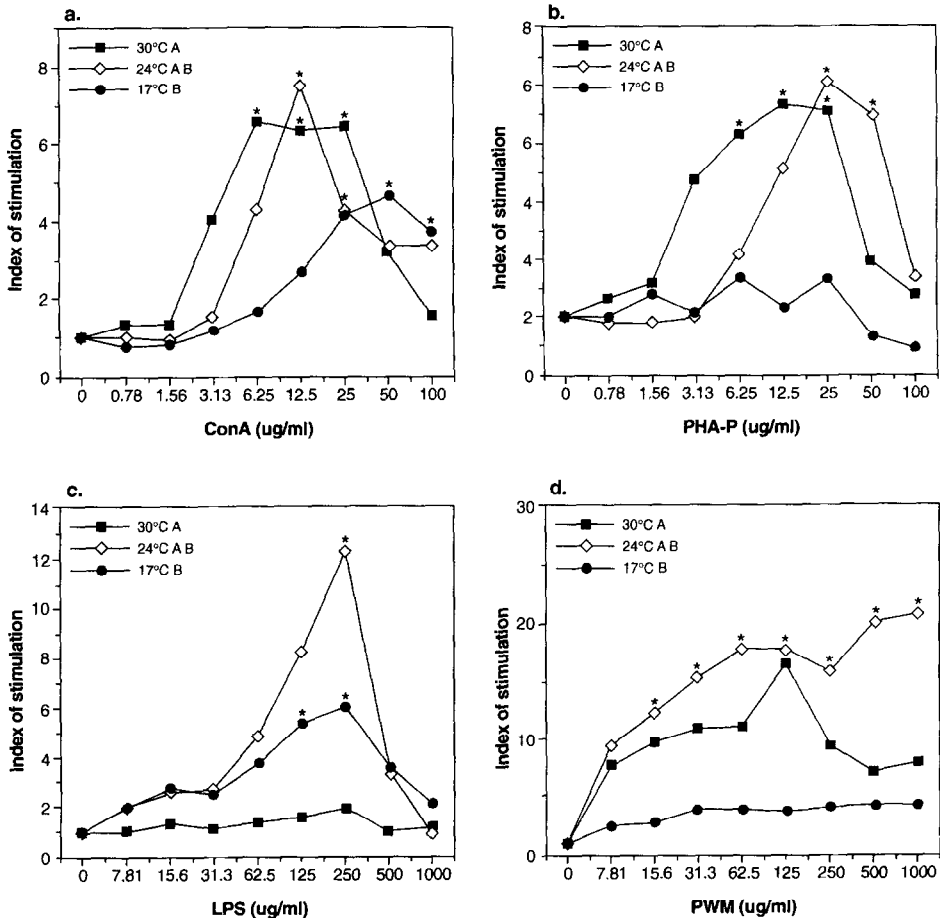


Fig. 2. SIs of whole blood samples from three hybrid bass that were stimulated with serially diluted mitogen and incubated for 3 days (ConA or PHA-P) or 5 days (LPS or PWM) at 17, 24 and 30°C, respectively, before ^3H -thymidine labelling. The effect of different temperatures was significant ($p < 0.05$) for all mitogens. Significant ($p < 0.05$) differences among treatment temperatures are indicated with different letters (A, B, or C). Treatments having the same letters (e.g., 'AB' vs 'B') were not significantly different. SIs labelled with an '*' were significantly different from their negative control (no mitogen).

5, or 7 days. Samples stimulated with ConA showed significantly higher SI after 3 days' incubation than those after 5 or 7 days' incubation ($p < 0.05$) (Fig. 1a). No significant difference was found between 5 and 7 days' incubation. For PHA-P, there were no significant differences among incubation periods (Fig. 1b). For LPS-stimulated cultures, peak SI appeared after 5 days' incubation, but the overall response of 5 days' incubation was not significantly different from that of 3 days' incubation. However, the SI of 7 days' incubation was significantly lower than that of 5 days' incubation (Fig. 1c). For PWM, the SI of 5 days' incubation was significantly higher than that of 3 or 7 days' incubation. No significant difference was found between 3 and 7 days' incubation (Fig. 1d).

3.1.2. *Effect of incubation temperature*

Whole blood samples from three hybrid bass were stimulated with serially diluted ConA, PHA-P, LPS, and PWM. Duplicate samples from each fish were incubated at 17, 24 or 30°C. In general, the response was lowest at 17°C (Fig. 2a, b and d) and improved at the higher temperatures (Fig. 2a–c). However, while a strong response was observed with LPS at 24°C, the response was abolished at 30°C (Fig. 2c).

3.1.3. *Optimal mitogen concentrations*

The optimal ConA and LPS concentrations were similar with different culture conditions (Fig. 1a and c, Fig. 2a and c) and while the optimal PHA-P and PWM concentrations varied with culture conditions, including incubation period and temperature (Fig. 1b and d, Fig. 2b and d).

4. Discussion

4.1. *Advantages of whole blood culture*

For mitogen blastogenesis experiments, blood is usually processed to obtain partially purified lymphocytes. A well-established technique for partial purification is density gradient separation with Ficoll-sodium diatrizoate (Tillitt et al., 1988; Luft et al., 1991). This technique is difficult to perform with large numbers of blood samples and is relatively expensive and time-consuming. Moreover, the separation procedure can give rise to selective depletion or enrichment of certain subpopulations of lymphocytes (Romeu et al., 1992) or monocytes, or can even lead to preactivation (Bloemena et al., 1989). Conventional culture techniques are performed with a standardized number of lymphocytes. Therefore, diminished responsiveness due to lymphocytopenia is not reflected by this method (Bloemena et al., 1989).

We developed a whole blood culture technique which was very simple and reproducible. Using this method, the peak SIs were always significantly higher than their controls. One possible disadvantage of the whole blood blastogenesis assay is that considerable variation exists in the number of lymphocytes in whole blood samples from different individuals, which might contribute to a large variation in response. However, in many of our experiments, the between-fish variation was not excessively high (data

not shown), taking into account the fact that ^3H -quantitated blastogenesis is usually associated with quite large variation. In cases where large variation did occur, it may not be due to the variation in lymphocyte counts since Leroux et al. (1985) found that lymphocyte numbers did not correlate with the proliferative response in healthy individuals.

It should also be noted that not all cell harvesters can be used with this technique. In preliminary experiments, we found that the PHD™ cell harvester (Cambridge Technology), which only produces 15 mm Hg vacuum, was not sufficient to effectively harvest the samples.

4.2. *Effects of different mitogens*

Relatively high SIs occurred in ConA and PWM-stimulated samples (Fig. 1d, Fig. 2d); PHA-P also stimulated PBL, inducing a relatively low SI (Fig. 1b, Fig. 2b). LPS was an effective mitogen only when extremely high concentrations were used (Fig. 1c, Fig. 2c). ConA is designated as a T-cell mitogen, which induces a high level of activation of CD8 cells in mammals. This activity may reflect the induction of T-suppressor cell activity by ConA. PWM activates only a low proportion of CD8 cells but stimulates predominantly CD4 cells in mammals, possibly reflecting the activation of T-helper cells in inducing immunoglobulin production (Schauer et al., 1989). Like ConA, PHA-P is also a T-cell mitogen but with a different carbohydrate specificity (Tizard, 1992). LPS is the classical B-cell mitogen (Tillitt et al., 1988). While all of these definitions and descriptions are based upon more well-defined cell types in mammals, the strong responses of hybrid bass leucocytes to classical mitogens is good indirect evidence for lymphocyte heterogeneity in *Morone*, and suggests T-cell heterogeneity.

4.3. *Effects of incubation period*

At 24°C, the optimal incubation periods for each mitogen were different. For best results, PWM-stimulated cells needed a longer culture period compared to those treated with ConA or PHA-P.

Similar results were observed for the chicken lymphocyte transformation test (Barta et al., 1992). LPS-treated cells resembled those treated with PWM, but the increase in SI was not as obvious as with PWM. After 7 days, the samples partially (LPS and PWM) or completely (ConA) lost the ability to respond as measured by ^3H -thymidine incorporation (Fig. 1a, c, and d), but the samples were still sensitive to PHA-P after 7 days' incubation (Fig. 1b). Viability examination with trypan blue dye exclusion after 7 days' incubation showed that most of the cells were still alive. It is possible that the nutrients in the culture medium were exhausted, or cell growth was stopped by inhibitors released from the cells.

4.4. *Effects of temperature*

Incubation temperature is the most important variable affecting mitogen blastogenesis of fish lymphocytes (Faulmann et al., 1983). In general, a 24°C incubation temperature was best for all mitogens (Fig. 2a–d). For hybrid bass, low temperature (17°C) reduced

the response of ConA and PWM-stimulated cultures (Fig. 2a and d) and abolished the response stimulated by PHA-P (Fig. 2b). Conversely, there was a significant stimulation by LPS at low temperature (17°C) (Fig. 2c). High temperature (30°C) reduced the stimulation by PWM to a lesser extent than low temperature (17°C) (Fig. 2d), and low temperature (17°C) did not abolish the stimulation by ConA (Fig. 2a). PHA-P stimulation was not reduced by high temperature (Fig. 2b), but high temperature abolished the stimulation by LPS (Fig. 2c). Low temperature increased the ConA or PWM concentrations needed to reach the maximum response (Fig. 2a and d). These results agree with the hypothesis by Faulmann et al. (1983), who found that low temperature immunosuppression in channel catfish may result from a preferential effect on T-like cells. In our studies, low-temperature abolished (Fig. 2b) or reduced (Fig. 2a and d) the response of T-like cells. In contrast, low temperature did not reduce the response of B-like cells significantly (Fig. 2c). It is tempting to speculate that low-temperature immunosuppression on T-like cells in *Morone* may result from a preferential effect on T helper-like (CD4) cells which are sensitive to PWM, since the effect of low temperature inhibition on T suppressor-like cells (CD8) (Fig. 2a), which are sensitive to ConA, is less pronounced than the effect on T helper-like cells (CD4) (Fig. 2d).

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