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IN VITRO MITOGENESIS OF PERIPHERAL BLOOD

LYMPHOCYTES FROM RAINBOW TROUT (Salmo gairdneri)

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Donald Edward Tillitt

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John P. Giesy
Major professor

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IN VITRO MITOGENESIS OF PERIPHERAL BLOOD LYMPHOCYTES FROM RAINBOW TROUT (*Salmo gairdneri*)

By

Donald Edward Tillitt

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ABSTRACT

IN VITRO MITOGENESIS OF PERIPHERAL BLOOD LYMPHOCYTES FROM RAINBOW TROUT (Salmo gairdneri)

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In vitro mitogenesis of rainbow trout peripheral blood lymphocytes (RBT PBL) was investigated to define the optimal culture conditions and repeatability of the assay for routine laboratory use. The assay variables of media, mitogen, serum supplementation, lymphocyte isolation procedure, and incubation period were assessed. Optimal proliferative response was obtained when RBT PBL were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and stimulated with 10 ug Concanavalin A/ml for between four and five days. I observed statistically significant variation among fish. Power analysis with variance estimates from this study reveal that sample size requirements of further studies under the given conditions could severely limit the applicability of this procedure for RBT health assessment. Further work in this area should center around standardization of culture conditions pertaining to the source of protein supplementation.

To my parents, Bart and Marvelle

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I would like to express my gratitude to my major professor, Dr. John Giesy, and the other members of my guidance committee, Dr. P. O. Fromm and Dr. Monte Mayes, for their support during the course of this study.

There are also a number of people to whom I would like to give special thanks for technical assistance. Dr. Robert Bull performed screening tests on ConA; Dr. Louis King for the assistance and use of the flowcytometer; Ken Weber, owner of Green River Trout Farm, for his kind donation of rainbow trout serum; Harry Westors, of the Michigan Department of Natural Resources, for permission to collect salmon sera and steelhead from the Little Manistee River; the Michigan State University Clinical Center for donation of human sera; and Dr. Robert Ringer for use of his automated cell harvester. Lastly, I would like to thank Christine Flaga and Elizabeth Bartels for preparation of this thesis.

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INTRODUCTION

The importance of hematology and immunology in health assessment of higher vertebrates has lead workers to adopt a variety of hematological and immunological techniques for fish health assessment (Hesser, 1960; Blaxhall, 1972; Hickey, 1976; Wedemeyer and Yasutake, 1977). Techniques adopted are classically descriptive in nature, which stress numbers and sizes of fish blood cells. Examples of descriptive techniques include: use of hematocrit (Soivio and Oikari, 1976; Munkittrick and Leatherland, 1983), hemoglobin (Sniesko, 1960), leukocyte counts or leucocrit (McLeay and Gordon, 1977; Wedemeyer et al., 1983), chromosomal aberrations (Al-Sabti, 1985), macrophage aggregates (Wolke et al., 1985) or combinations of these parameters.

Reports of the utilization of functional immunological tests for the evaluation of fish health are notably absent from the literature in the area of aquatic toxicology. In their review of reports dealing with the effects of toxic agents on the immune systems of fish Zeeman and Brindley (1981) concluded that such reports comprised merely sidelights and footnotes in the literature. This is not the situation in mammalian toxicology where a variety of functional studies have been recommended for routine screening for suppression of the immune system (Vos, 1977; Koller, 1979; Vos, 1981; Sharma, 1981; Bleavins and Aulrich, 1983). Vos (1977 and 1981) suggested tests for the immunological functions of cell-mediated immunity, humoral immunity, and phagocytoses by macrophages.

Cellular immune function is an important component of both cell-mediated and humoral immunity and is crucial in assessing immunocompetence of an organism. Lymphocyte proliferation or stimulation assay, commonly referred to as lymphocyte activation (LA), is an extremely useful test of cellular immune function. *In vitro* LA techniques are used in human medicine to assess cellular immunity in cases of immunodeficiency, autoimmunity, infectious diseases, and cancer (Stites et al., 1982). LA techniques have also been utilized to monitor the immunosuppressive activity of toxic compounds in mammals (Vos and Moore, 1974; Bleavins et al., 1983; Greenlee et al., 1985).

LA is the morphological and functional alteration process which occurs when immunocompetent lymphocytes are stimulated by antigens or nonspecific mitogens. This transformation occurs *in vivo* when lymphocytes are presented with properly processed antigens. The site of antigen presentation may be in the systemic circulatory system but more commonly occurs in mammalian lymph nodes (Spent, 1977) or the pronephros of teleosts (Chiller et al., 1969; Smith et al., 1970). These centers have concentrated numbers of lymphocytes in close proximity to one another so as to increase the likelihood of an antigen-presenting macrophage associating with a lymphocyte that has surface membrane immunoglobin receptors for that particular antigen. This transformation process occurs *in vitro* when lymphocytes are cultured with either specific antigens or, more commonly, with nonspecific mitogens (mitogenesis). Mitogens are: 1) lectins derived

from various plants or 2) polysaccarides from bacterial cell coats. They act to nonspecifically stimulate lymphocytes, without the requirement of a sensitized host.

A myriad of biochemical events occur in the lymphocyte upon activation by the mitogen. These events include changes in lipid components of the plasma membrane, increased permeability to divalent cations, adenylate cyclase and guanylate cyclase activation and resultant elevation of intracellular cAMP and cGMP in early and late phases, respectively. Synthesis of protein, RNA and shortly thereafter DNA occurs in activated cells (Hadden, 1981). The DNA synthesis is the basis for measuring cell proliferation. Morphologically, proliferating cells enlarge, form large pyroninophilic vesicles, fill with endoplasmic reticulum, polysomes, free ribosomes, and have marked increases in microtubule development. These changes give the cells the appearance of primordial blastlike cells from which the term blastogenesis is derived. The biochemical and morphological changes are a prelude to the production of antibodies in the case of B-cells or the synthesis soluble factors (i.e., lymphokines, prostaglandins) and cell-mediated activities with T-cells (Hume and Weideman, 1980).

A number of fish species have the ability to produce lymphocytes that respond to LA techniques (Ethlinger et al., 1976a; Cuchens and Clem, 1977; Sigel et al., 1978; Al-Sabti, 1983; Blaxhall, 1983b; Faulman et al., 1983; Warr and Simon, 1983; Caspi et al., 1984). Cells separated from lymphoid tis-

sues (spleen, thymus, and anterior kidney) and peripheral blood of fish have produced various levels of response to the same mitogens used in higher vertebrates. Authors of the above mentioned studies were attempting to establish lymphocyte heterogeneity in fish or develop methods for karyotyping fish.

Lymphocytes from teleosts, although not as clearly definable as those from higher vertebrates, appear to have discernible subpopulations resembling T and B-cells (Clem et al., 1981; Warr and Simon, 1983). Partial evidence for this fact comes from differential responsiveness to "classical" T-cell mitogens, phytohemagglutinin (PHA) and concanavalin A (ConA), as compared to B-cell mitogens lipopolysaccharide (LPS) and purified protein derivative of tuberculin (PPD) (Etlinger et al., 1976; Cuchens and Clem, 1977; Warr and Simon, 1983). Although in disagreement about the exact nature of tissue-specific mitogenic responsiveness, all of their studies demonstrated some degree of response to "T-cell" and "B-cell" type mitogens by cultures of lymphocytes which were prepared from peripheral blood samples. This is not unexpected based on the role of the circulatory system in transporting lymphoid cells during the ontogeny of cellular immunity in young fish and in adults (Tatner and Manning, 1983).

Adapting lymphocyte mitogenesis assay for routine use with fish in the laboratory or in the field, could provide a useful tool to workers interested in fish health. Such a tool could be used to assess the status of the immune system during immunotoxicological screening tests, in fish culturing systems, and in wild populations. To this end, my research was aimed at evalu-

ating the applicability of mitogenic responses of lymphocytes as a routine tool for fish health assessment. To address this problem, I conducted lymphocyte mitogensis assays on cells separated from peripheral blood of rainbow trout. The parameters of incubation time, culture media, serum supplement, mitogen type and concentration were evaluated to maximize the proliferative response.

The specific objectives of my stidues were to:

1. Optimize *in vitro* culture parameters of culture media, serum supplement, mitogen type, mitogen concentration and incubation time for peripheral blood lymphocyte mitogenesis assay in rainbow trout.
2. Test and define the repeatability of the assay as a tool for fish health assessment.

MATERIALS AND METHODS

Fish

The rainbow trout (*Salmo gairdneri*), RBT, was chosen as the experimental organism because it is a common freshwater fish about which an abundance of physiological, hematological and husbandry information is available. Previous studies have also demonstrated that lymphocytes from rainbow trout are capable of mitogenic transformation (Etlinger et al., 1976a; Warr and Simon, 1983). Rainbow trout of both sexes, 150-250g, were purchased from Balders Fish Farming Enterprise, Big Rapids, Michigan. Fish were held in 500 l fiberglass tanks, at 10 $\pm$ 1 C, with a continuous flow (5 turnovers/day) of activated charcoal filtered, aerated tap water. Twice a week the tanks were cleaned and the fish were fed (Purina Trout Chow) to satiation. The photoperiod was 16 h light, 8 h dark.

Media

Media were prepared fresh for each assay, generally 24 hours prior to use. RPMI 1640 (Gibco Laboratoreis, Grand Island, New York, Catalog No. 330-2511) with L-glutamine was prepared from 10x liquid concentrate and supplemented according to the method of Warr and Simon (1983, see Table 1). Medium 199 (Gibco Laboratories, Catalog No. 330-1181) with Hank's balanced salts and L-glutamine was prepared from 10x liquid concentrate and supplemented according to Blaxhall (1983a) with modifications as noted in Table 2. Media and supplements were diluted to volume with double distilled water. pH was adjusted to 7.3

with 25% NaOH and monitored by an Electro-Mark pH meter (Markson Science Inc., Del Mar, California). Osmolality of the media was checked by a 5100 B vapor pressure osmometer (Wescor Inc., Logan, Utah) and adjusted to 312 mOs/Kg with NaCl. Media were then sterilized by filtration with Type TC filter units (Nalgene Co., Rochester, New York) which were equipped with 0.2 micron membrane filters and stored at 2-8 C.

Serum Supplements

Fetal bovine serum (FBS) and heat inactivated FBS (Gibco Laboratories, Control Nos. 29P8834 and 29K9051, respectively) and human serum (HS) obtained from the Michigan State University Clinical Center were tested for their ability to support growth and mitogenic stimulation of RBT peripheral blood lymphocytes. Serum that was not already heat inactivated was treated at 56 C for 30 minutes to destroy the C3 component of the complement system. All serum was stored at -20 C. Serum was added to media before filter sterilization.

Mitogens

Concanavalin A, (ConA; Pharmacia Fine Chemicals, Uppsala, Sweden, Lot KA 35107), was obtained freeze dried, prepared by chromatography on Sephadex with less than 0.1% carbohydrate. Lipopolysaccharide, LPS (*Escherichia coli* 0111:B4-W, Control No. 721935 and *E. coli* 055:B5-W, Control No. 725525), pokeweed mitogen, PWM, (Control No. 13N7932), and phytohemagglutinin-P, PHA-P (Control No. 715136), were obtained in lyopholized form

from Difco Laboratories, Detroit, Michigan. All mitogens were rehydrated with sterile double distilled water and stored at -20 C. Dilutions of each of the mitogens were made with the appropriate media just prior to use.

ConA was screened for its ability to stimulate human lymphocytes by Dr. Robert Bull, College of Veterinary Medicine, Michigan State University. Stimulation was noted from all four individuals tested (Appendix A1).

Isolation of Lymphocytes

Lymphocytes were separated from peripheral blood by a modification of Boyum's (1968) method. Numerous variations of dilution volume, centrifuge time and cell suspension to gradient material ratios were tested. The purest separations resulted from the following procedure. Whole blood samples were diluted 1:3 with cold, complete medium to reduce viscosity. Two milliliters of whole blood suspension was carefully layered over 4 ml Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, New Jersey) in a 17 x 100 mm polystyrene tube (Falcon, Oxnard, California) so as not to mix the two phases. The gradients were centrifuged at 2000 RPM (500 x gravity) in an International Centrifuge Model SBV (International Equipment, Boston, Massachusetts) with a swing-bucket rotor at 10 C for 30 minutes. The overlying medium was removed by aspiration and the lymphocytes at the interface collected with a polyethylene transfer pipette. A typical blood sample was 3 ml whole blood, with a resultant 12 ml dilution volume. Therefore, six gradients were required for a single

blood sample (2 ml/gradient). The lymphocytes from these gradients were collected and placed in another 17 x 100 mm tube. Cells were washed twice with cold, complete medium at 100 x gravity (700 RPM in the International Model SBV), 10 C, for 10 minutes. Cells were enumerated using an Improved Neubauer hemacytometer (Arthur H. Thomas Co., Philadelphia, Pennsylvania) as described by Absher (1973). Determination of cell viability by trypan blue exclusion (0.4% trypan blue in 1% NaCl) was performed concurrently (Phillips, 1973).

Alternatively, lymphocyte isolation techniques described by Warr and Simon (1983) were assessed. This approach employs a continuous gradient consisting of Percoll (Pharmacia Fine Chemicals, Piscataway, New Jersey), 2.5 M sucrose, and distilled water in a 3:1:6 ratio. The sterile solutions were mixed and 8 ml placed in a 16 x 116 mm polycarbonate tube. The gradient was established by centrifuging for 35 minutes in a Sorvall RC-2 with a SS-24 angular head rotor at 20,000 RPM (49,000 x gravity) and 20 C. A 1 ml aliquot of 1:1 dilution of whole blood and complete media was carefully layered over the Percoll gradient and centrifuged for 8 minutes at 10 C in an International Centrifuge Model SBV with a swing-bucket rotor at 2000 RPM (500 x gravity). The band of lymphocytes was collected, transfered to a 17 x 100 mm polystyrene tube and washed twice with cold, complete medium as in the Ficoll-Paque procedure.

Flow cytometric and histological staining examination of the separated cells were done to characterize the population of

cells obtained from these procedures. Cells were stained with a Ralph's-Wright-Giesma procedure (Luna, 1968) and viewed under a light microscope. The cirterion for cell classification was as described by Ellis (1977) and Yasutake and Walles (1983). Cell collected from either of these procedures were composed of over 90% lymphocytes by morphologic criterion; the remaining cells were an approximately even distribution of thrombocytes, monocytes, and granular leukocytes. Red blood cells (RBC) were generally less than 5% of the total cells counted. Separations containing more than 5% RBC were discarded.

Flow cytometry was done on a Cytofluorograf equipped with a 2150 Ortho Diagnostic Systems (Westwood, Massachusetts) computer. Cells separated by both methods (Ficol-Paque and Percoll gradients) were analyzed for their forward red light scatter (a measure of cell size) and 90-degree blue light scatter (a measure of cell granularity). Data is expressed as relative light intensity (for each form of light scatter) and relative frequency of cells. Cytograms from both methods (Figures 1 and 2) show very similar patterns of 90 degree blue light scatter for both light intensity range and frequency of cells. The pattern of forward red light scatter for Ficol-Paque separated cells had a distinct peak narrower than that of Percoll separated cells.

The cytogram of forward red light scatter of Percoll separated cells has more of a bell shape, but the boundaries are similar to the Ficol-Paque separated cells. The cytograms of red-light versus blue-light-scatter further shows

the similarity in cell populations attained by these two gradient separation methods as well as the homogeneity of size and internal granulation within each of the populations.

Assay Procedure

Fish were quickly netted from holding tanks and immobilized by a blow to the head. This method is preferred over anaesthesia for giving samples representative of resting state (Oikari and Soivio, 1975). The ventral side of the peduncle, just posterior to the anal fin, was topically disinfected with 95% ethanol. Peripheral blood was taken into a heparinized syringe (approximately 100 units/ml final concentration). Blood samples were diluted with cold, complete medium and lymphocytes isolated as previously discussed. After enumeration and viability testing, cells were resuspended in complete medium to the desired concentration. All lymphocyte suspensions contained 10,000 viable cells/ml unless otherwise noted.

Cells were cultured in 96-well microculture plates (Falcon, Becton Dickinson and Co., Oxnard, California). Cells were delivered to culture wells in a 50 ul volume of complete medium that resulted in a cell concentration of 5×10^5 cells/well. In certain cases the initial seeding density of cells was less than 5×10^5 cells/well due to low recovery rates from separation. Mitogens at the desired dilution were also delivered to culture wells in a 50 ul volume of complete medium,

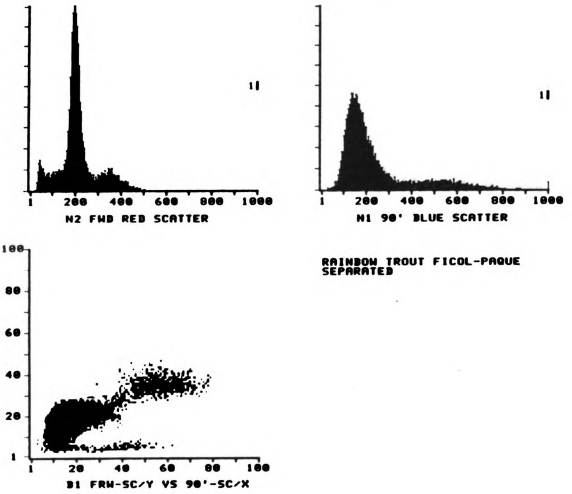

Figure 1. Cytogram of RBT PBL Separated on Ficol-Paque Gradient Material.

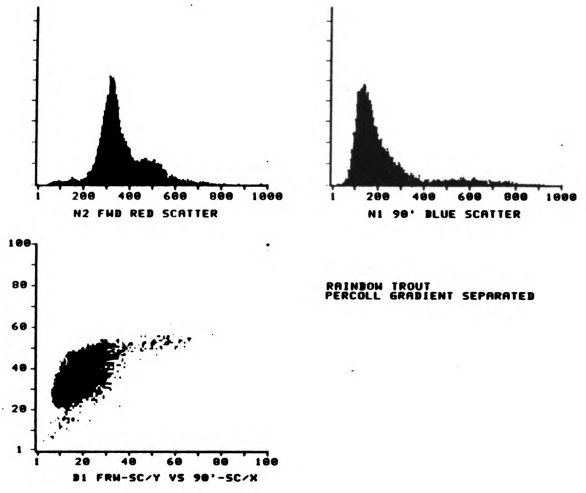

Figure 2. Cytogram of RBT PBL Separated on Percoll Gradient Material.

Table 1. RPMI 1640 Tissue Culture Medium and Supplements.

Supplement	Concentration
RPMI 1640 (10x)	10% (V/V)
Penicillin-G	10^5 units/l
Streptomycin sulfate	50 mg/l
2-Mecaptoethanol	50 uM
Sodium bicarbonate	25 uM

Table 2. Medium 199 Tissue Culture Medium and Supplements.

Supplement	Concentration
Medium 199 (10x)	10% (V/V)
Penicillin-G	2×10^5 units/l
Streptomycin sulfate	10^5 units/l
Nystatin	2.5×10^4 units/l
HEPES buffer (1)	5 uM
2-Mercaptoethanol	50 uM

(1) N-2-Hydroxyethylpiperczine-N'-2-ethanesulfonic acid, sodium salt.

resulting in a final culture volume of 100 ul. Cultures of each mitogen concentration were conducted in triplicate.

Microculture plates containing cells were incubated in an atmosphere of 95% air and 5% CO_2 at 20 C. Growth kinetics were studied by varying the period of incubation from 1 to 7 days. Proliferation of the lymphocytes was monitored by the uptake and incorporation of [methyl-3H]-thymidine (ICN, Irvine, California, specific activity 6.7 Ci/mmole, Lots 2235129 and 2614119, >99% purity) into DNA. Each culture well was dosed with 1 uCi [methyl-3H]-thymidine in 25 ul of complete media 24 h before harvesting. Etlinger et al. (1976a) demonstrated that radioactivity incorporated in this method was a valid measure of cellular proliferation.

Cell cultures were harvested using a multiple automated sample harvester (MASH) that was constructed specifically for Dr. R. Ringer, Animal Science Department, Michigan State University, and is not available commercially. MASH units were first introduced by Hartzman and coworkers (1971, 1972) to simplify harvesting in conjunction with microculture systems. Together, the microculture plates and MASH units afforded a simple technique that required fewer cells and allowed more replicate cultures. The MASH unit used in these assays harvested 24 culture wells simultaneously. Culture cells were rinsed with distilled water. Cells that were not disrupted by the suction action of the MASH were lysed by the distilled water. Cellular fragments, including DNA with incorporated [methyl-3H]-thymidine, were collected on glass microfiber filter

paper (Whatman 934-AH, Cambridge Technology, Cambridge, Massachusetts).

Filter disks were placed in liquid scintillation counting (LSC) vials and scintillation cocktail was added to the vial. The scintillation cocktail was prepared with toluene and 42 ml Liquifluor (New England Nuclear, Boston, Massachusetts) per liter toluene. Liquifluor is a PPO-POPOP concentrate that results in 4 g PPO/liter and 50 mg POPOP/liter when diluted with toluene. Radioactivity was quantitated by LSC on a Beta Tracor by ESR. The Beta Tracor contained an internalized quench curve for automatic converstion from counts per minute (CPM) to disintegrations per minute (DPM).

Statistical Analysis

All data was analyzed with the computer program Statistical Analysis Systems (SAS) (SAS Institute Inc., Cary, North Carolina). Means, standard deviation, range, variance, corrected sums of squares, uncorrected sums of squares, standard error of the mean, and the coefficient of variation for replicate samples and/or across fish means were calculated with the MEANS procedure for the dependent variables DPM, stimulation index (SI = experimental DPM/control DPM) and standardized DPM (SDPM = experimental DPM-control DPM).

Analysis of variance (ANOVA) of the variables DPM and SI was accomplished with the General Linear Models (GLM) procedure because of its flexibility to accept mixed models.

The models of response contained fixed main effects of mitogen concentration and incubation time, and random effects of fish. The GLM procedure gave results in the form of sums of squares for main effects, interactions, and error, F-values and associated probabilities. Models, formula and coefficients of expected mean squares for each of the experiments are presented in Appendix B.

Expected mean squares were calculated according to the procedures of Gill (1978). Comparison of mean response (DPM, SI, and SDPM) from controls and experimentals was by t-test (Gill, 1978) and least significant difference (LSD) multiple range tests was calculated according to Sokal and Rohlf (1969a) with t-values from Rohlf and Sokal (1969). Comparison of mean response was also performed by Tukey's Studentized Range (HSD) with the MEANS/GLM procedures of SAS.

RESULTS

Media

Cell viability was assessed with lymphocytes cultured in RPMI 1640 (Table 1) or TC 199 (Table 2) culture media. Either tissue culture medium supported lymphocytes when viability was measured by Trypan blue dye exclusion. Viability in all cases was greater than 90% when monitored daily through seven days of incubation.

Mitogens

Stimulation of rainbow trout peripheral blood lymphocytes (RBT PBL) was greatest when ConA was in the culture medium (Table 3). The data presented in Table 3 represents the mean responses of six or more fish with three or four replicate cultures per fish. All cultures were incubated four days of which the final 24 h was with radiolabelled thymidine. The proliferative response of RBT PBL to the classical mammalian mitogens PHA, LPS, or PWM was small, relative to that of mammals at all concentrations. The lymphocytes' lack of response to these three mitogens is noted when comparing both raw incorporation of labelled thymidine (DPM) and stimulation indices (SI). The maximal mean SI of RBT PBL to PHA, LPS, or PWM was 1.83, 1.78, and 1.40, respectively.

In contrast, the maximal mean SI of RBT PBL was 4.87 when cultured with 10 ug ConA/ml. However, during this series of experiments it became apparent that there was a high degree of variation between the response of individual

Table 3. Mean Uptake of ^{3}H-Thymidine by RBT PBL Cultured in RPMI 1640 with 10% FBS.[1]

	Mean Response ($n \geq 6$)		
Mitogen	DPM x 10^{-3} (SE)	SI (SE)[2]	SDPM x 10^{-3} (SE)
ConA			
3 ug/ml	4.51 (0.57)	1.22 (0.13)	0.77 (0.47)
10 ug/ml	17.72 (4.07)*[4]	4.87 (1.18)*	14.05 (4.05)*
15 ug/ml	2.92 (0.81)	1.11 (0.14)	0 (0.69)
PHA			
5 ug/ml	0.90 (0.12)	1.61 (0.23)	0.27 (0.10)
25 ug/ml	1.03 (0.14)	1.83 (0.29)*	0.41 (0.12)*
100 ug/ml	0.60 (0.08)	1.11 (0.15)	0 (0.10)
LPS			
50 ug/ml	0.93 (0.10)	1.53 (0.23)	0.24 (0.11)
100 ug/ml	1.13 (0.12)*	1.78 (0.22)*	0.44 (0.12)*
250 ug/ml	0.46 (0.05)	0.73 (0.09)	0 (0.08)
PWM			
5 ug/ml	0.65 (0.10)	1.11 (0.13)	0 (0.07)
50 ug/ml	0.78 (0.08)	1.40 (0.15)	0.10 (0.07)
100 ug/ml	0.69 (0.09)	1.25 (0.18)	0.02 (0.11)

1) All data represents an incubation period of four days, the last 24 hours of which was with ^{3}H-Thymidine with $n \geq 6$ and three to four replications per fish.

2) SI = Stimulation Index = $\frac{\text{DPM stimulated cells}}{\text{DPM control cells}}$

3) SDPM = DPM stimulated - DPM control.

4) * Significantly different from controls ($p=0.05$) according to Tukey's Studentized Range (HSD).

fish (Table 4). The range of stimulation indices was 24.37 when RBT PBL were cultured four days in RPMI 1640 with 10% FBS and 10 ug ConA/ml. The response of some individual fish are presented in Table 5 as an example of this variation between individual fish. The variation within individual fish was considerably less than among-fish variation. Coefficients of variation from triplicate samples were usually less than 20%. Analysis of variance was performed on all individual experiments. The models, estimate of mean squares, variance, percent of associated variance, F-values, and probability values are presented in Appendix B. The effect of fish was significant ($p = 0.05$) in all experiments conducted with ConA as the mitogen when assessed with the dependent variable DPM. The variation from fish to fish was significantly greater than that of replication sample from a fish. The variation in response to ConA by RBT PBL was directly related to the degree of stimulation (Table 4). The highest coefficients of variation are seen at what appears to be the optimal dose of ConA (10 ug/ml).

Kinetics of Response

RBT PBL were cultured for periods of one to seven days at doses of 1, 3, 10, 15, or 25 ug ConA/ml to determine the optimal culture time and dose-response kinetics of the proliferative response. Studies to optimize incubation period and mitogen dose were limited to the use of ConA because the results of preliminary experiments indicated that ConA possessed

Table 4. Mean, Standard Error, Standard Deviation, Variance, Range, and Coefficient of Variation of the Response of RBT PBL to ConA Across All Experiments.[1]

ConA	N	$\bar{X}$	SE	STD	VAR	RANGE	CV (%)
Control							
DPM (x 10^{-3})	40	3.84	0.41	2.60	6736	8.09	67.59
SI	40	1.00	0.05	0.32	0.10	1.68	31.45
3 ug/ml							
DPM (x 10^{-3})	37	4.51	0.57	3.47	432798	11.01	76.91
SI	37	1.22	0.13	0.79	0.63	3.10	64.44
10 ug/ml							
DPM (x 10^{-3})	38	17.72	4.07	25.00	629938	79.78	141.65
SI	38	4.87	1.18	7.25	52.6	24.37	149.07
15 ug/ml							
DPM (x 10^{-3})	24	2.97	0.81	3.95	15614	14.48	133.16
SI	24	1.11	0.14	0.73	0.53	2.30	65.50

1) Cells incubated four days in RPMI 1640 medium supplemented with 10% FBS. $\bar{X}$ = mean, N = replicates, SE = standard error of the mean, STD = standard deviation, VAR = variance, CV = coefficient of variation.

Table 5. Mean, Standard Deviation, Variance, Range, and Coefficient of Variation of the Response of Individual RBT PBL to 10 ug ConA/ml.[1]

Fish	N	$\bar{X}$	STD	VAR	RANGE	CV (%)
1						
DPM (x 10^{-3})	4	77.42	2.60	6740	6.03	3.35
SI	4	23.84	0.80	0.64	1.86	3.35
2						
DPM (x 10^{-3})	4	42.44	7.27	52842	15.65	17.13
SI	4	8.94	1.53	2.34	3.30	17.13
21						
DPM (x 10^{-3})	4	29.36	1.51	22.88	3.11	5.15
SI	4	7.36	0.38	0.14	0.78	5.15
52						
DPM (x 10^{-3})	3	1.57	0.10	91	0.18	6.06
SI	3	1.04	0.06	<0.01	0.12	6.06
55						
DPM (x 10^{-3})	3	8.04	0.66	440	1.16	8.26
SI	3	1.24	0.10	0.01	0.18	8.26
56						
DPM (x 10^{-3})	3	4.45	0.86	732	1.49	19.22
SI	3	0.61	0.12	0.01	0.21	19.22

1) Cells incubated four days in RPMI 1640 medium supplemented with 10% FBS. X = mean, N = replicates, STD = standard deviation, VAR = variance, CV = coefficient of variation.

the greatest ability to stimulate RBT PBL. The culture media in this set of experiments were supplemented with 10% FBS.

Those fish lymphocytes that responded well to ConA *in vitro* had distinctive kinetic patterns of both dose and incubation period. An example of RBT PBL kinetics of response to ConA over a five day period is given in Figures 3 and 4. These are the two fish from an experiment that tested ConA concentrations of 1, 3, 10, and 25 ug/ml. The greatest response in both cases was at 10 ug ConA/ml with an incubation period of five days. A concentration of 25 ug ConA/ml caused a greater initial proliferation of RBT PBL. In contrast, the activation of RBT PBL with 3 ug ConA/ml required a longer incubation to attain maximum stimulation. The response to 1 ug ConA/ml was not different from control cell cultures. No plateau or decline of incorporation of radiolabelled thymidine was observed at the optimal dose of ConA, additional experiments were done determine whether proliferation of the cells increased past day five.

These results indicate an extreme variation in LA response to ConA stimulation between the two fish tested even though the control cells of these fish responded similarly to the culture conditions. PBL from fish 21 (Figure 5) responded to ConA at 10 ug/ml under culture conditions, while PBL from fish 24 (Figure 6) failed to respond under the same culture conditions.

The second point taken from these results is that after five days of incubation the proliferative response of RBT PBL to ConA plateaus. At all concentrations of ConA, except the

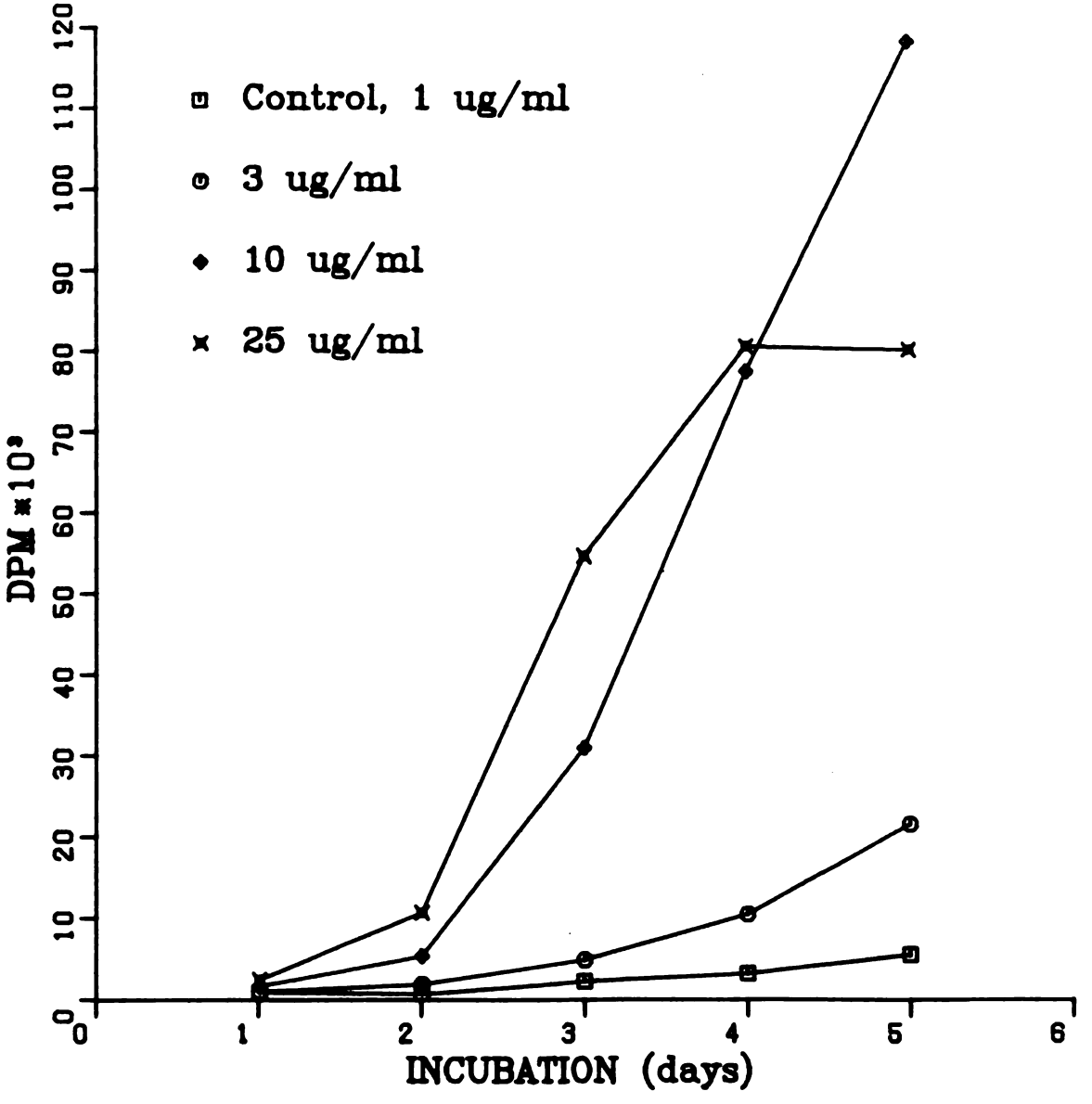

Figure 3. Kinetics of Response of Fish 1 PBL to ConA in Experiment 18.

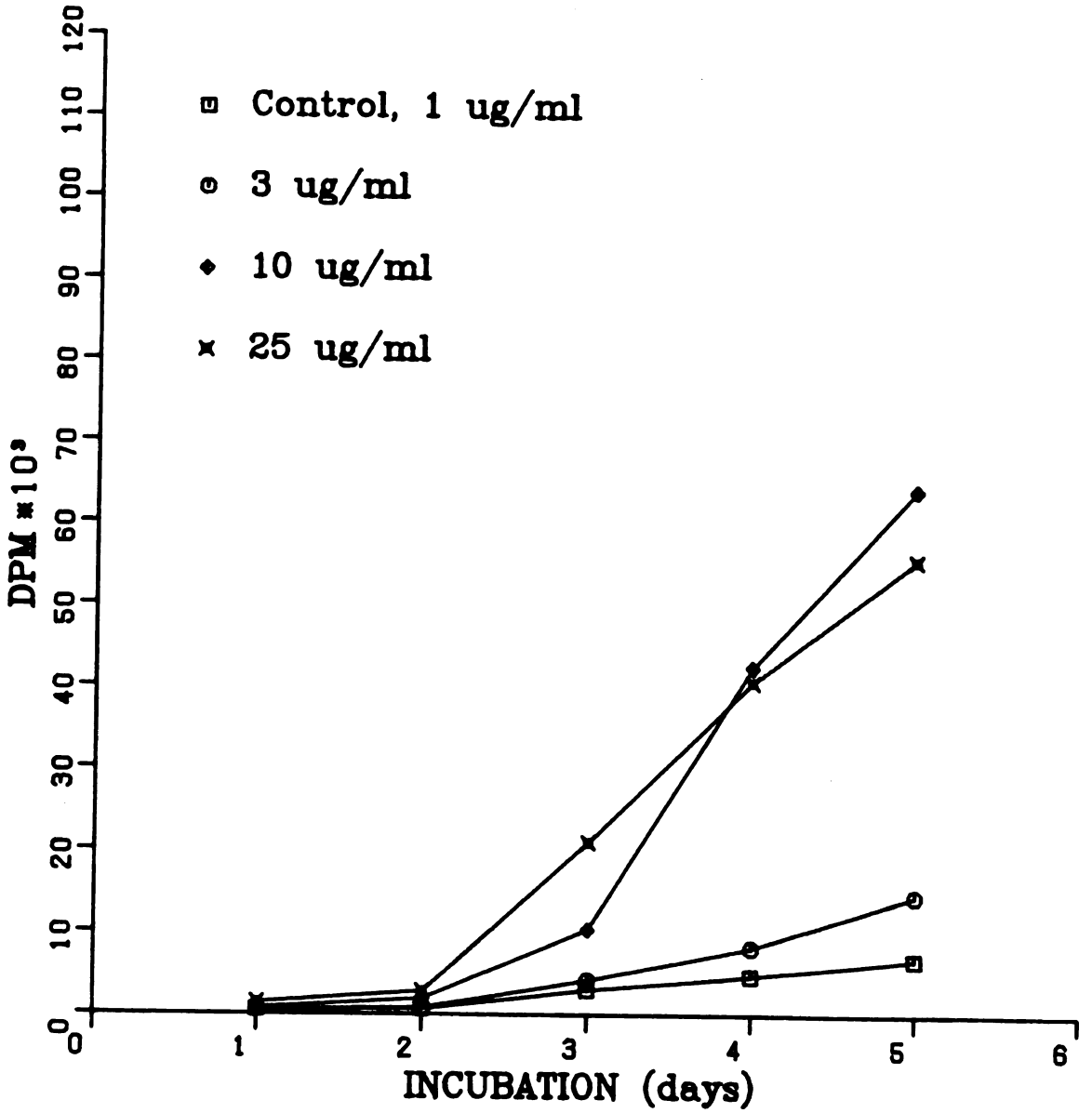

Figure 4. Kinetics of Response of Fish 2 PBL to ConA in Experiment 18.

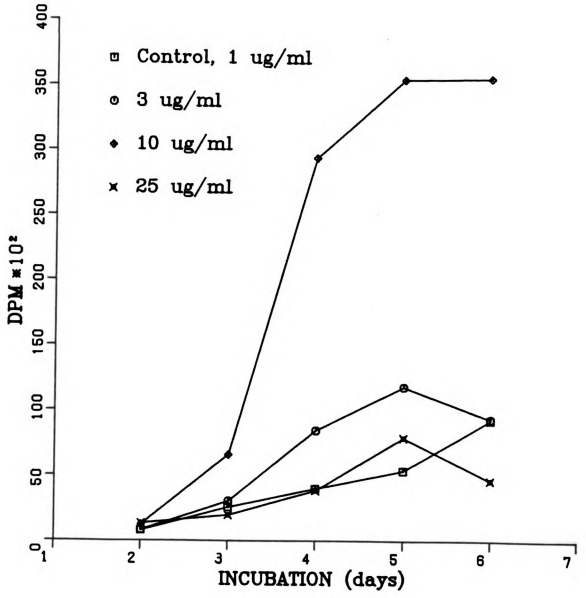

Figure 5. Kinetics of Response of Fish 21 PBL to ConA in Experiment 20.

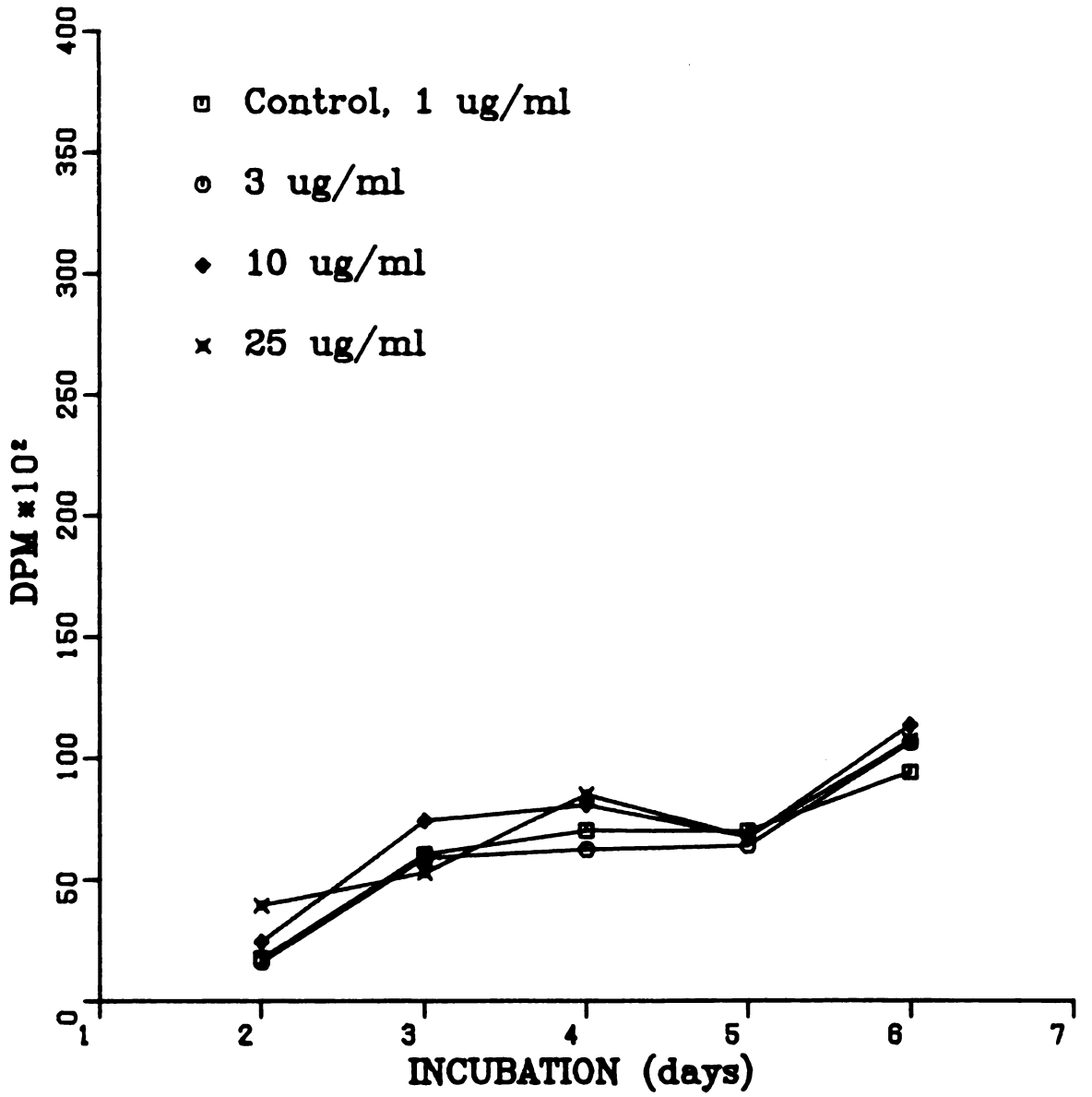

Figure 6. Kinetics of Response of Fish 24 PBL to ConA in Experiment 20.

smallest, the uptake and incorporation of radiolabelled thymidine either reached a plateau or began to decline by day six (Figure 5).

This experiment was repeated to further elucidate the effect of the incubation time on proliverative response and the variability in responsiveness among individual fish. Cells from eight fish were incubated for periods of two to seven days at ConA concentrations of 3, 10, and 15 ug/ml. The mean response (DPM) across fish is presented in Figure 7.

Kinetics of response of RBT PBL to ConA was similar to that of previous experiments, with the maximum amount of radiolabelled thymidine incorporated by day five at all concentrations of ConA. However, the rate of proliferation of the control cells was the same as that of ConA-stimulated cells. The optimal SI for individual fish ranged from 1.15 to 4.25 and varied with both incubation period and mitogen concentration. Analysis of variance, using the dependent variable DPM (Appendix B) resulted in significance of all main effects of fish ($p < 0.0001$), ConA concentrations ($p < 0.0001$), and incubation time ($p < 0.0001$), as well as all interaction terms ($p < 0.0001$). The variation in proliferation response is dose-dependent, with larger relative standard deviations seen at doses of ConA leading to greater lymphocyte activation (Figure 8). It is important to note that even though control cell cultures proliferated at the same rate as the optimal doses of ConA, they had much smaller relative standard deviations. Coefficients of

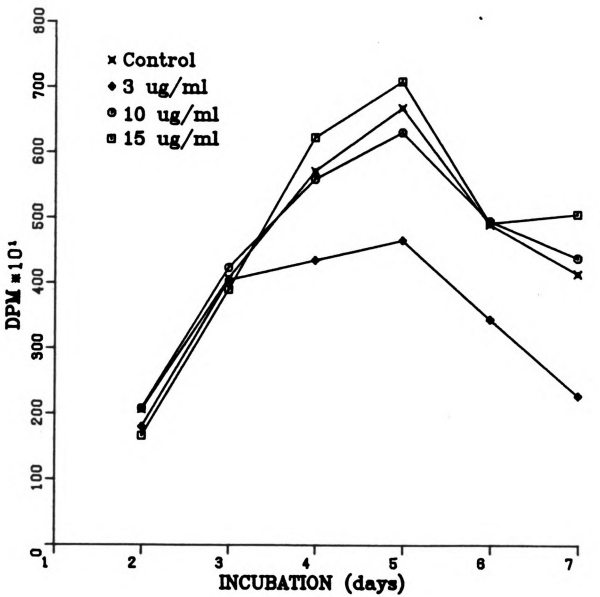

Figure 7. Mean Response of RBT PBL to ConA Across Fish in Experiment 23.

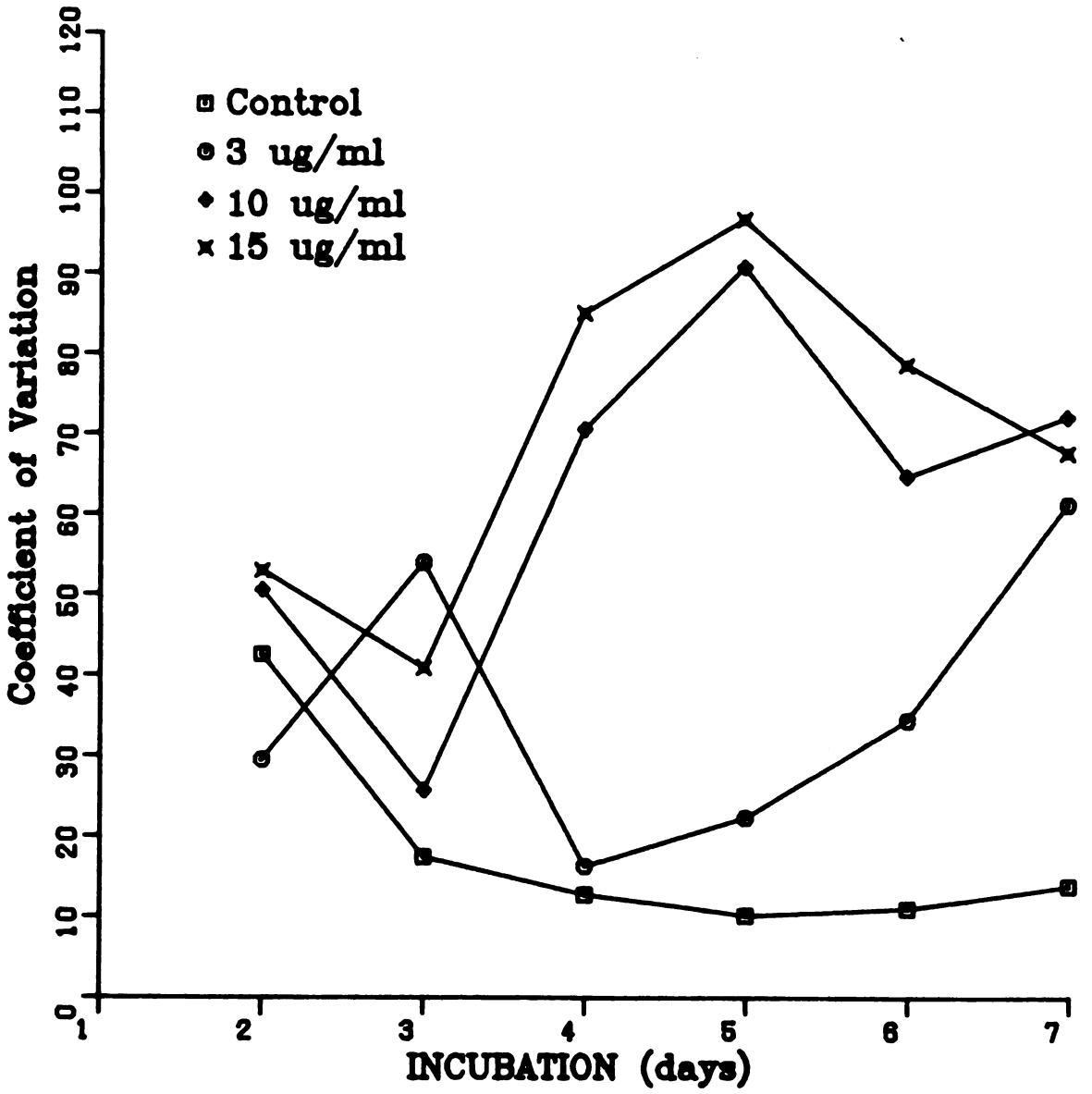

Figure 8. Coefficients of Variation of the SI Response Variable for Means Across Fish in Experiment 23.

variation for control cultures did not increase with the rate of cell proliferation. This same phenomena is true when CV of SI response are determined for across fish and experimental means for the optimal stimulatory dose of ConA (Figure 9).

Four points may be taken from this experiment: 1) The optimal incubation period for RBT PBL stimulated with ConA is five days. 2) The optimal dose of mitogen for proliferative response under the test conditions is in the range of 10-15 ug ConA/ml, however this dose range results in the greatest variation in proliferative response. 3) Activation of RBT PBL by ConA was slight as compared to control cultures with optimal SI for individual fish ranges from 1.15 to 4.25 and a mean optimal SI of 2.13. 4) The positive correlation of relative standard variation to rate of proliferation seen in ConA-stimulated cells is absent in control cultures.

The question raised by the last two points is whether the small amount of stimulation of lymphocytes treated with ConA relative to controls is due to an inhibition of mitogen-mediated proliferation or an artifact caused by the stimulation of controls by something in the culture medium. The next set of experiments was designed to determine how much of the variation was due to culture conditions, and how much was due to variation in experimental procedures.

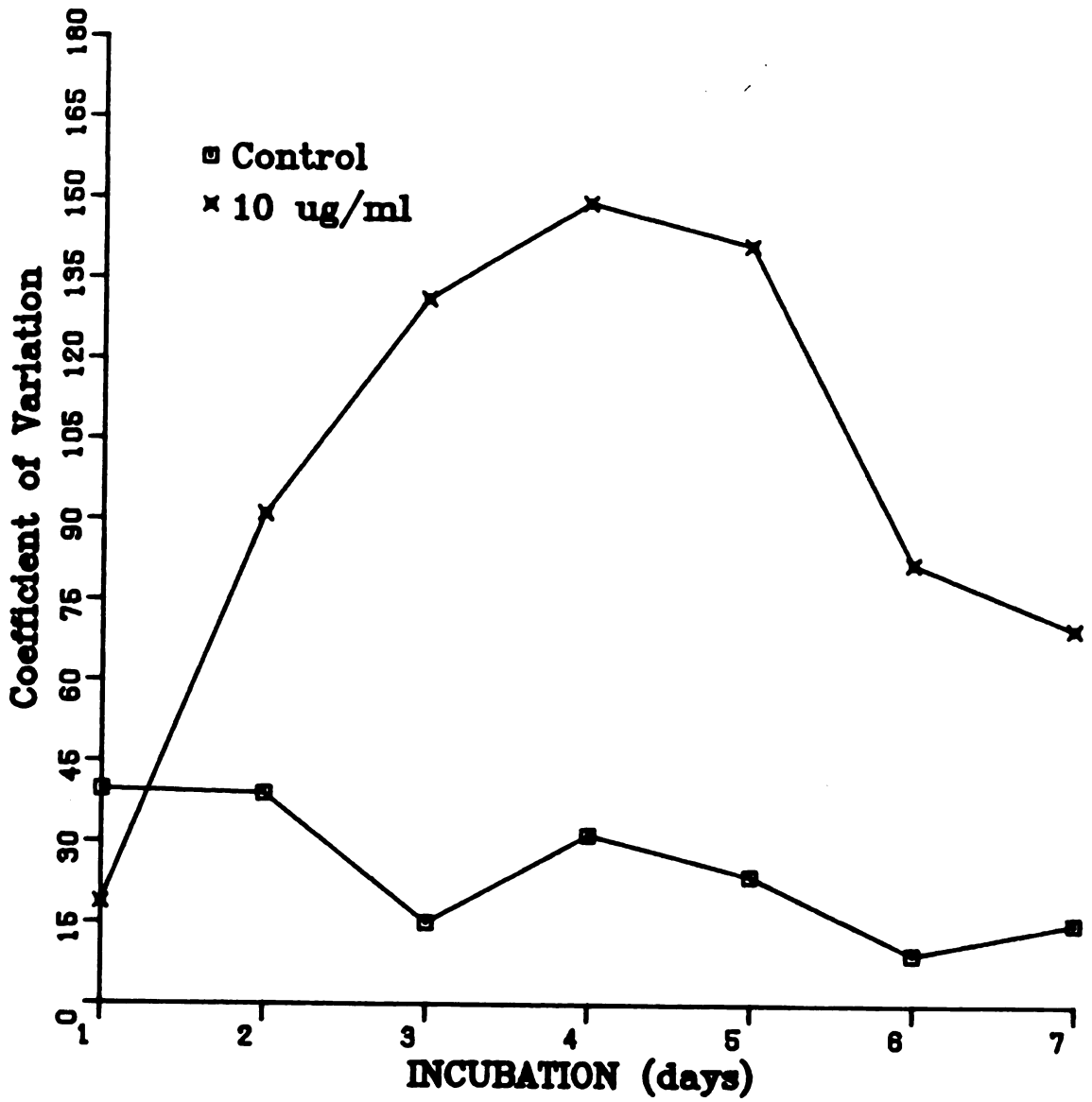

Figure 9. Coefficients of Variation of the SI Response Variable of Across Fish and Experiment Means for RBT PBL Stimulated with ConA.

Serum Supplement

Experiments were performed in which the culture medium was supplemented with human sera instead of FBS, to assess the effect of an alternate source of protein on RBT PBL proliferative response. All other parameters of the culture media and assay procedure were the same as in previous experiments with FBS supplementation. Heat inactivated human serum (HS) was added to the culture medium at 10% (v/v), lymphocytes were stimulated with ConA at 3, 10, or 15 ug/ml, and incubations were either 2, 3, 4, or 5 days.

The initial experimental design (Appendix B19) used lymphocytes from seven fish. Lymphocytes were exposed to three mitogen concentrations harvested after 3, 4, and 5 days of incubation. Maximum SI for ConA-stimulated lymphocytes ranged from 1.19 to 3.12. The mean response (DPM) across all fish in this experiment is presented in Figure 10. This experiment was repeated because it appeared that the optimal day for harvest may have been prior to day 3. The experimental design for this assay included a 2-day incubation period. In the second experiment maximal SI for individual fish ranged from 1.55 to 6.27. The mean response (DPM) at each dose across fish for this assay is presented in Figure 11. The mean maximal SI across both of these experiments was 2.95.

The kinetics of response of RBT PBL in culture medium supplemented with HS are slightly different from RBT PBL cultured with FBS. The greatest amount of incorporation of ^{3}H-thymidine into DNA was on day 3 (Figures 10 and 11) in both

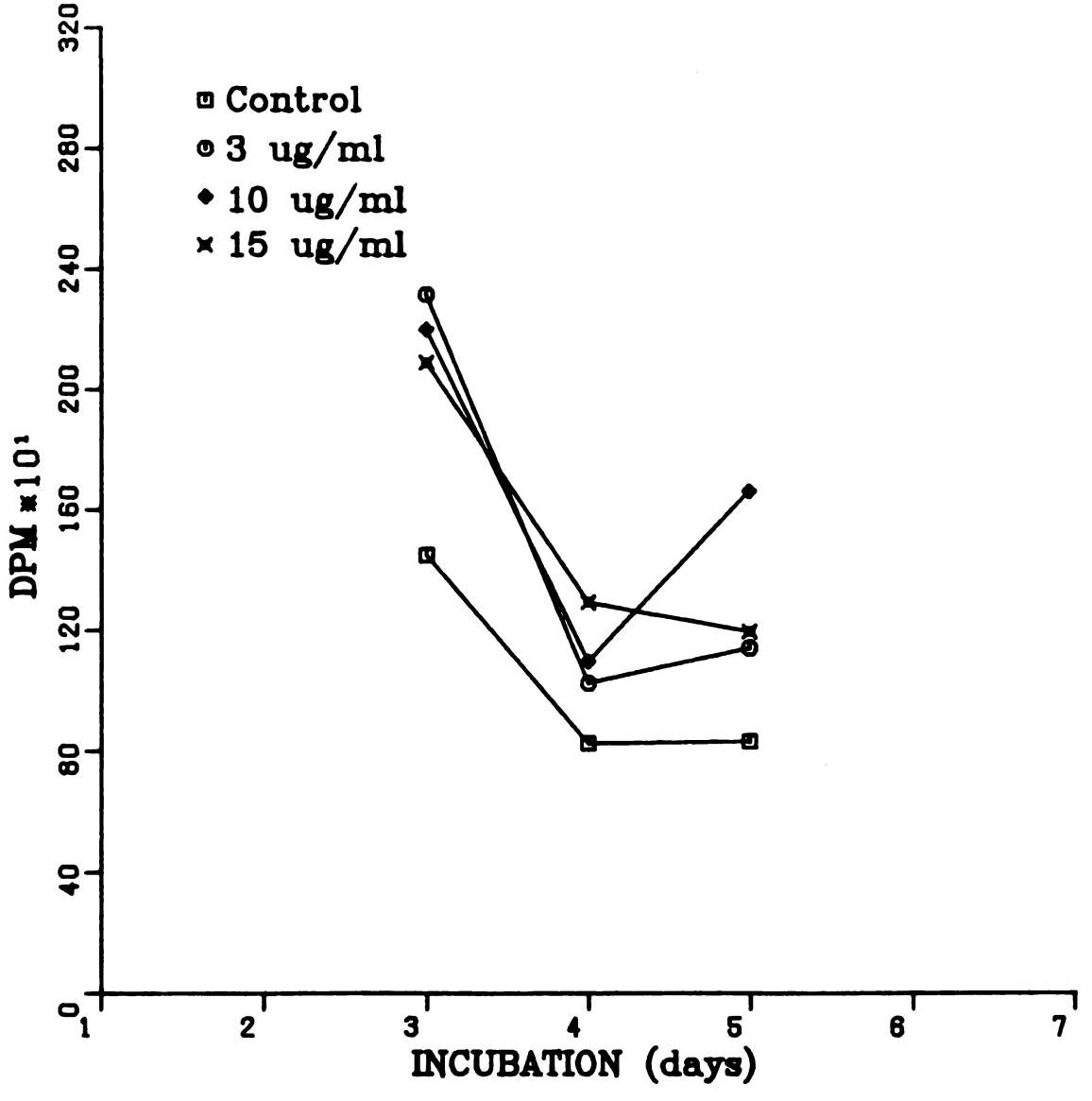

Figure 10. Mean Response (DPM) of RBT PBL to ConA with 10% Human Sera Supplementation in Experiment 28.

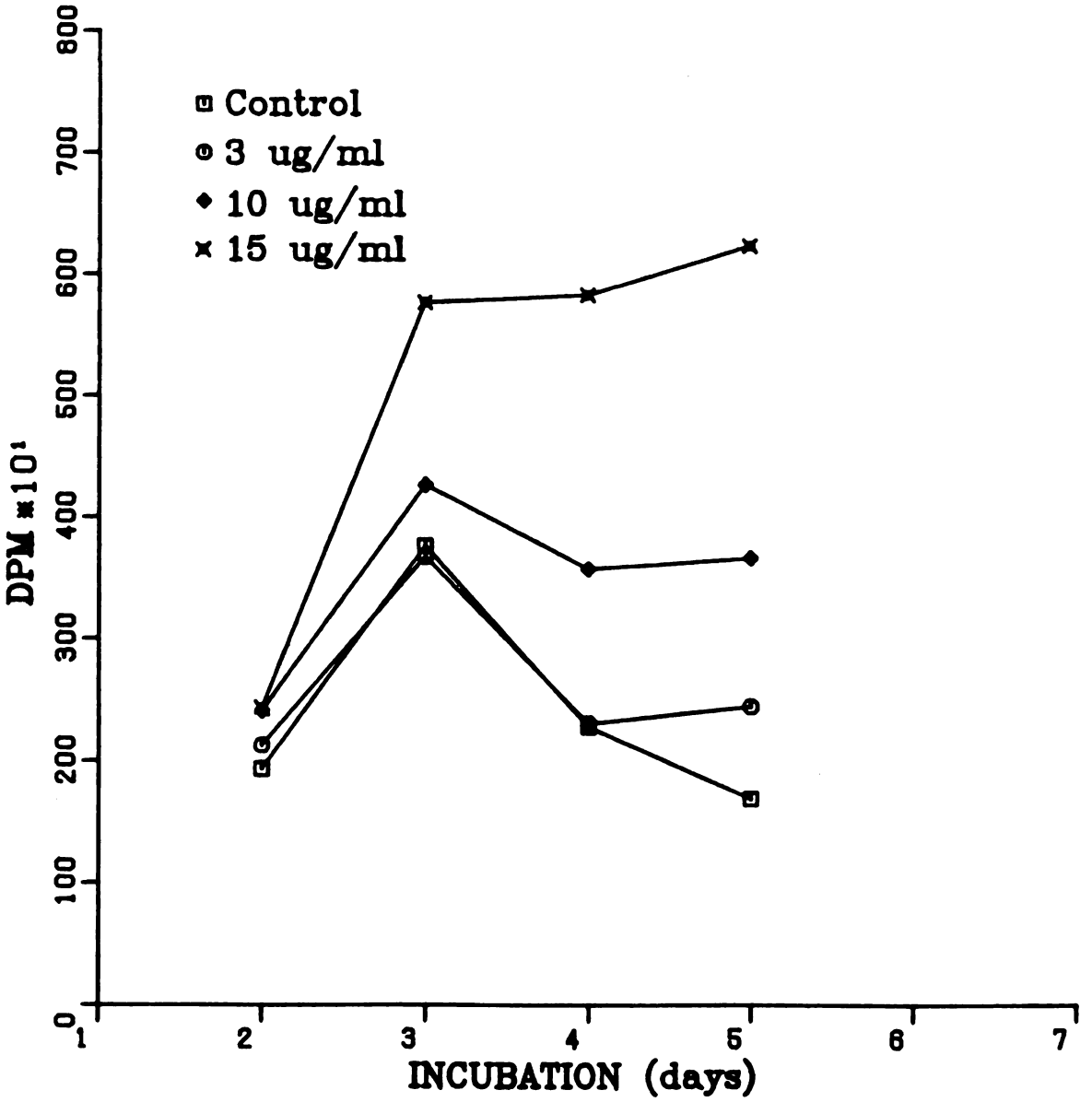

Figure 11. Mean Response (DPM) of RBT PBL to ConA With 10% Human Sera Supplementation in Experiment 31.

experiments. The mean response (DPM) across all fish PBL cultures, from both of these experiments, is presented in Figure 12. A dose-response is seen with increasing ConA concentration. The maximum average stimulation of RBT PBL was observed when lymphocytes were incubated with 15 ug ConA/ml for three days. Only those cultures harvested on days 4 or 5 had a mean response significantly greater than that of the control cultures. The pattern of greater variation in response associated with the treatment combinations that produce the greatest proliferative response (Figure 13) is similar to the results seen when FBS is used as a protein source.

General linear models were used to analyze and partition variance among the main effects (individual fish, ConA concentration, and duration of incubation) and their interactions for these experiments (Appendices B20-B21, B42-B43). All main effects contributed significantly to the total variance ($p < 0.001$) relative to unexplained residual variance when the dependent response variable DPM was analyzed. The interactions of fish * ConA concentration and fish * incubation period were also significant ($p < 0.005$) in both experiments with culture medium supplemented with human serum.

Procedural Variability

An experiment was performed in which a single blood sample from each of four fish was split into four portions and each of these portions was then assayed for lymphocyte proliferative response to determine how much of the variability in proliferative

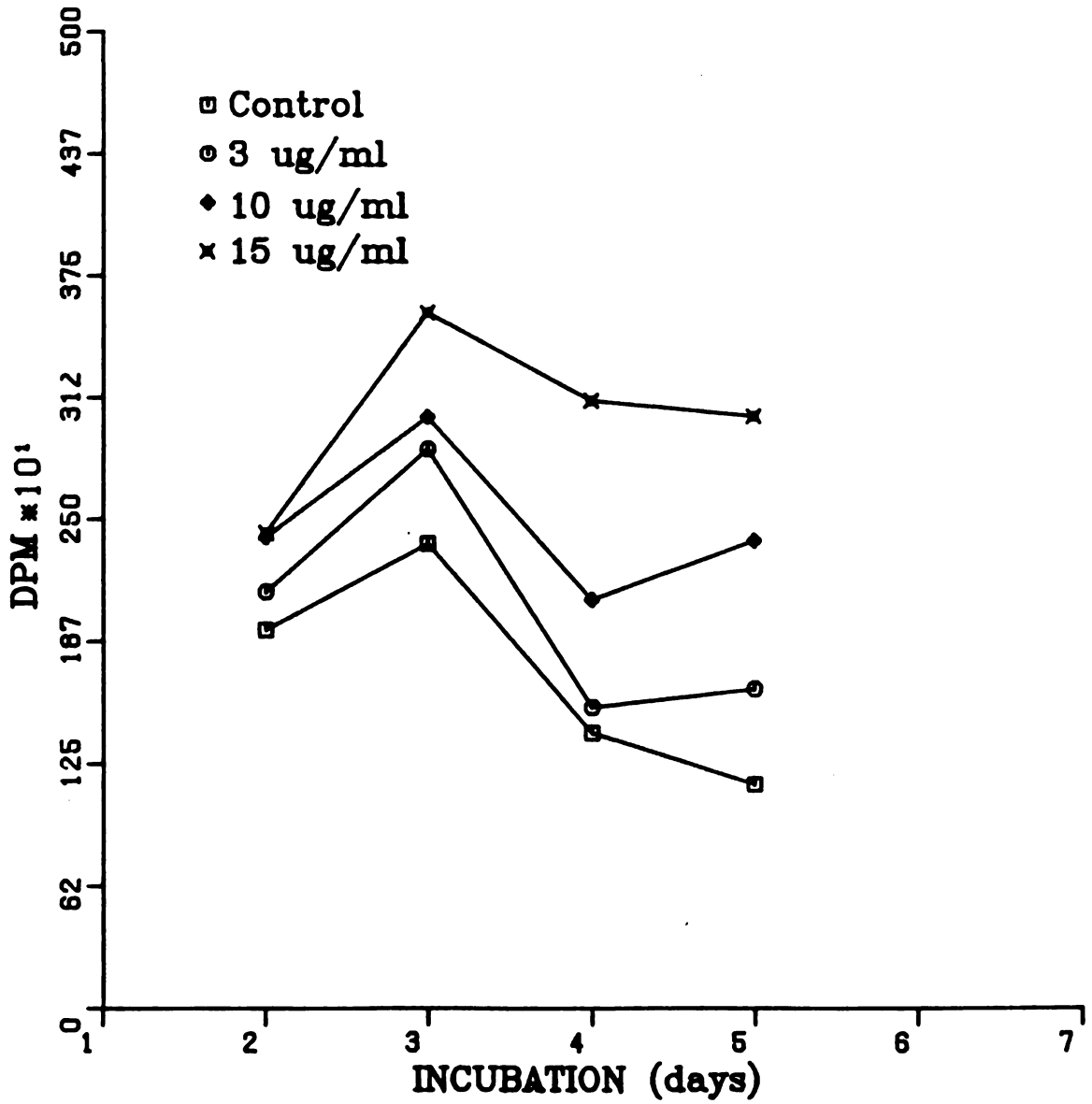

Figure 12. Mean Response (DPM) of RBT PBL to ConA With 10% Human Sera Supplimentation Across Experiments 28 and 31.

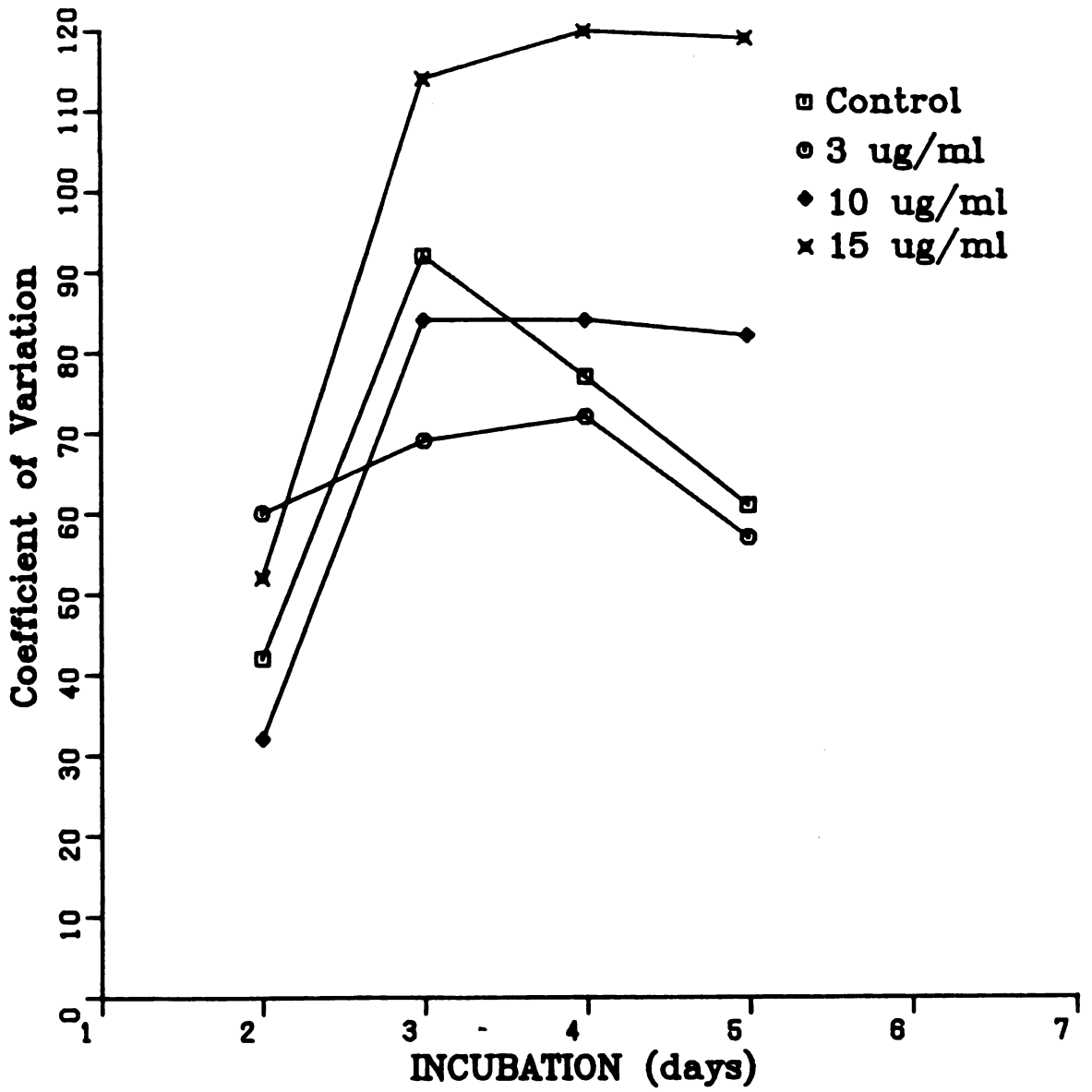

Figure 13. Coefficients of Variation for the DPM Response of Across Fish and Experiment Means of RBT PBL Stimualted With 10% Human Sera.

response was due to the separation of the cells and assay procedure. The results were analyzed with the GLM procedure of SAS. The model and formulation of expected mean squares are presented in Appendix B16. The estimates of variance, partitioning of variance, and F-values from a 3-way ANOVA are given for the dependent variables DPM (Appendix B17) and SI (Appendix B18). When the dependent variable DPM is used as the response, the main effects of individual fish, ConA concentration, and separation were all significant ($p < 0.05$) while none of the interaction terms explained significant portions of the variance. When the data was normalized to the proliferation observed in medium without mitogens (SI), the only main effect that was significant is the concentration of ConA, however, the interaction term between individual fish and separation was also significant ($p < 0.05$).

The percentage of the variance attributed to the separation procedure was 21.01% when using raw DPM response data. That is, 21.01% of the variance in DPM response was to lymphocyte isolation, cell counting, delivery volumes, and other procedural steps. When SI is used as the dependent variable, the variance due to these procedures is reduced to zero. This indicates that the procedure is not affecting the degree to which RBT PBL will be stimulated by ConA. Cells from an individual fish responded similarly to the mitogen. The variation in DPM response between samples from the same fish was likely due to error associated with cell counts.

Table 6. Mean and Range of Responses of Four Subsamples of PBL From Individual RBT to ConA.[1]

	Mean DPM	Range DPM	Mean SI	Range SI
Fish 61				
Control	1292	1124-1457	----	---
3 ug/ml	1745	1020-2409	1.37	0.78-2.10
10 ug/ml	1532	1166-1967	1.20	0.89-1.75
15 ug/ml	1250	813-1426	0.99	0.62-1.60
Fish 62				
Control	652	289- 996	----	---
3 ug/ml	1093	537-2179	1.68	0.86-2.21
10 ug/ml	723	355-1115	1.12	0.66-1.59
15 ug/ml	603	168-1091	0.99	0.27-1.65
Fish 63				
Control	1058	552-1507	----	---
3 ug/ml	2171	1516-2677	2.24	1.41-2.78
10 ug/ml	1398	1177-1896	1.49	0.78-2.21
15 ug/ml	952	603-1206	0.97	0.68-1.29
Fish 64				
Control	893	434-1642	----	---
3 ug/ml	1417	1273-1675	2.08	0.87-2.97
10 ug/ml	926	509-1609	1.22	0.52-1.67
15 ug/ml	726	402- 969	0.95	0.57-1.38

1) RBT PBL cultured in RPMI 1640 supplemented with 10% FBS, $10^{5.7}$ cells/well, incubated with ^{3}H-thymidine (1 uCi/well) for 24 hours prior to harvest, and harvested on day 5 of incubation. DPM = disintegrations per minute. SI = stimulation index (DPM treatment/DPM controls).

Fish Source

To determine whether the variability in response of RBT PBL to mitogens was due to laboratory fish health problems or holding conditions, PBL from fish taken at Balders Fish Farm were tested for their ability to respond to ConA in vitro. Three fish from Balders were bled and whole blood samples were diluted with complete medium (RPMI 1640/10% FBS). The samples were transported to the laboratory and lymphocytes were isolated from the diluted whole blood. Cells were assayed as in other experiments and stimulated with 1, 3, 10, 25 or 50 ug ConA/ml. Following a four day incubation, lymphocyte culture which had not been treated with ConA, had activities of 1580, 1585 and 1074 DPM and optimal stimulation indices in experimental cultures were 0.67, 1.19, and 0.83, respectively. The low degree of stimulation was similar to that seen in previous experiments with fish held in our facilities. Therefore our fish holding facilities did not appear to affect the *in vitro* mitogenesis of RBT PBL adversely.

DISCUSSION

Culture Conditions

RPMI 1640, a culture medium specifically designed for the *in vitro* culture of human lymphocytes (Moore et al., 1967), is well suited for the culture of RBT PBL. Viability tests resulted in >90% viability over a seven day period. The pH (7.3) and osmolality (312 mOs/kg) of the medium suggested by others for culture of salmonid cells (Wolfe and Quinby, 1969; Sigel et al., 1973; Warr and Simon, 1983) also appear to be suitable for growth of RBT PBL.

Mitogens

ConA in the culture media at 10 ug/ml provided optimal stimulation of RBT PBL with an incubation period of 4 to 5 days. The mean SI under these culture conditions was 4.85. The mitogens LPS, PHA, and PWM had only a slight stimulatory effect on RBT PBL under the same culture conditions. These results are similar to that of Etlinger et al. (1976a) who reported that maximal RBT PBL activation was obtained with 10 ug ConA/ml and a 4-5 day incubation period. When FBS was used as a protein source they reported SI between 2.2 and 3.1 that of controls. The results of Warr and Simon (1983) were somewhat different with ConA producing maximal stimulation at 3 ug/ml on day 4 only with RBT PBL. The major difference of these results and those reported here is the degree of stimulation. Warr and Simon reported SI of about 7.3 for RBT PBL activated with ConA, about twice that of the mean optimal SI in these studies. This

difference may be due to the fact that Warr and Simon did not report a mean across all fish tested. In the materials and methods section of their paper they state, "For reasons that are not understood lymphocytes from an occasional fish failed to respond or responded very poorly." Additionally, it was unclear how many fish were used in their study. From the results it appears that only a single fish with quadruplicate cultures was reported. If this were true, then their results would coincide with my own, for I had individual fish with SI equal to or greater than 7.3. Their objective was to define heterogeneity in RBT lymphocytes populations through mitogenic response, it was not to assess the precise degree of response.

ConA has often proven to be stimulatory to PBL from other fish species (Clem et al., 1977; Faulmann et al., 1983), however the source of protein supplementation for the media appears to be a strong covariate in these studies. For this reason a set of experiments were performed with an alternate source of serum supplementation.

Serum

Heat-inactivated human serum, used as an alternate source of protein for RBT PBL cultures in lymphocyte activation, resulted in a similar dose-response pattern and kinetic pattern as those studies with FBS. The optimal dose was slightly greater (15 ug ConA/ml) and the kinetic response was slightly accelerated (3 day optimal incubation) but the mean maximal SI across fish (SI = 2.95) was slightly less than that of experiments

conducted with FBS. Human serum was chosen because it successfully enhanced stimulation of PBL by ConA in other fish species (Faulmann et al., 1983), it had not been previously tested in this species, and is available commercially.

Finding the optimal serum supplement for lymphocyte activation studies in fish has been a major undertaking in previously reported studies and has only been determined through empirical testing of a wide variety of homologous and heterologous sera. Clem and his coworkers (Cuchens and Clem, 1977; Clem et al., 1981) tested human, calf, fetal calf, rabbit, alligator, bass, grouper, and homologous sera in an effort to find optimal conditions for bluegill (BG) and catfish (CF) PBL activation. Results obtained from these sera individually or in various combinations with BG PBL were "unrewarding" and pooled BG sera proved to be cytotoxic to BG PBL in some cases. They were able to solve this problem through dialysis of the BG sera overnight against 0.15 M NaCl. The reason for the loss of cytotoxic effects of the homologous serum after dialysis were not known or explained by Clem. A similar trial and error experimentation with CF PBL resulted in the unlikely combination of 10% human and 5% channel catfish sera as the preferred supplement. Again, the rationale for this choice is unknown and according to the authors "may seem absurd," however they report the synergistic effects of the mixture to be quite impressive (Faulmann et al., 1983).

Avtalion and his coworkers (Rosenberg-Wiser and Avtalion, 1982; Caspi et al., 1984) report similar problems when carp PBL

are cultured. There was only a low percentage of proliferation when 12% homologous serum was employed. Later studies with rat, rabbit, horse, calf, human, dog, chicken, Tilapia, and carp sera showed that manipulation of the sera was required for optimal results. Charcoal-absorbed pooled carp sera (2-4%) was optimal, although only with low efficiency and high individual variability. Further, they tested charcoal-absorbed carp sera from various individuals and found that certain fish served well as donors while others had sera that failed to support lymphocyte activation.

The only other sera previously tested (besides FBS) with RBT PBL is homologous rainbow trout sera (RBTS). Etlinger et al. (1976a) reported that SI of RBT PBL cultured with ConA, LPS, or purified protein derivative (PPD) were enhanced when 20% RBTS was employed as opposed to 10% FBS. A possible reason for the increased SI of cultures grown with RBTS comes from another report by these same authors. Etlinger et al. (1976b) cultured RBT PBL in media with 10% FBS, 30% FBS, 20% RBTS, or no serum at all. These experiments were conducted without the addition of mitogens and cultures were incubated either 4 or 5 days. There was little growth in cultures without serum or those supplemented with 20% RBTS, while those cultures supplemented with either 10% or 30% FBS had marked growth in the absence of any mitogens. Therefore, the smaller SI observed with FBS supplementation compared with RBTS supplementation (Etlinger et al., 1976a) was most likely due to the mitogenic effect of FBS

on control cultures. This stimulatory effect of FBS was noted in PBL cultures from some fish in this study (control cultures having counts up to 10,000 DPM), although not to the same extent reported by Etlinger who reported counts upwards of 45,000 CPM. A stimulatory effect of FBS on control cultures may have been a factor in the relatively small SI seen in this study, with the mean response of 3840 DPM on day 4. This is approximately twice that reported by Warr and Simon after the same incubation period. However, it seems more likely that inhibitory factors, such as hormones, present in the FBS were responsible for the low SI observed in these studies. Evidence for this comes from the fact that fish cells with the greatest SI often had greater incorporation of ^{3}H-thymidine in control cultures (Figures 3 and 4).

All of these studies reported above point out that serum supplements play a critical role in LA assays, yet they remain an unresolved problem. The concentrations of hormones and proteins have been observed to vary widely in commercially acquired lots of FBS (Horn et al., 1975), particularly those hormones which may have an effect on immunocompetence. In particular, fish cellular immune response has been shown to be suppressed by cortisone (Weinreb, 1959). Rasquin (1951) also demonstrated fishes immune system sensitivity to hormones with ACTH. Therefore, the development of assay procedures that utilize chemically defined media could help standardize <u>in vitro</u> LA techniques with fish PBL and enhance the possibilities of their use as a diagnostic tool.

Variation and Sample Size

Variance of SI among fish in a given experiment when the main effects of incubation period (day) and ConA concentration were considered ranged from 3.31 to 19.83% of the total variance (Appendix B). The variance components of incubation period (day) and ConA concentration in these same experiments had ranges of 0 to 6.77% and 6.00 to 29.48% of the total variance, respectively. These main effects and all the interaction terms (except the Day*ConA interaction in experiment 20, Appendix B6) were significant at the $p = 0.05$ level. The error variance was between 0.23 and 29.64% of the total variance in these experiments. These fractions were all increased in experiments that had only a single incubation period. The variance attributable to harvest day effects and interactions of this variable were folded into the other main effects and error terms.

Orthogonal contrasts incorporating experiment to experiment variation were impossible due to loss of samples in the lymphocyte isolation process and poor recovery efficiency on others. These problems led to varied numbers of fish or reduced harvest days in some of the experiments. A two-stage nested contrast of control cultures of fish across experiments was performed, however, and both experiment and fish were significant effects ($p < 0.001$) on any given harvest day. A possible explanation of these sources of variance is the differences in serum lots used in the various experiments.

Variation within a fish sample due to procedural handling of the cells (separation, counting, and platting) was 21.01% of the total variance when the dependent variable DPM was analyzed and 0% of the total when SI was used. The difference seen here was most likely attributable to the fact that error due to separation and cell counts is normalized when SI are the dependent variable. Any variation in DPM response of mitogen stimulated cultures that is caused by altered cell counts or cell separation on the gradient material is reduced when divided by the control cultures response from that same separation. The control cultures having been handled the same as the stimulated cells in a particular separation.

A useful piece of information that can be obtained from the estimates of variance is the sample sizes required in future experiments. Previous investigations of RBT PBL LA have either failed to report the number of fish used (Warr and Simon, 1983) or standard errors were not reported (Etlinger et al., 1976a). From the estimates of variance reported in this study, power analyses were conducted according to Gill (1977). The design of the studies to be conducted dictates the number of samples (fish) that are required to detect a specified difference or change in response. In an experimental design that included only a single mitogen treatment at the optimal dose (10 ug ConA/ml) and a single incubation period (4 days) the sample sizes required to detect various degrees of immunosuppression are presented in

Table 7. Variance estimated for this power analysis were taken from Table 4, Type I error (A) = 0.05, and Type II error (B) = 0.20. The number of fish that would be required, with three replications per fish, is prohibitively large even for the detection of a 50% reduction in response. An experimental design similar to a typical experiment from this study (4 ConA concentrations and 6 harvest days) reduces this requirement. Power analyses were performed with A = 0.05, B = 0.20, replication per fish = 3, and variance estimates from one of my experiments (Appendix B14). Detection of a 1000 DPM reduction (approximately 10%) in response would require a sample size of 41 fish, while a 0.2 SI reduction (approximately 10%) would require a 54 fish sample size. The requirements of holding facilities in a toxicological study for such a large number of fish in a given exposure would be prohibitive due to the size of fish necessary. Each fish blood sample is split into at least four tubes in the lymphocyte isolation procedure, therefore centrifuging would also be a limiting step in the process. The personnel needed to process the samples would also be inordinantly large.

Therefore, the use of this assay as a diagnostic tool in RBT is limited under conditions specified in the literature and this work.

Table 7. Sample Sizes Required to Demonstrate Reductions in Immunocompetence of RBT PBL Responses to 10 µg ConA/ml With An Incubation Period of 4 Days.[1]

Percent Reduction in Response	Dependent Response Variable	
	DPM	SI
50%	50	55
40%	77	86
30%	136	152
20%	310	342
10%	1240	1370

1) Type I error (A) = 0.05, Type II error (B) = 0.20, 3 replications/fish, and variance estimates from Table 4.

SUMMARY AND CONCLUSIONS

In vitro mitogenesis of RBT PBL, which had been demonstrated in the literature previously, was further investigated to define the optimal conditions for the procedure and the respeatability of the assay for routine laboratory use. The assay variables of media, mitogen, serum supplementation, lymphocyte isolation procedure, and incubation period were assessed.

RPMI 1640 culture media adjusted to pH 7.3 and 312 mOs/kg provided ample nutrients and tonicity for growth of RBT PBL. Isolation of lymphocytes from whole blood was by density gradient centrifugation. Both of the commercially available gradient materials tested (Percoll and Ficol-Paque) resulted in cell populations consisting of >90% lymphocytes and the remainder a distribution of moncytes, thrombocytes, and granular leukocytes. Cell classification was based on staining characteristics and flowcytometrics.

Of the mitogens tested (ConA, LPS, PHA, and PWM), the greatest degree of lymphocyte activation of RBT PBL was observed with ConA. The optimal dose of ConA in the culture wells was 10 ug/ml, however this treatment variable showed a significant interaction with both fish and incubation period as well as a three-way interaction ($p < 0.0001$) in most experiments. Kinetic studies of ConA stimulated cells resulted in optimal stimulation at 4 to 5 days, but as with mitogen concentration, incubation period had significant interactions ($p < 0.0001$) with the other main treatment

effects (fish and ConA concentration). Therefore, comments as to the optimal conditions of ConA concentration and incubation period must be kept to a qualitative or general nature. The optimal ConA concentration is 10 ug/ml with an incubation period of 4 to 5 days when across fish averages are considered, but individual fish may respond optimally at a different mitogen concentration in a different time frame. Mean SI across fish and experiments at 10 ug/ml incubated 4 days was 4.87 ($\pm$ 1.18 SE).

Human serum was tested as an alternative source of protein. Dose-response and kinetic pattern of ConA stimulated cells was similar to that of cultures supplemented with FBS, but the degree of stimulation was lower.

Variability in response was shown to be directly related to the degree of proliferation in mitogen stimulated cultures. Coefficients of variation for across fish means of SI response at optimal dose of ConA were in the range of 100%, and those for across fish and experiment means were up to 150%. Control cultures generally had coefficients of variation for across fish and experiment means of less than 30%. Procedural steps of cell isolation and fish holding facilities did not appear to account for the large amount of variation. It is suggested that serum supplement may account for a large portion of the variation. However, the optimal serum supplement or combination of sera was not established in this set of experiments. Development of assay procedures in completely defined media may provide a solution to this problem.

Power analysis with variance estimates from these studies reveal that with an experimental design consisting of a single ConA concentration (10 ug/ml) and incubation period (4 days), the sample sizes required to detect a 50% reduction in mitogen responsiveness of RBT PBL is approximately 50 if the DPM response variable is used or 55 if the SI response variable is evaluated. Requirements of sample size are reduced if a multi-stage nested design is used, however, the overall work load would not be reduced significantly because of the increase in harvest days, mitogen concentrations, and number of cells needed in such studies.

My specific conclusions are:

1) Cell separation with either Percoll or Ficol-Paque gradient materials provided relatively pure preparations of RBT PBL.
2) Optimal conditions of RBT PBL <u>in vitro</u> mitogenesis were determined to be 10 ug/ml ConA, 4-5 day incubation, and 10% FBS supplementation in this study.
3) Medium supplemented with 10% human serum did not enhance stimulation indicies of RBT PBL cultured with ConA or reduce the variability in response.
4) Variability in proliferative response of RBT PBL under optimal conditions of this study would preclude the use of this assay for laboratory screening of RBT immunocompetence.

5) Responsiveness of individual fish PBL suggests that *in vitro* mitogenesis of RBT PBL may be a useful technique if standardized culture conditions (i.e. serum supplementation) are established.

APPENDICES

APPENDIX A1

ConA screening with human lymphocytes from prospective organ donors. Assay preformed in Dr. Robert Bull's laboratory with 3H-thymidine as a monitor.

	Mean CPM	SI*
Donar C		
Control	1571	1.0
ConA 1 mg/ml	39308	25.0
ConA 0.1 mg/ml	125789	80.1
ConA 0.01 mg/ml	107431	68.4
ConA 0.001 mg/ml	17760	11.3
Donar D		
Control	2481	1.0
ConA 1 mg/ml	53482	21.6
ConA 0.1 mg/ml	81678	32.9
ConA 0.01 mg/ml	101962	41.1
ConA 0.001 mg/ml	30682	12.4
Control X		
Control	1056	1.0
ConA 1 mg/ml	5788	5.5
ConA 0.1 mg/ml	190166	179.9
ConA 0.01 mg/ml	57786	54.7
ConA 0.001 mg/ml	3340	3.2
Control Y		
Control	1732	1.0
ConA 1 mg/ml	5486	3.2
ConA 0.1 mg/ml	148285	85.6
ConA 0.01 mg/ml	62214	35.9
ConA 0.001 mg/ml	5318	3.1

* SI=Stimulation Index = CPM experimentals/ CPM controls.

APPENDIX B1

Model, Formula and Coefficients of Expected Mean Squares for the 3-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL in Experiment 18[1].

Model

$$Y = u + A_a + B_b + C_c + (AB)_{ab} + (AC)_{ac} + (BC)_{bc} + (ABC)_{abc} + E_{(abc)r}$$

Sources of Variation	df[2]	E[MS][2]
1.Fish (C)	1	$s^2 + 100s^2_c$
2.ConA (A)	4	$s^2 + 20s^2_{ac} + 10\Sigma A^2_a$
3.Day (B)	4	$s^2 + 20s^2_{bc} + 10\Sigma B^2_b$
4.Fish*ConA	4	$s^2 + 20s^2_{ac}$
5.Fish*Day	4	$s^2 + 20s^2_{bc}$
6.ConA*Day	16	$s^2 + 4s^2_{abc} + 0.5\Sigma\Sigma(AB)^2_{ab}$
7.Fish*ConA*Day	16	$s^2 + 4s^2_{abc}$
8.Error	143	s^2
9.Total	192	

---

1)Model for experiment is mixed with fixed treatment effects of ConA and Day, and random effect of Fish. The levels for each treatment are: Fish, c = 2; ConA, a = 5; Day, b = 5. The experimental design was completely cross-classified with r=4.

2)df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B2

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of DPM Response from RBT PBL Cultured in Experiment 18[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	1	0.4437×10^8	0.71	567.97	<0.0001
2.ConA	4	9.7607×10^8	15.53	1448.20	<0.0001
3.Day	4	8.9145×10^8	14.18	1206.14	<0.0001
4.Fish*ConA	4	0.8728×10^8	1.25	201.05	<0.0001
5.Fish*Day	4	0.2567×10^8	0.41	67.10	<0.0001
6.ConA*Day	16	4.2021×10^9	66.84	300.67	<0.0001
7.Fish*ConA*Day	16	0.6107×10^8	0.97	32.21	<0.0001
8.Error	143	0.0782×10^8	0.12	--	--
9.Total	192	6.2870×10^9	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B3

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of SI Response from RBT PBL Cultured in Experiment 18[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	1	7.58	3.31	1430.97	<0.0001
2.ConA	4	67.50	29.48	1727.69	<0.0001
3.Day	4	15.50	6.77	422.99	<0.0001
4.Fish*ConA	4	12.07	5.27	457.43	<0.0001
5.Fish*Day	4	3.42	1.50	130.32	<0.0001
6.ConA*Day	16	116.00	50.66	159.67	<0.0001
7.Fish*ConA*Day	16	6.37	2.78	49.34	<0.0001
8.Error	143	0.53	0.23	--	--
9.Total	192	228.98	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B4

Model,Formula and Coefficients of Expected Mean Squares for the 3-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL in Experiment 20[a].

Model

$$Y = u + A_a + B_b + C_c + (AB)_{ab} + (AC)_{ac} + (BC)_{bc} + (ABC)_{abc} + E_{(abc)r}$$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	2	$s^2 + 100s^2$
2.ConA (A)	4	$s^2 + 20s^2_{ac} + 15\Sigma A^2_a$
3.Day (B)	4	$s^2 + 20s^2_{bc} + 15\Sigma B^2_b$
4.Fish*ConA	8	$s^2 + 20s^2_{ac}$
5.Fish*Day	8	$s^2 + 20s^2_{bc}$
6.ConA*Day	16	$s^2 + 4s^2_{abc} + 0.75\Sigma\Sigma(AB)^2_{ab}$
7.Fish*ConA*Day	32	$s^2 + 4s^2_{abc}$
8.Error	208	s^2
9.Total	282	

---

a) Model for experiment is mixed with fixed treatment effects of ConA and Day, and random effect of Fish. The levels for each treatment are: Fish, c = 3; ConA, a = 5; Day, b = 5. The experimental design was completely cross-classified with r=4.

b) df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B5

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of DPM Response from RBT PBL Cultured in Experiment 20[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	2	8.6479×10^6	11.00	286.39	<0.0001
2.ConA	4	3.9192×10^6	4.98	136.18	<0.0001
3.Day	4	2.2615×10^7	28.76	169.48	<0.0001
4.Fish*ConA	8	1.7542×10^7	22.31	116.78	<0.0001
5.Fish*Day	8	8.5663×10^6	10.89	57.54	<0.0001
6.ConA*Day	16	0	0.0	12.34	<0.0001
7.Fish*ConA*Day	32	1.4319×10^7	18.21	19.90	<0.0001
8.Error	208	3.0302×10^6	3.85	--	--
9.Total	282	7.8640×10^7	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B6

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of SI Response from RBT PBL Cultured in Experiment 20[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	2	0.2447	8.46	29.55	<0.0001
2.ConA	4	0.5168	17.94	22.89	<0.0001
3.Day	4	0.0	0.0	2.62	<0.0359
4.Fish*ConA	8	0.5490	18.99	13.81	<0.0001
5.Fish*Day	8	0.2160	7.47	6.04	<0.0001
6.ConA*Day	16	0.0	0.0	1.35	<0.1781
7.Fish*ConA*Day	32	0.5060	17.50	3.34	<0.0001
8.Error	208	0.8569	29.64	--	--
9.Total	282	2.8911	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B7

Model,Formula and Coefficients of Expected Mean Squares for the 3-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL in Experiment 21[a].

Model

$Y = u + A_a + C_c + (AC)_{ac} + E_{(abc)r}$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	7	$s^2 + 12s^2$
2.ConA (A)	3	$s^2 + 3s^2_{ac} + 8\Sigma A^2_a$
3.Fish*ConA	21	$s^2 + 3s^2_{ac}$
4.Error	64	s^2
5.Total	95	

---

a) Model for experiment is mixed with fixed treatment effects of ConA, and random effect of Fish. The levels for each treatment are: Fish, c = 8; ConA, a = 4. The experimental design was completely cross-classified with r=3.

b) df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B8

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of DPM Response from RBT PBL Cultured in Experiment 21[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	7	0.6814×10^5	8.77	3.29	<0.0048
2.ConA	3	3.5242×10^5	45.36	8.79	<0.0001
3.Fish*ConA	21	0	0.0	0.88	<0.6155
4.Error	64	3.5631×10^5	45.86	--	--
5.Total	95	7.7688×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B9

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of SI Response from RBT PBL Cultured in Experiment 21[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	7	0.0267	2.63	1.65	<0.1380
2.ConA	3	0.4804	47.34	8.83	<0.0001
3.Fish*ConA	21	0.0122	1.20	1.07	<0.3973
4.Error	64	0.4955	48.83	--	--
5.Total	95	1.0148	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B10

Model,Formula and Coefficients of Expected Mean Squares for the 2-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL in Experiment 22[a].

Model

$Y = u + A_a + C_c + (AC)_{ac} + E_{(abc)r}$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	2	$s^2 + 12s^2_c$
2.ConA (A)	3	$s^2 + 3s^2_{ac} + 3\Sigma A^{\ 2}_a$
3.Fish*ConA	6	$s^2 + 3s^2_{ac}$
4.Error	24	s^2
5.Total	35	

---

a) Model for experiment is mixed with fixed treatment effects of ConA, and random effect of Fish. The levels for each treatment are: Fish, c = 3; ConA, a = 4. The experimental design was completely cross-classified with r=3.

b) df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B11

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of DPM Response from RBT PBL Cultured in Experiment 22[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	2	2.9130×10^4	39.67	3.29	<0.0048
2.ConA	3	0	0.0	8.79	<0.0001
3.Fish*ConA	6	3.0516×10^4	41.56	0.88	<0.6155
4.Error	24	1.3777×10^4	18.76	--	--
5.Total	35	7.3423×10^4	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B12

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of SI Response from RBT PBL Cultured in Experiment 22[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	2	0.0508	22.08	10.34	<0.0006
2.ConA	3	0.0	0.0	0.55	<0.6528
3.Fish*ConA	6	0.1141	49.55	6.24	<0.0005
4.Error	24	0.0653	28.37	--	--
5.Total	35	0.2302	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B13

Model,Formula and Coefficients of Expected Mean Squares for the 3-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL in Experiment 23[a].

Model

$$Y = u + A_a + B_b + C_c + (AB)_{ab} + (AC)_{ac} + (BC)_{bc} + (ABC)_{abc} + E_{(abc)r}$$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	7	$s^2 + 72s^2_c$
2.ConA (A)	3	$s^2 + 18s^2_{ac} + 36\Sigma A^2_a$
3.Day (B)	5	$s^2 + 12s^2_{bc} + 16\Sigma B^2_b$
4.Fish*ConA	21	$s^2 + 18s^2_{ac}$
5.Fish*Day	32	$s^2 + 12s^2_{bc}$
6.ConA*Day	15	$s^2 + 3s^2_{abc} + 1.6\Sigma\Sigma(AB)^2_{ab}$
7.Fish*ConA*Day	96	$s^2 + 3s^2_{abc}$
8.Error	358	s^2
9.Total	537	

---

a) Model for experiment is mixed with fixed treatment effects of ConA and Day, and random effect of Fish. The levels for each treatment are: Fish, c = 8; ConA, a = 4; Day, b = 6. The experimental design was completely cross-classified with r=3.

b) df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B14

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of DPM Response from RBT PBL Cultured in Experiment 23[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	7	2.3126×10^6	10.78	209.26	<0.0001
2.ConA	3	0	0.0	58.65	<0.0001
3.Day	5	1.2057×10^7	56.23	261.93	<0.0001
4.Fish*ConA	21	2.9221×10^6	13.63	66.79	<0.0001
5.Fish*Day	32	1.3094×10^6	6.11	20.65	<0.0001
6.ConA*Day	15	0	0.0	5.92	<0.0001
7.Fish*ConA*Day	96	2.0432×10^6	9.53	8.67	<0.0001
8.Error	358	0.7995×10^6	3.73	--	--
9.Total	282	2.1444×10^7	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B15

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of SI Response from RBT PBL Cultured in Experiment 23[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	7	0.1098	19.83	103.72	<0.0001
2.ConA	3	0.0332	6.00	46.83	<0.0001
3.Day	5	0.0	0.0	2.98	<0.0120
4.Fish*ConA	21	0.1295	23.38	31.28	<0.0001
5.Fish*Day	32	0.0929	16.78	15.49	<0.0001
6.ConA*Day	15	0.0	0.0	4.75	<0.0001
7.Fish*ConA*Day	96	0.1113	20.11	5.34	<0.0001
8.Error	358	0.0770	13.90	--	--
9.Total	537	0.5537	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B16

Model, Formula and Coefficients of Expected Mean Squares for the 3-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL in Experiment 26[1].

Model

$$Y = u + A_a + B_b + C_c + (AB)_{ab} + (AC)_{ac} + (BC)_{bc} + (ABC)_{abc} + E_{(abc)r}$$

Sources of Variation	df[2]	E[MS][2]
1.Fish (C)	3	$s^2 + 48s^2_c$
2.ConA (A)	3	$s^2 + 12s^2_{ac} + 16\Sigma A\ ^2_a$
3.Separation (B)	3	$s^2 + 12s^2_{bc} + 16\Sigma B\ ^2_b$
4.Fish*ConA	9	$s^2 + 12s^2_{ac}$
5.Fish*Sep	9	$s^2 + 12s^2_{bc}$
6.ConA*Sep	9	$s^2 + 3s^2_{abc} + .5\Sigma\Sigma(AB)\ ^2_{ab}$
7.Fish*ConA*Sep	27	$s^2 + 3s^2_{abc}$
8.Error	128	s^2
9.Total	191	

---

1)Model for experiment is mixed with fixed treatment effects of ConA and Separation, and random effect of Fish. The levels for each treatment are: Fish, c = 4; ConA, a = 4; Separation, b = 4. The experimental design was completely cross-classified with r=3.

2)df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B17

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of DPM Response from RBT PBL Cultured in Experiment 26[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	9.0173×10^4	5.74	14.06	<0.0001
2.ConA	3	2.9066×10^5	18.50	13.47	<0.0001
3.Separation	3	3.3012×10^5	21.01	15.11	<0.0001
4.Fish*ConA	9	0	0.0	0.87	<0.5547
5.Fish*Sep	9	0	0.0	0.79	<0.6254
6.ConA*Sep	9	0.0170×10^4	0.01	0.71	<0.7035
7.Fish*ConA*Sep	27	0	0.0	0.71	<0.8533
8.Error	128	8.6030×10^5	54.75	--	--
9.Total	191	1.5714×10^6	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value from General Linear Models procdure from SAS.

APPENDIX B18

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of SI Response from RBT PBL Cultured in Experiment 26[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0.86×10^{-2}	0.87	1.55	<0.0890
2.ConA	3	4.57×10^{-3}	46.41	13.47	<0.0001
3.Separation	3	0	0.0	1.31	<0.0676
4.Fish*ConA	9	0.10×10^{-2}	1.12	0.87	<0.2006
5.Fish*Sep	9	1.32×10^{-3}	13.81	3.98	<0.0001
6.ConA*Sep	9	0	0.0	0.71	<0.4103
7.Fish*ConA*Sep	27	2.30×10^{-2}	3.04	0.71	<0.1949
8.Error	128	3.43×10^{-3}	34.75	--	--
9.Total	191	9.86×10^{-3}	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B19

Model,Formula and Coefficients of Expected Mean Squares for the 3-Way ANOVA of ^{3}H-Thymidine Incorporation into RBT PBL Cultured with Human Sera in Experiment 28[a].

Model

$$Y = u + A_a + B_b + C_c + (AB)_{ab} + (AC)_{ac} + (BC)_{bc} + (ABC)_{abc} + E_{(abc)r}$$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	6	$s^2 + 36s^2_c$
2.ConA (A)	3	$s^2 + 9s^2_{ac} + 21\Sigma A^2_a$
3.Day (B)	2	$s^2 + 12s^2_{bc} + 42\Sigma B^2_b$
4.Fish*ConA	18	$s^2 + 9s^2_{ac}$
5.Fish*Day	10	$s^2 + 12s^2_{bc}$
6.ConA*Day	6	$s^2 + 3s^2_{abc} + 3.5\Sigma\Sigma(AB)^2_{ab}$
7.Fish*ConA*Day	30	$s^2 + 3s^2_{abc}$
8.Error	151	s^2
9.Total	226	

---

a) Model for experiment is mixed with fixed treatment effects of ConA and Day, and random effect of Fish. The levels for each treatment are: Fish, c = 7; ConA, a = 4; Day, b = 3. The experimental design was completely cross-classified with r=3.

b) df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B20

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of DPM Response from RBT PBL Cultured with Human Sera in Experiment 28[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	6	1.9260×10^5	10.54	10.61	<0.0001
2.ConA	3	1.2464×10^5	6.82	5.83	<0.0010
3.Day	2	4.0745×10^5	22.31	28.61	<0.0001
4.Fish*ConA	18	0.9670×10^5	5.29	2.21	<0.0051
5.Fish*Day	10	2.3349×10^5	12.78	4.88	<0.0001
6.ConA*Day	6	0.5041×10^5	2.76	0.72	<0.6332
7.Fish*ConA*Day	30	0	0.0	0.48	<0.9903
8.Error	151	7.2142×10^5	39.49	--	--
9.Total	226	1.8267×10^6	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B21

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of SI Response from RBT PBL Cultured with Human Sera in Experiment 28[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	6	0.2270	12.64	8.46	<0.0001
2.ConA	3	0.2596	14.45	7.21	<0.0002
3.Day	2	0.0	0.0	0.40	<0.6685
4.Fish*ConA	18	0.1497	8.34	2.23	<0.0046
5.Fish*Day	10	0.0	0.0	0.85	<0.5858
6.ConA*Day	6	0.0643	3.58	0.61	<0.7183
7.Fish*ConA*Day	30	0.0	0.0	0.41	<0.9973
8.Error	151	1.0953	60.99	--	--
9.Total	226	1.7959	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B22

Model,Formula and Coefficients of Expected Mean Squares for the 3-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL Exposed to ConA or LPS in Experiment 29[a].

Model

$Y = u + A_a + C_c + (AC)_{ac} + E_{(ac)r}$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	3	$s^2 + 12s^2_c$
2.ConA (or LPS) (A)	4	$s^2 + 3s^2_{ac} + 4\Sigma A^2_a$
3.Fish*ConA (or LPS)	9	$s^2 + 3s^2_{ac}$
4.Error	32	s^2
5.Total	47	

---

a) Model for both ConA and LPS stimulated cells in this experiment. Model is mixed with fixed treatment effects of ConA or LPS, and random effects of Fish. The levels for each of the treatments are: Fish, c = 4; ConA (or LPS), A = 4. The experimental design was completely cross-classified with r = 3.

b) d.f. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B23

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of DPM Response from RBT PBL Cultured with ConA in Experiment 29[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	7.9251×10^4	19.88	4.03	<0.0154
2.ConA	3	0.5490×10^4	1.38	0.49	<0.6895
3.Fish*ConA	9	0	0.0	0.42	<0.9127
4.Error	32	3.1398×10^5	78.75	--	--
5.Total	47	3.9872×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B24

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of SI Response from RBT PBL Cultured with ConA in Experiment 29[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0.0998	16.34	3.68	<0.0220
2.ConA	3	0.0642	10.52	1.05	<0.3825
3.Fish*ConA	9	0	0	0.48	<0.8784
4.Error	32	0.4466	73.14	--	--
5.Total	47	0.6105	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B25

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of DPM Response from RBT PBL Cultured with LPS in Experiment 29[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	1.3965×10^4	2.55	1.60	<0.2088
2.LPS	3	2.5487×10^5	46.49	3.97	<0.0163
3.Fish*LPS	9	0	0.0	0.32	<0.9616
4.Error	32	2.7941×10^5	50.96	--	--
5.Total	47	5.4825×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B26

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of SI Response from RBT PBL Cultured with LPS in Experiment 29[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0.0353	4.10	2.15	<0.1138
2.LPS	3	0.4550	52.92	5.36	<0.0042
3.Fish*LPS	9	0.0	0.0	0.43	<0.9080
4.Error	32	0.3695	42.98	--	--
5.Total	47	0.8598	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B27

Model,Formula and Coefficients of Expected Mean Squares for the 2-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL Exposed to PWM or PHA in Experiments 29 and 30[a].

Model

$Y = u + A_a + C_c + (AC)_{ac} + E_{(ac)r}$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	3	$s^2 + 12s^2_c$
2.PWM (or PHA) (A)	3	$s^2 + 3s^2_{ac} + 4\Sigma A^{2}_{a}$
3.Fish*PWM (or PHA)	9	$s^2 + 3s^2_{ac}$
4.Error	32	s^2
9.Total	47	

---

a) Model for both PWM and PHA stimulated cells in these experiments. Model is mixed with fixed treatment effects of PWM or PHA, and random effects of Fish. The levels for each of the treatments are: Fish, c = 4; PWM (or PHA); a = 4. The experimental design was completely cross-classified with r = 3.

b) df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B28

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of DPM Response from RBT PBL Cultured with PHA in Experiment 29[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	1.8564×10^5	20.38	10.70	<0.0001
2.PHA	3	4.7827×10^5	52.52	9.56	<0.0001
3.Fish*PHA	9	0.1714×10^5	1.88	1.22	<0.3153
4.Error	32	2.2965×10^5	25.21	--	--
5.Total	47	9.1068×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B29

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of SI Response from RBT PBL Cultured with PHA in Experiment 29[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0.4241	13.01	6.40	<0.0016
2.PHA	3	1.4383	44.13	7.47	<0.0006
3.Fish*PHA	9	0.4545	13.95	1.36	<0.2452
4.Error	32	0.9420	28.91	--	--
5.Total	47	3.2589	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B30

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of DPM Response from RBT PBL Cultured with PWM in Experiment 29[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	2.6780×10^5	48.50	12.32	<0.0001
2.PWM	3	0.0058×10^5	0.11	0.46	<0.7134
3.Fish*PWM	9	0	0.0	0.45	<0.8967
4.Error	32	2.8377×10^5	51.39	--	--
5.Total	47	5.5214×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B31

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of SI Response from RBT PBL Cultured with PWM in Experiment 29[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0.0937	17.83	4.37	<0.0109
2.PWM	3	1.0985	18.74	1.98	<0.1368
3.Fish*PWM	9	0	0.0	0.80	<0.6206
4.Error	32	0.3334	63.43	--	--
5.Total	47	0.5257	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B32

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of DPM Response from RBT PBL Cultured with PHA in Experiment 30[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	2.1324×10^4	10.10	2.39	<0.0873
2.PHA	3	0.5464×10^4	2.59	0.46	<0.7096
3.Fish*PHA	9	0	0.0	0.35	<0.9522
4.Error	32	1.8440×10^5	87.32	--	--
5.Total	47	2.1119×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B33

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of SI Response from RBT PBL Cultured with PHA in Experiment 30[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0.0483	7.96	2.08	<0.1227
2.PHA	3	0.0209	3.44	0.57	<0.6361
3.Fish*PHA	9	0.0	0.0	0.42	<0.9153
4.Error	32	0.5383	88.60	--	--
5.Total	47	0.6075	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B34

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of DPM Response from RBT PBL Cultured with PWM in Experiment 30[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0	0.0	0.14	<0.9331
2.PWM	3	3.1254×10^4	15.51	1.34	<0.2795
3.Fish*PWM	9	0	0.0	0.60	<0.7847
4.Error	32	1.7024×10^5	84.49	--	--
5.Total	47	2.0150×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B35

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of SI Response from RBT PBL Cultured with PWM in Experiment 30[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0.1198	15.92	3.75	<0.0204
2.PWM	3	0.1104	14.68	1.68	<0.1904
3.Fish*PWM	9	0	0.0	0.84	<0.5886
4.Error	32	0.5220	69.40	--	--
5.Total	47	0.7521	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B36

Model,Formula and Coefficients of Expected Mean Squares for the 2-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL Exposed to ConA or LPS in Experiment 30[a].

Model

$Y = u + A_a + C_c + (AC)_{ac} + E_{(ac)r}$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	1	$s^2 + 12s^2_c$
2.ConA (or LPS) (A)	3	$s^2 + 3s^2_{ac} + 2\Sigma A\ ^2_a$
3.Fish*ConA (or LPS)	3	$s^2 + 3s^2_{ac}$
4.Error	16	s^2
5.Total	23	

---

a) Model for both ConA and LPS stimulated cells in this experiment. Model is mixed with fixed treatment affects of ConA or LPS, and random effects of Fish. The levels for each of the treatments are: Fish, c = 2; ConA (or LPS), A = 4. The experimental design was completely cross-classified with r = 3.

b) df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B37

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of DPM Response from RBT PBL Cultured with ConA in Experiment 30[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	1	1.2256×10^5	26.51	16.07	<0.0010
2.ConA	3	1.3992×10^5	30.27	7.01	<0.0032
3.Fish*ConA	3	1.0225×10^5	22.12	4.14	<0.0237
4.Error	16	0.9758×10^5	21.11	--	--
5.Total	23	4.6231×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B38

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of SI Response from RBT PBL Cultured with ConA in Experiment 30[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	1	0.0	0.0	0.04	<0.8477
2.ConA	3	0.3878	59.25	6.76	<0.0037
3.Fish*ConA	3	0.0869	13.28	2.45	<0.1010
4.Error	16	0.1798	27.47	--	--
5.Total	23	0.6545	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B39

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of DPM Response from RBT PBL Cultured with LPS in Experiment 30[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	1	0	0.0	0.23	<0.6410
2.LPS	3	3.0406×10^5	69.97	5.00	<0.0123
3.Fish*LPS	3	0	0.0	0.34	<0.7965
4.Error	16	1.3048×10^5	30.03	--	--
5.Total	23	4.3451×10^5	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B40

Estimate of Variance, Partition of Variance, and 2-Way ANOVA of SI Response from RBT PBL Cultured with LPS in Experiment 30[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	1	0.1387	5.81	3.11	<0.0969
2.LPS	3	1.4600	61.51	4.33	<0.0205
3.Fish*LPS	3	0	0.0	0.63	<0.6081
4.Error	16	0.7889	33.04	--	--
5.Total	23	2.3876	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B41

Model,Formula and Coefficients of Expected Mean Squares for the 3-Way ANOVA of ^{3}H-Thymidine Incorporation into Cultured RBT PBL Cultured with Human Sera in Experiment 31[a].

Model

$$Y = u + A_a + B_b + C_c + (AB)_{ab} + (AC)_{ac} + (BC)_{bc} + (ABC)_{abc} + E_{(abc)r}$$

Sources of Variation	df[b]	E[MS][b]
1.Fish (C)	3	$s^2 + 48s^2_c$
2.ConA (A)	3	$s^2 + 12s^2_{ac} + 16\Sigma A^2_a$
3.Day (B)	3	$s^2 + 12s^2_{bc} + 16\Sigma B^2_b$
4.Fish*ConA	9	$s^2 + 12s^2_{ac}$
5.Fish*Day	9	$s^2 + 12s^2_{bc}$
6.ConA*Day	9	$s^2 + 3s^2_{abc} + 1.33\Sigma\Sigma(AB)^2_{ab}$
7.Fish*ConA*Day	27	$s^2 + 3s^2_{abc}$
8.Error	128	s^2
9.Total	191	

a) Model for experiment is mixed with fixed treatment effects of ConA and Day, and random effect of Fish. The levels for each treatment are: Fish, c = 4; ConA, a = 4; Day, b = 4. The experimental design was completely cross-classified with r=3.

b) df. = degrees of freedom, E[MS] = expected mean squares

APPENDIX B42

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of DPM Response from RBT PBL Cultured with Human Sera in Experiment 31[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	4.2308×10^6	24.95	236.56	<0.0001
2.ConA	3	2.5453×10^6	15.01	80.13	<0.0001
3.Day	3	1.1018×10^6	6.50	43.37	<0.0001
4.Fish*ConA	9	2.2909×10^6	13.51	32.89	<0.0001
5.Fish*Day	9	1.5748×10^6	9.29	22.92	<0.0001
6.ConA*Day	9	3.6213×10^6	21.35	9.13	<0.0001
7.Fish*ConA*Day	27	0.7318×10^6	4.32	3.55	<0.0001
8.Error	128	0.8621×10^6	5.08	--	--
9.Total	191	1.6959×10^7	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

APPENDIX B43

Estimate of Variance, Partition of Variance, and 3-Way ANOVA of SI Response from RBT PBL Cultured with Human Sera in Experiment 31[1].

Sources	df	s^2	%Tot. s^2	F	PR>F
1.Fish	3	0.1450	3.51	32.02	<0.0001
2.ConA	3	0.7142	17.29	67.67	<0.0001
3.Day	3	0.5776	13.98	48.26	<0.0001
4.Fish*ConA	9	0.2938	7.11	16.72	<0.0001
5.Fish*Day	9	0.1132	2.74	7.06	<0.0001
6.ConA*Day	9	1.8225	44.11	15.03	<0.0001
7.Fish*ConA*Day	27	0.2409	5.83	4.22	<0.0001
8.Error	128	0.2243	5.43	--	--
9.Total	191	4.1314	100%		

1)df = degrees of freedom, s^2 = estimated variance, F-value, and probability value from General Linear Models procdure from SAS.

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