

## Arachidonic Acid Metabolism in Neutrophils from Patients with Rheumatoid Arthritis

### INTRODUCTION

This study is to allow for the important collaboration between Drs. Richard Franson and Duane Smith for analysis of phospholipase A<sub>2</sub> activity in conjunction with arachidonic acid metabolism studies on the neutrophils of patients with rheumatoid arthritis both before and after the completion of therapy with Clotrimazole. Dr. Franson will be doing his studies measuring the phospholipase A<sub>2</sub> activity under a separate grant. Dr. Smith will be performing the arachidonic acid metabolism studies of 10 patients with the blood to be drawn at visit 1 before the patients have begun taking either Clotrimazole or placebo (wash-out period) and at visit 12 when they are completing twelve weeks of therapy with either of these agents. Dr. Smith will separate up to 60 ml of blood for Dr. Franson and ship the cells to him for his studies. Dr. Smith will also obtain 60 ml for his studies. This protocol will not involve an extra stick for these patients but will only involve the addition of an extra blood drawing syringe.

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Protocol

A. Cell isolation

The techniques for harvesting human polymorphonuclear leukocytes (neutrophils) from peripheral blood have previously been established [J. Reticuloendothel. Soc. 25:575-583(1979)]. Briefly, cells are obtained after plasma gel sedimentation of erythrocytes from heparinized whole blood, and layered over a lymphoprep gradient for removal of mononuclear cells. Greater than 95% of the cells have been found to be granulocytes. Contaminating erythrocytes are lysed by a brief hypotonic treatment. Neutrophils are then resuspended in phosphate buffered saline (PBS), pH 7.4. Cells obtained by this procedure have generally been found to retain greater than 95% viability as evidenced by trypan blue exclusion.

B. Incorporation of radiolabel, cell challenge and lipid extraction

Neutrophils  $3.5 \times 10^7$  in 1ml PBS are incubated with [ $^3\text{H}$ ] arachidonic acid (0.5  $\mu\text{Ci}$  0.2 $\mu\text{M}$ ) dissolved in a small amount of 100% ethanol. The final ethanol concentration constitutes less than 0.5% of the total volume. Following a 2 hr incubation at 37°C the cells were centrifuged for 5 min (250xg), washed once with PBS to remove any unbound label, and resuspended to 0.5 ml in PBS.

Opsomized zymosan is prepared as previously described by Waite et al [Biochem. Biophys. Res. Commun. 90; 985-992(1979)]. Stock solution of calcium ionophore A23187 in dimethyl sulfoxide (DMSO) is diluted to 4 $\mu\text{M}$  with PBS. Aliquots (0.5 ml) are added to 0.5 ml of prelabeled cells to give a final concentration of 2 $\mu\text{M}$  A23187. All agents are pre-warmed to 37°C before addition to the cells. The reaction mixture containing cells and PBS buffer (control) or stimuli (zymosan or A23187) are incubated for 5 min at 37°C in a shaker  $\text{H}_2\text{O}$  bath. The incubations are terminated by the addition of 3 ml chloroform/methanol (1:2, v/v). The reaction mixture is acidified with 90% formic acid (0.2 ml) for the efficient extraction of HETEs and prostaglandins [J. Biol. Chem. 242:5344-5354 (1967); and J. Biol. Chem. 251:7816-7820 (1976)], and the lipids extracted into the lower chloroform phase by the procedure of Bligh and Dyer [Can. J. Biochem. Physiol. 37:911-917 (1959)]. The lipids are dried under  $\text{N}_2$ , resuspended in a small amount of chloroform/methanol (1:1, v/v) and chromatographed on Silica Gel H thin-layer chromatography plates as described below.

C. Chromatography of lipids

Neutral lipids and phospholipids are separated by a two-step TLC procedure I developed in the laboratory. Phospholipids are first separated on the lower half of the TLC plate by allowing the plate to chromatograph halfway in a phospholipid solvent system (System I) containing chloroform/-methanol/glacial acetic acid/water (75:50:10:6 by vol). The plate is taken out and allowed to briefly air dry. The neutral lipids are then allowed to effectively separate on the top half of the plate by allowing the plate to chromatograph completely in a neutral lipid solvent system (System II) containing hexane/diethyl ether/90% formic acid (90:60:6, by vol). Several lipoxygenase products tend to co-migrate with some of the neutral lipids and the following two-dimensional system can be used: first system: chloroform/methanol/acetic acid (98:2:1, by vol); second system: chloroform/methanol/ammonium hydroxide (98:2:1, by vol). [Cyclooxygenase products may also be separated using the organic (lower) phase of ethyl acetate/isooctane/acetic acid/water (55:25:10:50, by vol)]. The compounds are

visualized by  $I_2$  vapors, identified by co-migration with authentic lipid standards, scraped from the plates into mini-vials, and the radioactivity counted in Budget Sol: scintillation cocktail.

## ESTIMATED BUDGET

R-1120A

## ARACHIDONIC ACID METABOLISM IN NEUTROPHILS FROM PATIENTS WITH RHEUMATOID ARTHRITIS

RHEUMATOID DISEASE FOUNDATION

Dr. Duane Smith

Blood donations	\$ 200.00
Radioactive fatty acids, phospholipid precursors and other supplies for metabolic studies	1,200.00
Use of the core membrane facility for phospholipid analysis by gas liquid chromatography and high pressure liquid chromatography	700.00
Supplies for cell isolation and cultures, and solvents for extraction, chromatography and liquid scintillation	2,400.00
Tech & Cler. Support Charges	1,000.00
M.D., PH.D. Support	2,833.00
Institutional Overhead	<u>1,667.00</u>
Total	\$10,000.00