Int. J. Immunopharmac., Vol. 11, No. 1, pp. 45–55, 1989.
Printed in Great Britain.

0192–0561/89 $3.00 + .00
International Society for Immunopharmacology.

PHOSPHOLIPID METABOLISM IN POLYMORPHONUCLEAR LEUKOCYTES FROM RHEUMATOID ARTHRITIS PATIENTS: EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS AND CLOTRIMAZOLE

DUANE M. SMITH JR,* HERMANN GONZALES,* JUNE A. JOHNSON,* RICHARD C. FRANSON† and ROBERT A. TURNER*

*Department of Medicine, Section on Rheumatology, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina; †Department of Biochemistry, Medical College of Virginia, Richmond, Virginia, U.S.A.

(*Received* 1 *March* 1988 *and in final form* 22 *July* 1988)

Abstract — Arachidonic acid (AA) metabolism and phospholipase A_2 (PLA_2) activity were measured in the peripheral blood polymorphonuclear leukocytes (PMNL) from ten patients with rheumatoid arthritis (RA) on treatment with various non-steroidal anti-inflammatory agents (NSAIA). AA metabolism and PLA_2 activity were measured both initially and after treatment with either placebo or Clotrimazole, a broad spectrum anti-mycotic agent, as a possible anti-rheumatic drug. AA metabolism was also measured in PMNL from ten patients with active RA untreated with any NSAIA and ten normal volunteers. Using ^{3}H-AA prelabeled cells, we show that there was a significantly higher ($P<0.025$) production of ^{3}H-LTB_4 in response to stimulation with the calcium ionophore A23187 in untreated RA patients than in normal volunteers (mean ± S.D.:4.8 ± 1.6% and 3.1 ± 1.0%, respectively). The production of ^{3}H-LTB_4 by PMNL from patients on NSAIAs was less elevated (mean ± S.D.:4.1 ± 1.5%) and was not significantly different from normal controls. Concurrently we examined PLA_2 activity in PMNL-sonicates from ten of our study patients using autoclaved [^{14}C]oleate-labeled *E. coli* biomembranes as an exogenous substrate. Using linear regression analysis, we demonstrate a significant correlation between *in vitro* PLA_2 activity and the release of ^{3}H-AA from the cellular phospholipids (deacylation) in response to A23187 stimulation ($r = -0.526$, $P<0.025$). We also demonstrate significant correlations between the overall clinical state of the RA patient, as evaluated by a modified rheumatoid activity index (MRAI), and both the release of ^{3}H-AA from the cellular phospholipids and its production of total [^{3}H]eicosanoids ($r = -0.557$, $P<0.025$ and $r = 0.644$, $P<0.005$, respectively). This data suggests that: PLA_2 activity may, in part, account for the higher generation of LTB_4 by RA PMNL; NSAIAs may be capable of modulating this abnormality; and Clotrimazole may affect the clinical or laboratory data of RA patients already on treatment with NSAIA.

Arachidonic acid (AA) and its various metabolites, including prostaglandins, thromboxanes and the mono- and dihydroxy-eicosatetraenoic acids (HETE and leukotrienes, respectively), have been shown to affect various metabolic and immunologic activities (Bass, O'Flaherty, Goetzl, DeChatelet & McCall, 1981; Stenson & Parker, 1980; Hafstrom, Palmblad, Malmsten, Radmark & Samuelsson, 1981; Goetzl, Brash, Tauber, Oates & Hubbard, 1980; Goetzl & Pickett, 1980; Wedmore & Williams, 1981; Bray, Cunningham, Ford-Hutchinson & Smith, 1981). Elevated levels of prostaglandins E_2 and F_2 (PGE_2, PGF_2), thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) have been found in synovial fluids (Trang, Granström & Lövgren, 1977; Klickstein, Shapleigh & Goetzl, 1980), while elevated levels of 5-HETE have been found in synovial tissues (Klickstein, Shapleigh & Goetzl, 1980) from patients with rheumatoid arthritis (RA).

The release of AA from neutrophil membrane phospholipids, presumably by a phospholipase A_2 (PLA_2) mechanism (Bills, Smith & Silver, 1976; Walsh, DeChatelet, Chilton, Wykle & Waite, 1983), is believed to be the rate-limiting step in the biochemical activation of this family of eicosanoids. Recent studies have demonstrated increased phospholipase activities in various inflammatory exudates (Franson, Dobrow, Weiss, Elsbach & Weglicki, 1978; Pruzanski, Vadas, Stefanski & Urowitz, 1985), and in both peripheral blood monocytes (Bomalaski,

Clark & Zurier, 1986) and neutrophils (Bomalaski, Clark, Douglas & Zurier, 1985) from RA patients. Recent studies have since demonstrated an increased production of LTB_4 in peripheral blood neutrophils from RA patients (Elmgreen, Nielson & Ahnfelt-Ronne, 1987; Smith & Turner, 1987).

PLA_2 activity has been identified in both human and rabbit neutrophils (Elsbach, 1966; Franson, Patriarca & Elsbach, 1974; Franson, Weiss, Martin, Spitznagel & Elsbach, 1977) using autoclaved [^{14}C]oleate-labeled *E. coli* and purified radiolabeled phospholipid substrates (Bomalaski, Clark, Douglas & Zurier, 1985). Using [^{14}C]oleate-labeled *E. coli*, this enzyme activity was shown to be Ca^{2+}-dependent, with optimal activity at neutral pH, and enriched in both granular and plasma membrane fractions (Franson & Waite, 1978; Victor, Weiss, Klempner & Elsbach, 1981). Also when using the [^{14}C]oleate-labeled *E. coli* substrate, the primary lipids shown to be degraded are phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). However, when human neutrophils have been used to demonstrate the release and further metabolism of ^{3}H-AA in response to either opsonized zymosan (Waite, DeChatelet, King & Shirley, 1979) or the calcium ionophores A23187 and ionomycin (Walsh, DeChatelet, Thomas, O'Flaherty & Waite, 1981a; Walsh, Waite, Thomas & DeChatelet, 1981b), the primary lipids shown to be degraded were phosphatidylinositol (PI) and phosphatidylcholine (PC).

In the present studies phospholipid metabolism was determined both directly, by measuring *in vitro* PLA_2 activity in PMNL sonicates using autoclaved [^{14}C]oleate-labeled *E. coli* as substrate, and indirectly, by following the release of ^{3}H-AA from prelabeled PMNL and its subsequent metabolism in response to cell stimulation with the calcium ionophore A23187. The *in vitro* PLA_2 and [^{3}H]arachidonic acid metabolism assay procedures were further compared to determine how well the results from these two assays correlate with each other. The results from these two assays were also compared with various clinical parameters to determine which assay, if either, might be more indicative of the disease activity in RA patients on various nonsteroidal anti-inflammatory agents (NSAIA) studied before and after treatment with clotrimazole (Mycelex, Miles Laboratories).

EXPERIMENTAL PROCEDURES

Materials

Gelatin, used in preparing 3% (*w/v*) plasma gel solution, and Dulbecco's phosphate-buffered saline (PBS), were obtained from Gibco, Grand Island, NY. Isolymph (lymphoprep) used for gradient removal of blood monocytes was obtained from Gallard-Schlesinger, Carle Place, NY. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (80 – 120 Ci/mmol) for cell labeling experiments was obtained from Amersham, Arlington Heights, IL. Autoclaved, [^{14}C]oleate-labeled *E. coli* were prepared as previously described (Franson *et al.*, 1974). Calcium ionophore A23187 was obtained from Calbiochem, Behring Diagnostics, LaJolla, CA. Commercial silica gel F-254 thin layer chromatography plates were obtained from E.M. Laboratories, Elmsford, NY, and Silica gel H, used for preparing homemade thin layer chromatography plates, was obtained from Sigma, St Louis, MO. Lipid standards, used for identification of separated lipid species, was obtained from Serdary Research laboratories (London, Ontario, Canada). Organic solvents, used for lipid extractions and thin layer chromatography, were of either certified ACS or better and obtained from Fisher Scientific, Fairlawn, NJ.

Clinical study protocol

These studies were part of a clinical drug trial investigating the safety, efficacy, and tolerability of clotrimazole, a broad-spectrum, anti-mycotic drug in the treatment of patients with rheumatoid arthritis. (Clinical protocol approved by the Bowman Gray Clinical Practices Review Committee.) All patients had definite or classical rheumatoid arthritis according to American Rheumatism Association criteria (Ropes, Bennett, Cobb, Jacox & Jesser, 1958). Ten patients were taking various types of NSAIA (Table 1). After the initial visit, patients were randomly assigned to either a placebo group or the clotrimazole group. Those on active drug received Clotrimazole at 20 mg/kg/day in four divided doses for two consecutive days per week for 12 weeks. These patients also continued their previous NSAIA therapy. Ten additional RA patients (untreated) were also studied during a washout period while not taking any nonsteroidal anti-inflammatory agent (NSAIA) for at least 3 – 7 days prior to blood donation. Ten normal volunteers not taking any medications were also studied. Blood was drawn only from patients and normals who gave informed consent. Clinical and laboratory data, including joint count, Westergren erythrocyte sedimentation rate (ESR), and rheumatoid factor were obtained to calculate a modified rheumatoid activity index (MRAI) as previously described (Davis, Turner, Collins, Ruchte & Kaufmann, 1977). Routine blood

Table 1. Clinical and laboratory data

Patient	NSAIA	Joint count (0–60)	ESR (mm/hr)	MRAI (0–204)	^{3}H-AA metabolism* Deacylation	[^{3}H]eicosanoids	^{3}H-LTB4	PLA_2 activity†
1	Naproxen	11	40	80	−8.4	8.2	3.8	85.8
2	Indomethacin	6	6	81	−12.1	9.1	4.7	90.0
3	Salsalate	30	35	127	−19.2	23.9	7.0	83.8
4	Naproxen	9	7	38	−17.9	15.7	5.0	55.2
5	Naproxen	32	28	100	−6.4	4.7	1.8	74.4
6	Naproxen	8	52	43	−2.5	3.6	2.5	14.1
7	Aspirin	33	52	125	−10.3	15.2	5.9	47.5
8	Naproxen	27	16	63	−11.5	8.0	3.6	53.9
9	Tolmetin sodium	12	50	57	−14.8	14.4	4.0	55.2
10	Diflunisal	8	15	53	−9.5	8.4	3.8	52.6
Mean ± S.D.		17.6 ± 11.3	30.1 ± 18.3	76.7 ± 32.0	−11.3 ± 5.1	11.1 ± 6.1	4.2 ± 1.5	61.2 ± 22.9

*Change in percent distribution of the [^{3}H]label in a particular lipid fraction in the ionophore-stimulated cells minus control (PBS-treated) cells.

†pmol substrate hydrolyzed/h/10^7 cell equivalents.

Values for the clinical and laboratory data were obtained from ten RA patients including their NSAIA at the time of study. ^{3}H-AA metabolism and phospholipase A_2 activity were determined as described in Experimental Procedures. Deacylation refers to the release of [^{3}H]label from the cellular phospholipids PC and PI in response to A23187 stimulation. The production of total [^{3}H]eicosanoids includes the free ^{3}H-AA and its eicosanoid metabolites. Abbreviations used include: ESR, Westergren erythrocyte sedimentation rate; ^{3}H-LTB_4, ^{3}H-leukotriene B_4; MRAI, modified rheumatoid activity index; NSAIA, nonsteroidal anti-inflammatory agent; and PLA_2, phospholipase A_2.

chemistry, hematologic studies and urinalyses were also performed.

Cell isolation

Peripheral blood PMNL were isolated from whole heparinized blood (10 units/ml) following plasma gel sedimentation of erythrocytes and lymphoprep gradient centrifugation for removal of monocytes as previously described (Smith & Waite, 1986). Contaminating erythrocytes were removed by a brief hypotonic lysis. For prelabeling studies with ^{3}H-AA, cells were resuspended (3.5×10^7 PMNL/ml) in Dulbecco's phosphate-buffered saline (PBS), pH 7.4. (Cells were consistently found to be greater than 95% PMNL and greater than 95% viable, as evidenced by trypan blue exclusion.)

Radiolabeled ^{3}H-arachidonic acid metabolism

PMNL were prelabeled with 0.5 μCi (0.2 μM) ^{3}H-arachidonic acid at 3.5×10^7 PMNL/ml in PBS for 2 h at 37°C. The cells were then washed once with PBS to remove any unbound label and gently resuspended to 3.5×10^7 PMNL/ml in PBS. The cells were divided into 0.5 ml aliquots and incubated with either 0.5 ml PBS (control) or 10 μM A23187 for 5 min at 37°C. The reaction was stopped by the addition of 3 ml chloroform:methanol, 1:2 (v/v) and the lipids extracted using a Bligh and Dyer (Bligh & Dyer, 1959) procedure as modified by the addition of 0.2 ml 90% formic acid for the efficient extraction of HETEs and prostaglandins (Hamberg & Samuelsson, 1967; Borgeat, Hamberg & Samuelsson, 1976). The lower chloroform phase was removed, dried under N_2, and resuspended in a small amount of chloroform:methanol, 9:1 (v/v). The lipids were spotted on Silica gel H thin layer chromatography plates and separated by a two-step phospholipid/neutral lipid chromatography procedure as previously described (Smith & Waite, 1986). Lipid species were visualized by exposure of the plates to iodine vapors, fractions scraped directly into scintillation vials, and the radioactivity determined by liquid scintillation counting. Data for ionophore A23187-stimulated ^{3}H-AA metabolism in PMNL from normals and RA patients were determined and results are expressed as the difference in the percent distribution of the [^{3}H]label among the individual cellular lipids between A23187-stimulated minus PBS-treated (control) cells. Data for the release of ^{3}H-AA from the cellular phospholipids (deacylation) is given for both the individual phospholipids PC and PI and total deacylation (PC + PI). Data is also shown for both the production of total [^{3}H]eicosanoids (free ^{3}H-AA + any lipoxygenase and/or cyclooxygenase [^{3}H]metabolites) and the specific production of ^{3}H-LTB_4.

Table 2. AA metabolism in normal and RA PMNL*

	Deacylation	[^{3}H]eicosanoids	^{3}H-LTB$_4$
Normals	−7.2 ± 3.1	6.8 ± 3.8	3.1 ± 1.0
RA-untreated	−9.0 ± 2.5	8.7 ± 5.1	4.8 ± 1.7‡
RA-NSAIA	−11.3 ± 5.1†	11.1 ± 6.1	4.1 ± 1.5

*Change in percent distribution of the [^{3}H]label in that particular lipid fraction in the ionophore-stimulated cells minus control (PBS-treated) cells
Data was obtained from ten normal volunteers, ten RA patients untreated with any NSAIA and the ten RA patients treated with various NSAIA as indicated in Table 1. Data for phospholipid deacylation, total [^{3}H]eicosanoids, and ^{3}H-LTB$_4$ production were determined as explained in Table 1. Values shown represent the mean ± S.D. for each of the parameters examined. († and ‡ indicate that these values are significantly different from normals at $P<0.05$ and $P<0.025$, respectively).

Phospholipase A_2 assay

To measure *in vitro* PLA$_2$ activity using autoclaved, radiolabeled *E. coli*, the PMNL pellet was resuspended in distilled water (1 × 10^9 PMNL/ml) and sonicated twice for 15 sec in a Biosonic Sonifier. PLA activity (EC 3.1.1.4) was determined by measuring the release of ^{14}C-oleic acid from autoclaved ^{14}C-oleate-labeled *E. coli* cells as previously described (Franson *et al.*, 1974, 1977). Briefly, incubation mixtures contained 2.5 × 10^8 autoclaved *E. coli* cells (approximately 5 nmol of phospholipid), 5 μmol CaCl$_2$, 25 μM HEPES buffer, pH 7, and an aliquot of cell sonicate (representing 1 × 10^7 cell equivalents) in a total volume of 0.5 ml. Reaction mixtures were incubated for 3 – 6 h at 37°C in a shaking water bath. The reaction was stopped by the addition of 3 vol. chloroform:methanol, 1:2 (*v/v*), and the lipids extracted by a Bligh and Dyer (Bligh & Dyer, 1959) procedure. [^{14}C]Labeled free fatty acid was separated from the cellular phospholipids by thin layer chromatography on commercial silica gel F-254 plates in a solvent system consisting of petroleum ether:ethyl ether:glacial acetic acid, 80:20:1 (by vol.). Data for *in vitro* PLA$_2$ activity is calculated as pmol of *E. coli* phospholipid hydrolyzed/h/10^7 cell equivalents.

Statistical analysis

Comparisons between data for ionophore A23187 stimulation of ^{3}H-AA metabolism in PMNL from normals and RA patients, either on washout or various NSAIAs, were analyzed by an analysis of variance, followed by Tukey's test for critical difference. Correlation coefficients between data for A23187-induced ^{3}H-AA metabolism and the *in vitro* PLA$_2$ activity in PMNL from RA patients on NSAIAs were analyzed using linear regression analysis. Correlation coefficients between data for both A23187-induced ^{3}H-AA metabolism and *in vitro* PLA$_2$ activity in PMNL from RA patients on NSAIAs and their various clinical parameters, e.g. joint count, ESR and MRAI, were also analyzed using linear regression analysis. Comparisons for the individual effects of either clotrimazole or placebo on the various clinical and laboratory data in the RA patients on NSAIA, both initially and after 12 week treatment (or at time of dropout), were analyzed using Student's paired *t*-test. Comparisons between the effects of clotrimazole and/or placebo on the percent change of the final laboratory and clinical data over the initial values, for the two patient populations as a whole, were compared using Student's non-paired *t*-test.

RESULTS

Clinical and laboratory data in RA patients on treatment with various NSAIAs

Table 1 shows clinical data from ten RA patients studied prior to treatment with clotrimazole while on treatment with various NSAIA, listing their NSAIA, and the results of the laboratory assays. AA metabolism was measured by following the metabolic fate of ^{3}H-AA in prelabeled PMNL in response to stimulation with the calcium ionophore A23187. Results include the deacylation (release) of ^{3}H-AA from the cellular phospholipids PC + PI (mean ± S.D.: −11.3 ± 5.1%), and the production of both total [^{3}H]eicosanoids (mean ± S.D.: 11.1 ± 6.1%) and the specific production of ^{3}H-LTB$_4$ (mean ± S.D.: 4.2 ± 1.5%). We concurrently examined *in vitro* PLA$_2$ activity in PMNL sonicates using autoclaved ^{14}C-oleate-labeled *E. coli* as an exogenous substrate. These results show that the *in vitro* PLA$_2$ activity in these RA PMNL was

Table 3. Effects of clotrimazole on clinical and laboratory data

A. Placebo (n = 5)				Percent change over initial visit*					
				^{3}H-AA metabolism†			Deacylation‡		
Patient activity	JC	ESR	MRAI	[^{3}H]eicosanoids	^{3}H-LTB_4	PC	PI	Total PLs	PLA_2
1	127	225	136	312	182	179	389	294	124
4	33	457	116	86	168	103	61	69	88
6	38	48	79	194	148	329	272	288	382
8	22	294	65	58	78	10	15	14	74
10	188	7	126	67	66	38	125	82	41
Mean ± S.D.	82 ± 73	206 ± 184	104 ± 31	143 ± 109	128 ± 53	132 ± 128	172 ± 155	149 ± 132	142 ± 138
B. Clotrimazole (n = 4)				^{3}H-AA metabolism†			Deacylation‡		
Patient activity	JC	ESR	MRAI	[^{3}H]eicosanoids	^{3}H-LTB_4	PC	PI	Total PLs	PLA_2
3	107	129	98	86	86	91	93	92	70
5	94	111	65	247	294	144	85	102	17
7	6	27	22	39	71	28	48	40	178
9	42	34	60	45	112	50	31	36	88
Mean ± S.D.	62 ± 47	75 ± 52	61 ± 31	104 ± 97	141 ± 104	78 ± 51	64 ± 30	68 ± 34	88 ± 67
	NS	NS	NS	NS	NS	NS	NS	NS	NS

*Values for percent change over initial visit were calculated by dividing each value for the clinical and laboratory data obtained at the completion of the study, whether on placebo or clotrimazole, by the initial value obtained at the initiation of the study × 100.

†‡Values and calculations for ^{3}H-AA metabolism and deacylation were performed as described in Table 1.

Values <100 indicate that these parameters improved at the completion of the study, whereas, values >100 indicate that these parameters got worse. NS indicates that there was no significant difference between the clotrimazole-treated group and the placebo group using Student's t-test for statistical significance.

61.2 ± 22.9 (mean ± S.D.) pmol substrate hydrolyzed/h/10^7 cell equivalents.

AA metabolism in PMNL from normals and RA patients

Comparing normals, untreated-RA, and NSAIA-treated RA patients, results shown in Table 2 demonstrate that PMNL from untreated RA patients produce significantly more ^{3}H-LTB_4 ($P<0.025$) than normals (mean ± S.D.:4.8 ± 1.6% and 3.1 ± 1.0%, respectively). PMNL from RA patients on various NSAIAs produce slightly less ^{3}H-LTB_4 (4.1 ± 1.5%) such that the difference between normals and NSAIA-treated RA patients is no longer significant. Because of the limited number of patients and the wide variety of NSAIAs, there was too little information to compare the efficacy of any one particular drug.

Effect of clotrimazole on the clinical and laboratory data in NSAIA-treated RA patients

This study also examines the efficacy of clotrimazole on the clinical and laboratory data in RA patients. After the initial screening visit, patients presently on treatment with various NSAIA, were randomly assigned to either a placebo group or the clotrimazole group. Patients were studied at the time of study initiation and at their final visit, either at the end of the 12 week trial period or at the time of dropout. (Unfortunately, one patient dropped out of the study after his initial visit and we were unable to obtain follow-up data.) Results shown in Table 3 indicate the percent change in the final data, for each of the clinical and laboratory parameters examined, compared to the initial visit data. (Values <100 indicate that these parameters improved at the completion of the study, whereas, values >100 indicate that these parameters deteriorated.) Results indicate that there was a wide variability of responses for the individual clinical and/or laboratory parameters of the RA patients either on placebo or Clotrimazole. While some patients demonstrated improvements in certain parameters, other patients in the same group also demonstrated a worsening in the same or other parameters. Although the mean values for both the laboratory assays and the clinical

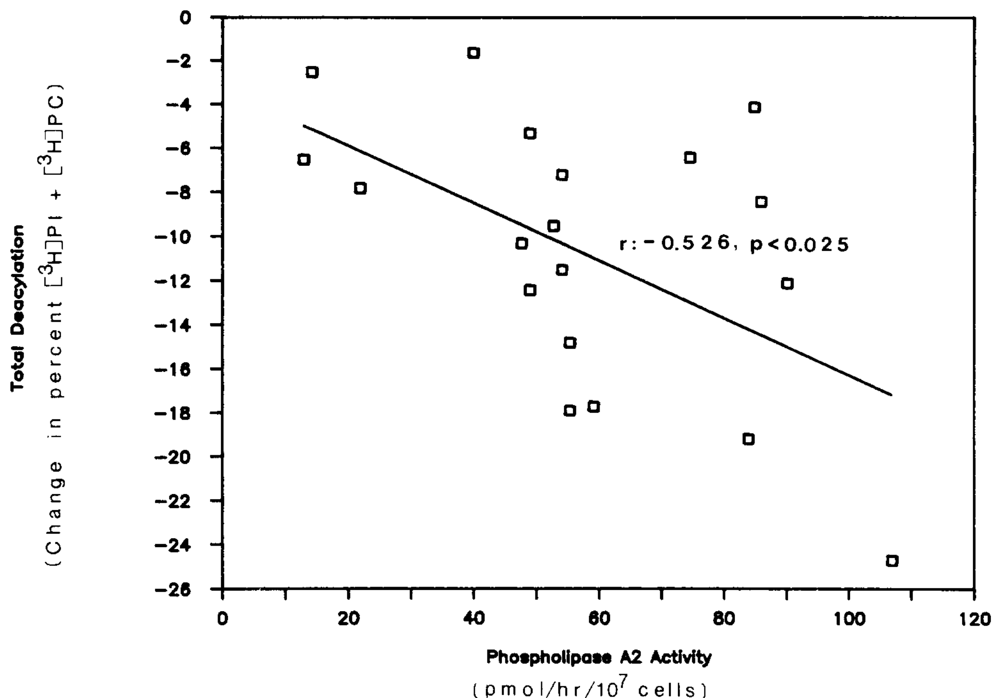

Fig. 1. Linear regression analysis of the correlation between the release of ^{3}H-AA from the cellular phospholipids (deacylation) and *in vitro* phospholipase A_2 activity in PMNL from RA patients on NSAIAs, using both initial and final sets of data. Deacylation is expressed as the decreased percent distribution of [^{3}H]label from PC + PI in response to ionophore stimulation as described in the Experimental Procedures. Phospholipase A_2 activity is expressed as pmol substrate hydrolyzed/h/10^7 cell equivalents (n = 19).

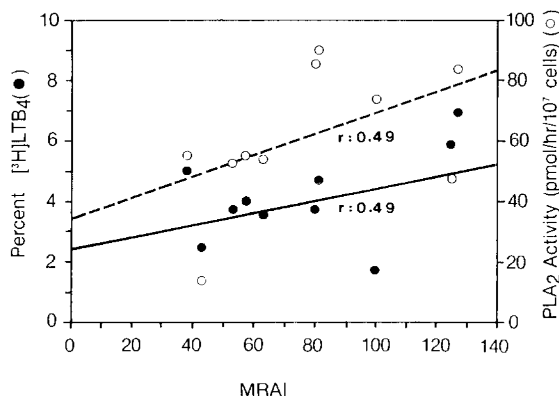

Fig. 2. Linear regression analysis of the correlations between the clinical parameters and the laboratory assay procedures in PMNL from RA patients on NSAIAs, as shown in Table 1. The overall clinical state of the RA patients was evaluated by a modified rheumatoid activity index (MRAI). ^{3}H-LTB_4 production (●) is expressed as the increased percent distribution of [^{3}H]label into LTB_4 in response to ionophore stimulation. *In vitro* phospholipase A_2 activity (○) is expressed as pmol substrate hydrolyzed/h/10^7 cell equivalents (n = 10 for both ^{3}H-LTB_4 production and phospholipase A_2 activity).

assays (excluding joint counts) in the placebo group are considerably greater than or equal to 100, and the mean values for the laboratory assays (excluding the production of total [^{3}H]eicosanoids and ^{3}H-LTB_4) and clinical assays in the clotrimazole group are less than 100, there was no statistically significant difference between the two groups for any of the parameters examined.

Relationship between ^{3}H-AA metabolism and PLA_2 activity

Since the release of AA from the cellular phospholipids, presumably by a PLA_2 mechanism, appears to be the rate-determining step in the further metabolism of AA into its lipoxygenase and/or cyclooxygenase metabolites, we examined the ability to release ^{3}H-AA from the cellular phospholipids (deacylation) in response to A23187 stimulation) versus *in vitro* PLA_2 activity (pmol/h/10^7 cell equivalents) using linear regression analysis in RA patients on NSAIA. There was a significant correlation ($r = -0.526$, $P<0.025$) between PLA_2 activity versus deacylation (Fig. 1).

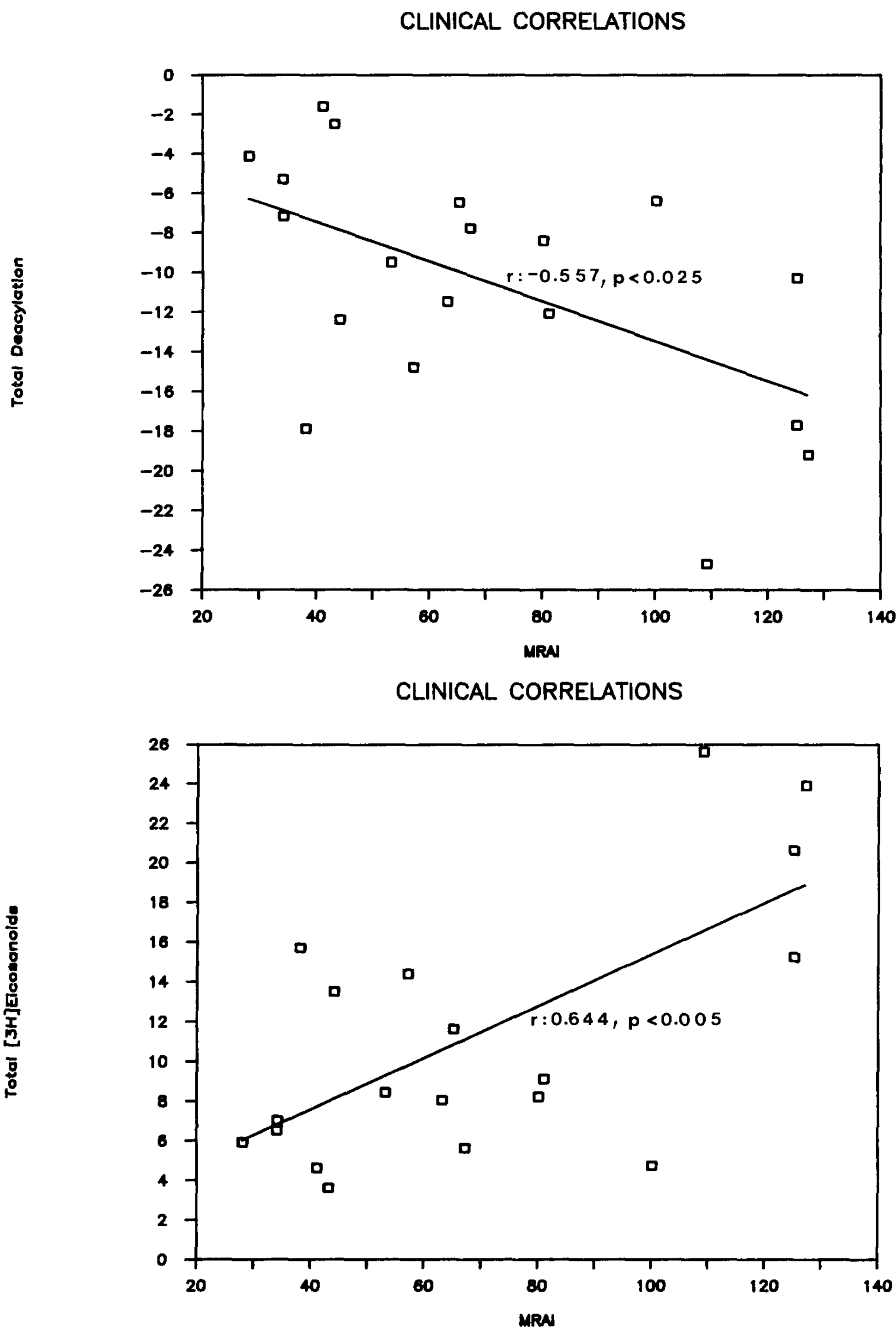

Fig. 3. Linear regression analysis of the correlations betwen the overall clinical state of the RA patients on NSAIAs (MRAI) and their PMNL ^{3}H-AA metabolism. (A) Linear regression analysis between MRAI and total deacylation (change in percent distribution of [^{3}H]label from PC + PI) in response to ionophore stimulation (n = 19). (B) Linear regression analysis between MRAI and production of total [^{3}H]eicosanoids (change in percent distribution of [^{3}H]label into free AA and its lipoxygenase and/or cyclooxygenase metabolites) in response to ionophore stimulation (n = 19).

Clinical correlations

Finally, we compared both LTB_4 and PLA_2 measurements with various clinical parameters to determine which assay procedure, if either, might give a better indication of the clinical state of the RA patient. The data found in Table 1, shows that there was a positive correlation between the overall clinical state of the NSAIA-treated RA patients, as assessed by MRAI, and both PMNL ^{3}H-LTB_4 production (n = 10, r = 0.49) and PLA_2 activity (n = 10, r = 0.49) (Fig. 2). In addition, using both initial and final sets of data, we show that there were significant correlations between the MRAI and both total deacylation (n = 19, r = 0.557, $P<0.025$) and production of total [^{3}H]eicosanoids (n = 19, r = 0.644, $P<0.005$) (Fig. 3).

DISCUSSION

The present results are in agreement with our previous report that shows that ^{3}H-AA-labeled PMNL from untreated RA patients produce significantly more ($P<0.025$) ^{3}H-LTB_4 in response to ionophore A23187 stimulation than normal volunteers (Smith & Turner, 1987). However, the present results also show that PMNL from NSAIA-treated RA patients produce slightly less ^{3}H-LTB_4, such that the difference between normals and RA patients is no longer statistically significant. Since LTB_4 and other eicosanoids can affect various metabolic activities, the lowered production of these inflammatory compounds might be one of the benefits of NSAIA treatment in RA patients. Several previous studies have suggested that the primary action of some NSAIAs was to block the further metabolism of AA by inhibition of the cyclo-oxygenase pathway (Lands & Rome, 1976; Humes, Bonney, Pelus, Dahlgren, Sadowski, Keuhl & Davies, 1977), however, more recent work has been directed toward the development of agents that block AA metabolism by inhibition of the lipoxygenase pathway (Walker & Dawson, 1979; Koshishara, Murota, Petasis & Nicolaou, 1982). Using highly purified mammalian phospholipases, Franson, Eisen, Jessi & Lanni (1980) showed that some NSAIAs might also affect PLA_2 activity by Ca^{2+} antagonism.

Free AA does not normally exist in the cell but is found esterified into various cellular lipids, including triglycerides and phospholipids. Therefore, the release of free AA from cellular lipids appears to be the initiating step in its subsequent metabolism into various eicosanoids. Several mechanisms have been proposed for the release of free arachidonic acid from cellular phospholipids, one of which is a direct release via a phospholipase A_2 enzyme. Although the action of a PLA_2 enzyme would also result in the formation of a 1-acyl,2-lysophospholipid, little evidence has been found to support the formation of lysophospholipids indicating either the presence of very active lysophospholipases, or a very active deacylation – reacylation pathway. While Walsh *et al.* (1983) demonstrated that ^{3}H-AA-labeled human neutrophils stimulated with the calcium ionophore A23187 to release free ^{3}H-AA would reincorporate exogenously added ^{14}C-AA back into the cellular lipids, Phillips, Mossmann & Ferber (1986) showed that the A23187-stimulated neutrophils preferred to reincorporate significantly higher amounts of other exogenously added unsaturated fatty acids over arachidonic acid, indicating that there may be some selective reacylation. Walsh *et al.* (1981b) had also shown that up to one-third of the [^{3}]HETEs produced upon A23187 stimulation were reincorporated back into cellular lipids, primarily the phospholipids. Previously, Stenson & Parker (1979) demonstrated that the inclusion of bovine serum albumin (BSA) into the assay medium at 1 mg/ml at the time of cell stimulation, would significantly diminish the re-uptake of arachidonic acid and its HETE metabolites by the cells. However, these present deacylation studies were performed in the absence of any exogenous protein to serve as a trapping agent for the released ^{3}H-arachidonic acid and/or metabolites in order to allow better comparison with recently reported studies most of which were done in the absence of exogenous protein.

Recent studies have demonstrated increased phospholipase A_2 activities in various inflammatory exudates (Franson *et al.*, 1978; Pruzanski *et al.*, 1985), and in both peripheral blood monocytes (Bomalaski *et al.*, 1986) and neutrophils (Bomalaski *et al.*, 1985) from RA patients. This increased phospholipase activity would result in an increased availability of the free AA for subsequent metabolism into HETEs and leukotrienes. This is consistent with our results since we also observed an increase in deacylation (release of ^{3}H-AA from cellular phospholipids) from both PC and PI in RA-PMNL such that this difference was statistically significant at $P<0.05$ between normals and NSAIA-treated RA patients (Table 2).

Further tests were designed to compare the results from two previously reported assay procedures described here. PLA_2 activity was assayed in human

PMNL either directly, by measuring the release of ^{14}C-oleic acid from autoclaved ^{14}C-oleate-labeled *E. coli* (Franson *et al.*, 1974, 1977), or indirectly, by measuring the release of ^3H-AA from cellular phospholipids in response to ionophore A23187 stimulation and subsequent metabolism to ^3H-LTB_4 (Walsh *et al.*, 1981a,b). These studies demonstrated that there was a significant relationship ($r = -0.526$, $P<0.025$) between an increased *in vitro* PLA_2 enzyme activity with an increased release (deacylation) of ^3H-AA from the cellular phospholipids PC and PI (presumably a more direct measure of endogenous PLA_2 activity). Although these two assay systems may be involved in some aspect of the inflammatory process, either collectively or individually, they do not necessarily exhibit a significant cause-and-effect relationship since it has not yet been clearly shown if the same ^{14}C-oleate-labeled *E. coli*-hydrolyzing PLA_2 can hydrolyze radiolabeled arachidonate-labeled *E. coli* or other arachidonate-labeled phospholipid substrates. Recent studies from this lab have since indicated that there are at least two separate PLA_2 present in RA synovial fluids which can be differentiated by their substrate specifically for either ^{14}C-oleate-labeled *E. coli* or ^3H-arachidonate-labeled *E. coli* (Gonzalez, Smith & Turner, submitted for publication).

We also examined which procedure (^3H-AA metabolism or ^{14}C-oleate-labeled *E. coli* PLA_2) might give a better indication of the clinical state of the NSAIA-treated RA patients. Using the data obtained in Table 1, we showed that there were definite correlations of $r = 0.49$ ($n = 10$) for both ^3H-LTB_4 production and PLA_2 activity with the overall clinical evaluation of the RA patients, as indicated by their MRAI score (Fig. 2); although these again were not statistically significant enough to prove a definite cause-and-effect relationship. Although both types of assay procedures described here have been well characterized in both human (Waite, *et al.*, 1979; Walsh *et al.*, 1981a,b, 1983; Smith & Waite, 1986) and rabbit neutrophils (Elsbach, 1966; Franson *et al.*, 1974; Franson & Waite, 1978), only the ^3H-AA metabolic studies showed significant correlations with the overall clinical state (MRAI) of the patients when the results are expressed in terms of either total deacylation ($n = 19$, $r = -0.557$, $P<0.025$) or the production of total [^{3}H]eicosanoids ($n = 19$, $r = 0.644$, $P<0.005$) (Fig. 3).

The present study was part of a comprehensive effort examining the efficacy of clotrimazole, a broad-spectrum, anti-mycotic drug on the clinical and laboratory data in RA patients, as a possible anti-rheumatic agent. In 1970, Abd-Rabbo first reported the use of a new anti-amoebic nitro-imidazole derivative "BT, 985. E. Merck AG" in the treatment of a patient with amoebic colitis, as also giving beneficial effects to the patient's acute systemic lupus erythematosus (Abd-Rabbo, 1970). Since then, several uncontrolled studies with this nitro-imidazole (Abd-Rabbo, Abaza, Hillal, Moghazy & Asser, 1972), and other imidazole-containing anti-protozoal drugs: levamisole (Schuermans, 1975; Runge & Rynes, 1983) and clotrimazole (Wyburn-Mason, 1976), showed at least promising effects in the treatment of rheumatoid arthritis. This led to several subsequent controlled studies (Runge, Pinals, Lourie & Tomar, 1977; Wojtulewski, Gow, Walter, Grahame, Gibson, Panayi & Mason, 1980; Multicentre Study Group, 1982) suggesting their efficacy in the treatment of rheumatoid arthritis. Unfortunately, however, the results presented here (Table 3) indicate that clotrimazole treatment has moderate, yet statistically insignificant effects on the clinical parameters, whole cell PMNL ^3H-AA metabolism, or *in vitro* PMNL PLA_2 activity in those RA patients currently on treatment with NSAIAs. This study while illustrating the complexity of attempting to correlate drug-related effects to clinically relevant biochemical parameters provides interesting data concerning possible drug effects on the increased AA metabolic activity of RA patients' PMNL.

Acknowledgements — This work was supported in part by a grant from the Rheumatoid Disease Foundation.

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