RELEASE OF ARACHIDONIC ACID FROM HUMAN LYMPHOCYTES IN RESPONSE TO MITOGENIC LECTINS*

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A variety of agents activate blast transformation and increase DNA synthesis in lymphocytes, including phaseolus vulgaris phytohemagglutinin (PHA)[1] (1), concanavalin A (Con A), sodium metaperiodate, trypsin, Hg^{2+}, Zn^{2+}, and calcium ionophore A-23187 (reviewed in 1, 2). Because the activation process is long and complex, involving a large number of different metabolic processes, a number of groups, including our own, have concentrated primarily on early activation events. Lymphocytes undergo a number of metabolic changes within the 1st 15–20 min of stimulation, including increases in cyclic AMP, phosphatidylinositol turnover, protein phosphorylation, and histone acetylation and phosphorylation; and increases in the transport of glucose, Ca^{++}, and nucleosides (1). One metabolic process that might be involved in activation that has received little attention is fatty acid release from phospholipids. Mammalian cells contain several different phospholipid-cleaving enzymes including phospholipase A_2 which acts selectively at the 2 position of the glycerol moiety of phospholipids releasing free fatty acids (3). Generation of arachidonic acid (AA) that is esterified primarily at the 2 position of phospholipids could be of particular significance because it is enzymatically converted to a variety of biologically active fatty acids (for example, the thromboxanes and prostaglandins) which may be important in the control of cellular metabolism (4). Moreover, once fatty acids have been removed from phospholipids, reacylation can occur (3, 5), providing a mechanism for changing the fatty acid composition of phospholipids and possible changes in membrane fluidity (6, 7). Some years ago, Resch and his colleagues (8) attempted to demonstrate increases in phospholipase activity in partially purified plasma-membrane preparations from human lymphocytes stimulated previously by lectin. When purified phosphatidylcholine was used as a substrate, lectin-activated cells showed no change in enzyme activity; although, with an erythrocyte plasma membrane substrate, a modest (up to 30%) increase in phosphatidylcholine-cleaving activity was seen.

In the present report, the possibility of early phospholipase activation in lectin-stimulated lymphocytes has been reexamined. Lymphocytes were preincubated with [^{3}H] or [^{14}C]fatty acids (particularly AA) to incorporate radioactivity into endogenous phospholipids and studied for release of radioactivity in response to lectin. Evidence

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[1] *Abbreviations used in this paper:* AA, arachidonic acid; acyltransferase, lymphocyte-acyl-CoA-lysophospholipid acyltransferase; Con A, concanavalin A; E-PHA, erythroagglutinating PHA; P-PHA, a mixture of erythroagglutinating and leukoagglutinating PHA's; PHA, phaseolus vulgaris phytohemagglutinin.

for the rapid release of sizable quantities of AA (and to a lesser extent, other fatty acids) from activated lymphocytes will be presented.

Materials and Methods

Reagents and Their Sources. [1-^{14}C]arachidonic acid ([^{14}C]AA) (sp act 60 mCi/mmol, Amersham Corp., Arlington Heights, Ill.), and [^{3}H]oleic acid (sp act 2.2 Ci/mmol, Amersham Corp.); phospholipids (Sigma Chemical Co., St. Louis, Mo.); fetal calf serum (heated at 56°C for 30 min to inactivate complement) and Eagle's medium with Earle's salts (Gibco Diagnostics, Gibco Invenex Div., Chagrin Falls, Ohio); unlabeled fatty acids (>99% pure, NuChek Inc., Elysian, Minn.); phytohemagglutinin-P (P-PHA), a mixture of erythroagglutinating and leukoagglutinating PHA's, Difco Laboratories, Detroit, Mich.), phytohemagglutinin-E (E-PHA, purified erythroagglutinating PHA, Burroughs Wellcome & Co., Greenville, N. C.), concanavalin A (Con A, Sigma Chemical Co.), bovine serum albumin and bovine gamma globulin (Sigma Chemical Co.), lipid-poor albumin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), archidonyl CoA (Avanti Biochemicals, Inc., Birmingham, Ala.). Human serum was obtained from the clotted blood of a donor with AB-positive erythrocytes, heated at 56°C for 30 min and stored at −20°C. Fatty acids were dissolved in hexane, dried, neutralized with Na_2CO_3 in water, and diluted in medium or buffer; the pH was adjusted to neutrality if necessary.

Purified Cell Preparations. Lymphocytes were purified from 250 or 500 ml of heparinized human peripheral venous blood as described previously by dextran sedimentation followed by isopycnic centrifugation over a mixture of Ficoll (Pharmacia Fine Chemicals Inc., Div. of Pharmacia Inc., Piscataway, N. J.) and Hypaque (Radiopaque Media, Winthrop Laboratories, New York) (9, 10). An average of 95% of the nucleated cells were lymphocytes as judged morphologically after staining with Wright's stain and by the failure of the cells to ingest latex particles. Contamination of platelets and erythrocytes was minimal (less than one platelet or erythrocyte per two nucleated cells). In selected experiments, the few contaminating nucleated cells were removed by filtration through a 4-g nylon column (10), providing a preparation in which 99–100% of the nucleated cells were lymphocytes. By immunofluorescence staining (11, 12) with a mixture of rabbit anti-human IgG, IgM, and IgA antibodies, 9 ± 4% (SEM) of filtered cells are B lymphocytes as compared with 23 ± 4% (SEM) of the unfiltered cells. Because 50–80% of the lymphocytes were lost on the nylon, this procedure was not used routinely.

Human peripheral blood platelets (99% pure), polymorphonuclear leukocytes (>99% pure with >90% neutrophiles, termed "neutrophiles," henceforth), and erythrocytes (>99% pure) were obtained from human peripheral blood by previously described methods (13, 14).

An enriched population of human monocytes was obtained by incubating prelabeled, unfiltered lymphocytes (see below) in plastic Petri dishes (Fisher Scientific Co., St. Louis, Mo.) (15, 16) for 40 min and followed by washing to remove nonadherent cells. After the addition of Eagle's 2% AB serum, the adherent cells (≅75% monocytes) were evaluated for the release of radioactivity in response to PHA as described above for nonadherent cells, but the supernates were removed by aspiration instead of by centrifugation.

Labeling of Cells. In a typical experiment, lymphocytes were labeled with [^{14}C]AA by incubation of a suspension of cells (1×10^7 cells/ml) in Eagle's medium containing 2% (vol/vol) AB serum and ≅2.25 μM [^{14}C]AA (≅2×10^5 cpm/ml or 0.63 μg/ml). (The [^{14}C]AA concentration varied by as much as 25% with different cell preparations.) After the cells had been maintained for 60 min at 37°C, they were centrifuged at 120 *g* and washed three times with Eagle's 2% AB medium at room temperature. They were then resuspended in the same medium at a density of 1×10^7 cells/ml and used within 15 min for studies of [^{14}C]AA release. Uptake of radioactivity was determined by counting 1 aliquot of sonicated cells in Scintiverse (Fisher Scientific Co.) on a Searle liquid scintillation counter (Searle Radiographics Inc., Des Plains, Ill.). Neutrophiles and erythrocytes were labeled under the usual conditions for labeling lymphocytes at 1×10^7 cells/ml. Platelets were labeled in the final resuspension buffer used in the platelet purification (140 mM NaCl, 15 mM Tris, 5 mM glucose, pH 7.5) (13) at a density of 2×10^9 cells/ml in the presence of [^{14}C]AA, 4×10^6 cpm/ml. Erythrocytes were labeled under the same conditions as platelets with the exception that the incubation medium was Eagle's 2% AB serum.

Studies of AA Release. Lymphocytes labeled with [^{14}C]AA were ordinarily incubated in the final resuspension medium (Eagle's 2% AB medium, see above) in a total incubation vol of 0.27 or 1.1 ml for various time periods at 37°C (usually 10 or 15 min). The incubation mixture contained 0.1 vol of 0.1 M NaCl (control) or reagent (usually a lectin) in 0.1 M NaCl. Each experimental condition was evaluated in duplicate or triplicate. Some experiments were carried out in Gey's medium which is a balanced salt solution containing 0.9 mM $CaCl_2$ and glucose (17). After incubation, the cells were centrifuged for 2 min at 250 *g* at room temperature. The supernates were saved for counting and, frequently, chromatography. In experiments in which the cell pellets were also studied, the cells were washed two–three times with ice-cold, phosphate-buffered saline (0.15 M NaCl and 0.01 M $PO_4^=$, pH 7.4) and extracted by the method of Bligh and Dwyer (18) as modified by Masuzawa et al. (19). In brief, the cell pellet was incubated with 3.8 ml of a mixture of $CHCl_3$:methanol:H_2O (1:2:0.8) with or without 4 mM $CaCl_2$ for 30 min at room temperature. The supernate was removed and 2.0 ml of a mixture of equal vol of $CHCl_3$ and H_2O was added. Aliquots from both the upper (aqueous) and lower (lipid) layers were removed, counted, and evaluated by thin layer chromatography.

Thin Layer Chromatography. Chromatography was performed in the presence and absence of unlabeled purified phospholipids and fatty acids as standards on silica-gel-G plates (Brinkmann Instruments, Inc., Westbury, N. Y. or Arthur H. Thomas Co., Philadelphia, Pa.) at room temperature in four different solvent systems, chloroform:methanol:13.5 M ammonia:water (70:30:4:1, vol/vol/vol/vol) (20), solvent system C of Nugteren and Hazelhof (21), the organic phase of ethyl acetate:2,2,4-trimethylpentane: water (50:100:100, vol/vol/vol) (22), and on argentaffin plates (prepared with silica gel G equilibrated with 5% $AgNO_3$) in ethyl acetate: methanol:isooctane:water (110:35:30:10:200, vol/vol/vol/vol/vol) (23). Areas containing lipids were identified by iodine staining, radioautography, radioactive scanning (Varian Associates, Instrument Div. Palo Alto, Calif.), or scraping and counting. In selected experiments, samples were also evaluated by two-dimensional thin layer chromatography in a system that provides a wider separation of phosphatidylinositol from phosphatidylserine and from phosphatidic acid than the one-dimensional systems (19).

Absolute fatty acid concentrations were determined on supernates of stimulated and unstimulated cells by gas liquid chromatography (24) (Varian Associates, Instrument Div., model 3700) on a 50-cm stainless steel column packed with 5% OV-101 on chrom G-HP using a hydrogen flame ionization detector. Before gas liquid chromatography, samples were esterfied with diazomethane and partially purified by preparative thin layer chromatography in solvent system C. Aliquots were chromatographed in the presence and absence of an internal standard (8, 11, 14-eicosatrienoic acid).

Statistical analysis was by the two-tailed *t* test.

Results

Determination of Optimal Conditions for Labeling Cells. Initially, an attempt was made to define the optimal incubation conditions for the incorporation of radiolabel from [^{14}C]AA into phospholipids, particularly phosphatidylinositol. In three experiments under incorporation conditions similar to those described in the Methods section, but at a somewhat higher [^{14}C]AA concentration, the overall uptake of [^{14}C]AA by cells increased from 5% (range 4–7%) at 5 min to 50% (range 45–53%) at 60 min (Fig. 1). Similar results were obtained in two experiments at 2×10^5 cpm/ml (not shown). The largest amount of radioactivity was in phosphatidylcholine which contained 60% of the cell-bound radioactivity, whereas phosphatidylinositol contained 15–20% of the cell-bound radioactivity. Because the percentage of cell-bound radioactivity in phosphatidylinositol was similar at 5 and 60 min (17 and 16%, respectively, in this set of experiments) and a longer incubation time than 1 h did not appear to provide more efficient utilization of label, 1 h was selected as the standard incubation time for labeling, although as shown below, other preincubation times were also studied. The ability to rapidly incorporate radioactive AA into lymphocyte phospholipids is in accord with previous studies in brain, platelets, and other tissues (3).

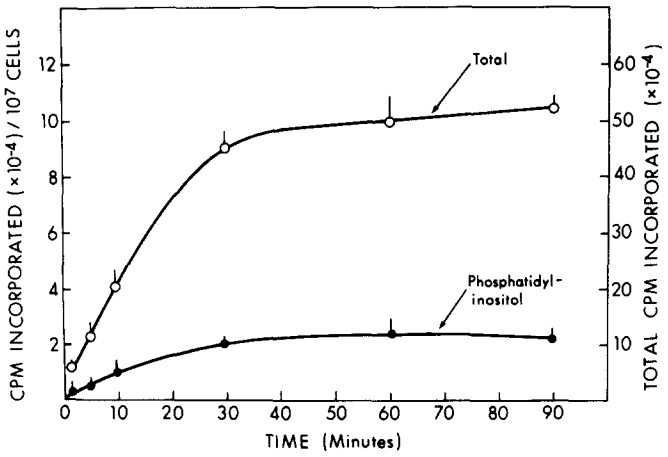

FIG. 1. Effect of preincubation time on incorporation of [^{14}C]AA into total lymphocyte lipids and phosphatidylinositol; 50 × 10^6 lymphocytes (unfiltered) were suspended in Eagle's 2% AB serum and incubated with [^{14}C]AA (1 × 10^6 cpm/ml) for the indicated time periods; they were then washed three times and counted. Mean ± SEM of three experiments.

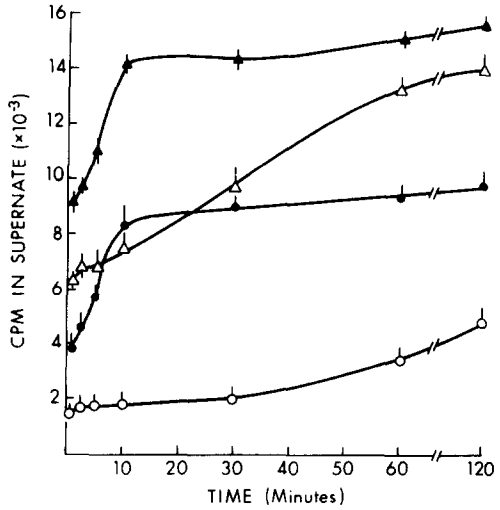

FIG. 2. Time course of release of radioactivity from prelabeled lymphocytes with P-PHA. Unfiltered lymphocytes were prelabled with [^{14}C]AA, washed three times and resuspended in 1 ml Eagle's 2% AB serum at a cell density of 1 × 10^7/ml. Some of the cells were incubated directly at 37°C in the absence (open triangles) and presence (closed triangles) of P-PHA (20 μg/ml) for the indicated time periods. Other cells were incubated further in medium at 37°C for 30 min, washed, resuspended in medium at a density of 1 × 10^7 cells/ml, and incubated at 37°C in the absence (open circles) and presence (closed circles) of PHA (20 μg/ml). At the start of the incubation with lectin, 1 × 10^7 cells contained an average of 117,000 cpm and 106,000 cpm, respectively. Mean ± SEM of three experiments. PHA produced significant increases in release ($P < 0.05$) at all of the early time points (30 min or less). In the experiment with preincubated and washed cells, a significant response was also obtained at later times.

Release of Radioactivity from Prelabeled Lymphocytes. When prelabeled cells were washed three times for 1–2 min and incubated further in Eagle's 2% AB medium at 37°C in the absence of lectin, ≅5% of the cell-bound radioactivity was released into the medium (Fig. 2, open triangles). A further slow release of cell-bound radioactivity

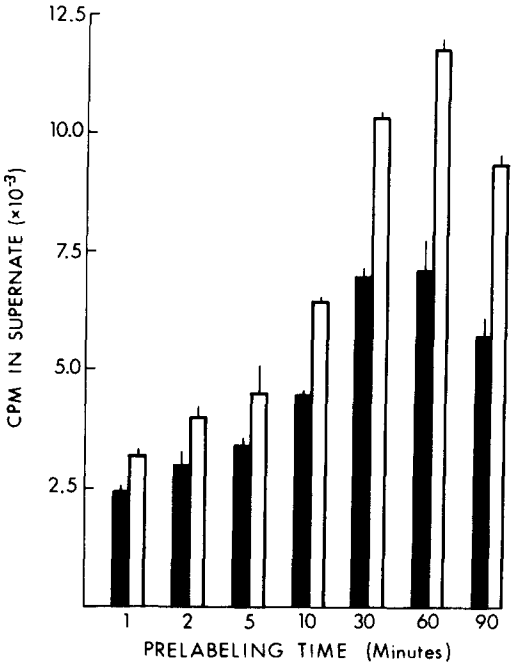

FIG. 3. Effect of prelabeling time on the release of radioactivity from prelabeled lymphocytes by PHA. Lymphocytes were prelabeled with [^{14}C]AA as described in the legend to Fig. 1, washed, and incubated for 10 min at 37°C in the presence (open bars) and absence (closed bars) of P-PHA (20 μg/ml) under the conditions given in the legend to Fig. 2. For the level of incorporation at the start of the incubation with PHA, see Fig. 1 (same three experiments). Mean ± SEM is given.

occurred over the next hour. PHA-P increased the amount of radioactivity in the cell supernate with changes occurring as early as 60 s (Fig. 2, closed triangles). (Because cells are centrifuged for 2 min at the completion of the incubation, the rapidity of the early response may be somewhat exaggerated.) Phosphate-buffered saline washes of stimulated centrifuged cells also demonstrated increases in radioactivity but they were smaller than in the initial supernates. A difference between PHA-stimulated and control cells was demonstrable for at least 30 min after the addition of lectin, although the most marked changes were in the first 10 min. Some of the early release of radiolabel in control cells may involve a relatively labile pool of incorporated fatty acid because incubating labeled, washed cells in medium at 37°C for 30 min, followed again by washing and incubation with PHA, resulted in considerably lower spontaneous release and a higher stimulation ratio with PHA (Fig. 2, closed circles [open circles, without PHA]).

In cells prelabeled with ^{14}C for 30 and 120 min, comparable stimulation ratios (PHA:control) for release of radioactivity were observed (Fig. 3). Significant responses were obtained with prelabeling times as short as 1 min, although the stimulation ratio was reduced, suggesting that the radiolabel incorporated at this time is not as susceptible to release as at later times.

The response to P-PHA was dose-related. In cells incubated for 10 min with lectin, P-PHA concentrations as low as 0.6 μg/ml were stimulatory, although the most marked response was at 20–100 μg/ml P-PHA (Fig. 4). Because binding equilibrium with PHA is not approached for many minutes (25) and is concentration-dependent,

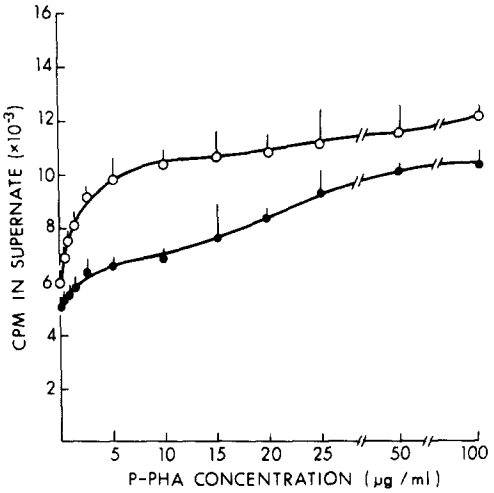

FIG. 4. Dose dependency of the release reaction to P-PHA. Prelabeled cells were incubated for 2 (closed circles) or 10 (open circles) min at various concentrations of P-PHA at a final cell density of 1×10^7/ml (83,400 cpm/ml). Mean ± SEM of two experiments is given.

the greater response to low concentrations to P-PHA at 10 min rather than at 1–2 min is easily explained, as is the greater effect of lectin concentration at early rather than at later times. The mitogenic optimum for P-PHA ranges from ≅1 to 10 μg/ml, depending on the culture conditions, but for metabolic changes occurring within the first several hours of the response, higher concentrations of lectin are ordinarily more effective (1).

The rapidity of the release reaction to P-PHA suggested that the response was being produced at the lymphocyte surface, where receptors are available and cell activation is presumably initiated. Since P-PHA readily agglutinates lymphocytes, even under relatively short incubation conditions, the possible role of agglutination in the response was considered. However, erythroagglutinating PHA (E-PHA), which agglutinates lymphocytes considerably less readily than P-PHA, was comparable to P-PHA in its potency as a releasing reagent with a response at concentrations as low as 0.8 μg/ml, even in short incubation experiments (Table I, Eagle's 2% medium). Con A, a mitogenic lectin with a different carbohydrate binding specificity than PHA, also was stimulatory. The response to Con A was almost completely blocked by α-methyl mannoside (50 mM) (Fig. 5), a monosaccharide with specificity for Con A that markedly reduces its binding to cells. By contrast, the response to P-PHA was unaffected by α-methyl mannoside, indicating that the inhibition is specific. Thus, the release reaction is initiated through specific carbohydrate sites on the cell surface and agglutination, per se, is probably not required, although a contributory role is not excluded.

Studies in Filtered Lymphocytes. Even though the lymphocytes used in the above studies were on average ≅95% pure, <10% of the cell-bound radioactivity was released by the various stimuli and possible contributions by contaminating cells in the response had to be considered. In our initial studies with filtered (essentially pure) lymphocytes little or no increase in AA release was obtained with P-PHA. However, filtration through nylon has been reported to nonspecifically alter both lymphocytes and polymorphonuclear leukocytes (26, 27). Although it is clear that filtered lympho-

TABLE I

Release of Radioactivity by PHA from Prelabeled Lymphocytes in Various Media

Medium	Control	E-PHA			
		20 μg/ml	4 μg/ml	0.8 μg/ml	0.16 μg/ml
			cpm		
Eagle's 2% AB	4,490	8,280‡	5,738‡	5,134§	4,268
Gey's	3,354	4,936‡	3,420	3,442	3,024
Gey's, no Ca^{++}	3,422	3,776	3,310	3,030	3,114
Gey's, EGTA,* no Ca^{++}	3,080	3,218	3,246	3,132	2,798
Gey's, 0.3% wt/vol, albumin	4,850	10,670‡	7,734‡	5,650§	5,236

Washed, unfiltered lymphocytes labeled with [^{14}C]AA (6.72×10^5 cpm/10^7 cells) were incubated in 1.1 ml of the indicated medium for 10 min at 37°C in the presence and absence of E-PHA at a cell density of 8.5×10^6 cells/ml.

* Mean of three experiments, 1 mM EGTA.

‡ Statistically higher than PHA alone ($P < 0.03$).

§ Statistically higher than PHA alone ($P < 0.05$).

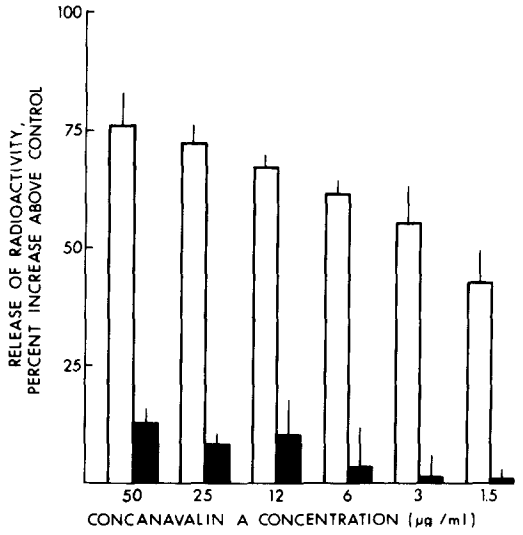

FIG. 5. Release of radioactivity from prelabeled lymphocytes in the presence and absence of Con A and α-methyl mannoside. Conditions for incubation of cells with lectin were the same as those given in the legend to Fig. 3. At the start of the incubation with lectin, labeled cells contained an average of 94,150 cpm. Mean ± SEM is given. Open bars, Con A alone; closed bars, Con A + α-methyl mannoside (50 mM). Spontaneous release was 6,200 cpm and was unaffected by α-methyl mannoside. 50 mM N-acetylglucosamine failed to diminish the increase in release with Con A (not shown).

cytes are subject to mitogenic activation they might, nonetheless, be temporarily damaged, helping explain their failure to release AA rapidly in response to PHA. In accord with this possibility, when filtered lymphocytes were allowed to "recover" by incubation at 37°C in Eagle's 2% AB medium for 2 h before exposure to [^{14}C]AA and then labeled in the usual way, a response to P-PHA was again demonstrable (usually at about 30–50% of the level seen in unfiltered cells, Table II). When unlabeled, unfiltered cells were added to labeled, filtered cells, responses to lectin were not enhanced, suggesting that reduced response was not due to the loss of contaminating

TABLE II

Release of Radioactivity by PHA from Filtered and Unfiltered Lymphocytes

	Time after filtration before labeling	Labeling time	Cells/10^7 cells	P-PHA increase above control*
	min	*min*	*cpm* × 10^{-4}	%
Unfiltered cells	—	60	83	89 ± 9‡
Filtered cells	0	60	44	8 ± 7
	0	15	15	7 ± 4
	120	60	53	38 ± 5‡
	1,080	60	45	29 ± 10§

Unfiltered lymphocytes were prepared, labeled, and evaluated for release of radioactivity as described in the legend to Fig. 2 with the exception that the incubation vol was 0.25 ml (because of limitations in cell number) and P-PHA concentrations of 4.0, 0.8, and 0.2 μg/ml, as well as 20 μg/ml, were examined. The remainder of the cells were filtered, labeled immediately for either 15 or 60 min, or resuspended in sterile medium and incubated for 120 or 1,080 min before labeling. They were then evaluated for release of radioactivity into the supernate by PHA as described for filtered cells. Mean ± SEM of three experiments, each comparing filtered and unfiltered lymphocytes from the same donor.

* Presented as the largest response seen at any of the PHA concentrations (in all instances, 20 μg/ml P-PHA).

‡ Significantly higher than control ($P < 0.02$); significant increases ($P < 0.05$) also occurred at 4 and 0.8 μg P-PHA/ml.

§ Significantly higher than control ($P < 0.05$); a significant increase also occurred at 4 μg P-PHA/ml.

cells (neutrophiles or monocytes) that interact with lectins and, under favorable circumstances, promote lymphocyte activation (1). Incubating the filtered cells for longer periods (up to 16 h) at 37°C in sterile Eagle's 2% AB medium (Table II) or in RPMI medium supplemented with pyruvate and glutamine (not shown) did not further increase the response.

Studies in Other Cell Types. The reason for the decreased AA response in filtered, as compared with unfiltered, lymphocytes is not presently clear. The possibility is not excluded that the cells fail to recover completely from the nylon filtration step, even when incubated for long periods. Another possible explanation, that contaminating cells enhance the response by lymphocytes by binding the lectin and by presenting it in the proper perspective to lymphocytes, seems unlikely because in the three experiments in which this possibility was evaluated, unlabeled, unfiltered lymphocytes failed to enhance release from labeled, filtered lymphocytes. Alternatively, in as much as the contaminating cells themselves are stimulated metabolically by lectins (28, 29) and they presumably contain [^{14}C]AA label, some of the release AA may be coming from these cells. However, in studies with prelabeled platelets and neutrophiles, little or no increase in AA release in response to PHA was seen (Table III). Moreover, erythrocytes incorporated very little radiolabel even when preincubated at unusually high ^{14}C concentrations and also failed to respond to PHA (not shown). Thus, based on the available evidence in the numbers present in our lymphocyte preparation, platelets, granulocytes, and erythrocytes probably do not contribute significantly to the response. In four experiments with mononuclear cells adherent to plastic (predominately monocytes and, to a lesser extent, B lymphocytes, T lymphocytes, and neutrophiles) there was significant release of radioactivity to response to PHA in two

TABLE III

Release of Radioactivity from Prelabeled Platelets and Neutrophiles

Cell type	P-PHA			
	Control	20 μg/ml	4 μg/ml	0.8 μg/ml
	cpm			
Platelets	8,426 ± 204	8,862 ± 176	8,498 ± 312	8,266 ± 264
Neutrophiles	2,040 ± 112	2,096 ± 102	1,878 ± 38	1,812 ± 74

Labeled, washed platelets (see text) containing 3.85×10^4 cpm/10^9 cells were suspended in Eagle's 2% AB serum at a density of 2×10^9 cells/ml and incubated in the presence and absence of P-PHA for 15 min at 37°C. Labeled, washed neutrophiles (7.6×10^4 cpm/10^7 cells) were resuspended at 1×10^7 cells/ml in Eagle's 2% serum and incubated as described for platelets. Mean ± SEM of two experiments with each of the cell types. (None of the changes is statistically significant.) Labeled, unfiltered lymphocytes from the same cell donor were incubated under identical conditions and significant increases in medium radioactivity were shown to occur at at least two concentrations of PHA. Platelets were also studied in Eagle's medium without serum and the final platelet resuspension medium (14) with similar results.

(not shown). However, even in the positive experiments, the response (on a per cell basis) was <50% of that in the filtered cells. Thus, while nylon filtration removes monocytes and partially depletes B-lymphocyte subpopulations in lymphocytes (10, 30), the major effect is probably on the T lymphocytes themselves that appear to account for the majority of the response. Nonetheless, the possibility cannot be excluded that monocytes respond more effectively in suspension than when attached to the dishes.

Release in Different Media. Table I also illustrates the considerable variation in the release reaction in different media. There was a marked reduction of the response in Gey's balanced salt solution and no response at all in Gey's solution without Ca^{++}, both in the presence and absence of EGTA, indicating that Ca^{++} must be present in the medium to obtain a response. By contrast, in Gey's solution containing both Ca^{++} and bovine serum albumin (either dialysed or undialysed), the response was as good or better than in Eagle's 2% AB serum. Bovine gamma globulin, which binds lipids to a much lesser extent than bovine serum albumin, had little or no enhancing effect on release (not shown).

Effect of Various Fatty Acids. The improved response in Gey's medium containing albumin compared to Gey's medium alone raised the possibility that the albumin might be enhancing the response by binding released AA, and reducing reuptake by cells. In accord with this possibility, the addition of lipid-poor albumin to Gey's solution increased the release of radioactivity in response to PHA. There were also less-marked effects on basal AA release and on uptake of [^{3}H]AA, added at the same time as lectin. Thus, the enhancing effect of albumin on [^{14}C]AA release is probably due to trapping of released AA. Serum albumins are known to contain binding sites which interact effectively with long-chain fatty acids.

The effect of exogenous fatty acids on release also was studied. Various fatty acids were added to ordinary Eagles 2% AB medium or Gey's solution; AA and, probably, 8,11,14-eicosatrienoic acid, but not other long-chain fatty acids; increased the release of radioactivity (Table IV) (Shown for Eagle's 2% AB serum only). Various AA analogues, including arachidonylmethylester and arachidonyl CoA were inactive

TABLE IV

Effect of AA and Other Fatty Acids on Release of Radioactivity from Prelabeled Lymphocytes

	cpm released	Percentage of increase above no PHA control	Percentage of increase above increase with PHA alone
		%	%
Control	4,058 ± 200		
PHA-P, 4 μg/ml	6,622 ± 276	63	
+ AA, 10 μM	9,024 ± 271*	121	91
+ AA, 1 μM	8,568 ± 330*	110	75
+ Stearic acid, 10 μM	6,720 ± 201	65	3
+ Stearic acid, 1 μM	6,518 ± 823	60	0
+ Palmitic acid, 10 μM	6,814 ± 173	68	7
+ Palmitic acid, 1 μM	6,448 ± 331	59	0
+ Oleic acid, 10 μM	6,570 ± 414	61	0
+ Oleic acid, 1 μM	6,612 ± 217	63	0
+ Linoleic acid, 10 μM	7,022 ± 086	73	16
+ Linoleic acid, 1 μM	6,876 ± 824	69	9
+ Linolenic acid, 10 μM	6,018 ± 507	49	0
+ Linolenic acid, 1 μM	6,418 ± 628	58	0
+ Eicosatrienoic acid, 10 μM	7,768 ± 376‡	92	46
+ Eicosatrienoic acid, 1 μM	7,124 ± 304	76	20

Washed, unfiltered lymphocytes labeled with [^{14}C]AA 1.2 × 10^5 cpm/10^7 cells) were incubated for 15 min at 37°C in 0.8 ml of Eagle's 2% AB serum medium at a cell density of 8 × 10^6/ml in the presence and absence of P-PHA (4 μg/ml), AA or another long-chain fatty acid. Mean ± SEM of three experiments.

* Statistically higher than PHA alone ($P < 0.02$).

‡ Statistically higher than PHA alone ($P < 0.05$).

(Table V). Thus, to effectively enhance release (or prevent reuptake) of AA, a fatty acid with a free carboxylic acid group and >18 carbons is needed. Even the loss of a single double bond at the 5 position (in going from AA [5,8,11,14-eicosatetraenoic acid] to 8,11,14-eicosatrienoic acid) is associated with a considerable diminution of the response.

The basis for the effect of AA is uncertain. Certainly, AA is being taken up from medium into phospholipids and would be expected to gradually exchange with cell-bound AA. However, in three experiments in which cells were prelabeled with [^{14}C]AA, little or no effect of the lectin on [^{3}H]AA uptake by cells was observed. Thus, under the conditions of our experiments, PHA did not appear to be promoting the exchange of cellular AA with AA in the medium. Moreover, in the absence of lectin, exogenous AA, at best, only modestly increased the accumulation of [^{14}C]AA in the medium (a mean increase of 15 ± 8% [SEM] in six experiments). Exchange alone could not be the sole basis for release of cell-bound radioactivity in any case because the absolute amount of arachidonate in the medium is increased by lectin (see below).

Studies of Phospholipids. To determine the source of the released radioactivity, lymphocytes that had been stimulated with PHA were extracted and the major intracellular lipids containing radioactivity were separated by thin layer chromatography. In four experiments, there was a 40% decrease in phosphatidylinositol radioactivity as compared with little or no change in phosphatidylcholine or phosphati-

TABLE V

Effect of AA and its Analogues on Release of Radioactivity from Prelabeled Lymphocytes

	cpm released	Percentage of increase above no PHA control	Percentage of increase over increase with PHA alone
		%	%
Control	3,162 ± 140		
P-PHA, 4 μg/ml	5,522 ± 205	75	
+ AA, 10 μM	7,156 ± 262*	137	82
+ AA, 1 μM	6,540 ± 165*	108	44
+ Arachidonyl alcohol, 10 μM	5,648 ± 428	77	3
+ Arachidonyl alcohol, 1 μM	5,554 ± 132	75	0
+ Arachidonyl CoA, 10 μM	5,348 ± 376	69	0
+ Arachidonyl CoA, 1 μM	5,550 ± 225	76	1
+ Arachidonyl OMe, 10 μM	5,698 ± 198	80	6
+ Arachidonyl OMe, 1 μM	5,162 ± 478	64	0

Washed, unfiltered lymphocytes labeled with [^{14}C]AA (8.8×10^5 cpm/10^7 cells) were incubated for 15 min at 37°C in 0.8 ml of Eagle's 2% AB serum medium at a cell density of 8×10^6 cells/ml in the presence and absence of P-PHA (4 μg/ml), AA, or an AA analogue. Mean ± SEM of two experiments.

* Statistically higher than P-PHA alone ($P < 0.02$).

dylethanolamine radioactivity (Table VI). Phosphatidic acid and AA radioactivity were increased but accounted for only a small percentage of the total. Losses of radioactivity from phosphatidylinositol could account for at least 70% of the increase in arachidonate radioactivity seen in supernates of PHA-stimulated cells (Table VI). However, other phospholipids and triglycerides are not excluded as significant sources of released AA.

Characterization of the Released Radioactivity. To determine the nature of the released radioactivity, supernates and washes of PHA-stimulated cells were chromatographed in four different solvent systems. In all of these systems >70% of the ^{14}C radioactivity in the supernate comigrated with reference long-chain fatty acids (arachidonic, linolenic, and oleic acids, which have the same R_f's in these systems). Washed, extracted cells exposed previously to lectin also contained increased quantities of fatty acid radioactivity, although the amount was <10% of that in the supernate. Considering that the original label was [^{14}C]AA and that even very short prelabeling times were associated with subsequent release of radioactivity into the supernate with PHA, it seemed likely that much or all of the released radioactivity in fatty acid was in AA itself. This was confirmed by argentaffin thin layer chromatography (not shown) which separates long-chain fatty acids on the basis of their degree of unsaturation (23).

Quantitative Studies of Unlabeled Fatty Acid Release. The release of AA by PHA was also shown by quantitative gas liquid chromatography measurements on partially purified supernates of stimulated and unstimulated lymphocytes incubated in medium with lipid-poor albumin to keep the fatty acid content of the medium to a minimum. P-PHA (20 g/ml, 10 min at 37°C) produced an approximately eightfold increase in the amount of AA in the supernates (Table VII). Smaller increases occurred in C16:1, C16:0, and C18:1 fatty acids. (C16:0 and C16:1 are not completely resolved on our

TABLE VI

Effect of Stimulation of Lectin on Lipid Radioactivity

	Lipid fraction	Control cpm	Percentage of pellet counts*	P-PHA cpm*	Percentage of pellet counts*
			%		%
Pellet	Total‡	85,998 ± 5,381		79,100 ± 4,610	
	Phosphatidyl-choline	44,800 ± 2,120	58.5	42,500 ± 2,066	61.2
	Phosphatidyl-inositol§	11,712 ± 408	15.3	7,108 ± 276‖	10.2
	Phosphatidyl-ethanola-mine	14,800 ± 411	17.2	14,211 ± 129	20.4
	Phosphatidic acid	472 ± 30	0.55	729 ± 14	0.92
	Free AA	121 ± 42	0.14	277 ± 44	0.35
	Triglyceride	1,208 ± 118	1.4	1,178 ± 42	1.5
Supernate		5,920 ± 376		11,315 ± 300‖	

For incubation conditions, see legend to Fig. 2. Before incubation with lectin, the cells contained 91,120 ± 4,864 cpm/10^7 cells. At the completion of the incubation, the cells and the supernates were separated. The cells were extracted as described in the text and the organic phase was chromatographed on silicic acid plates with chloroform:methanol:13.5 M ammonia:water (70:30:4:1) as the developing solvent, together with known phospholipid standards. Lipid-containing bands were localized with I_2 vapor, eluted, and counted. Mean ± SEM of four experiments.

* Based on the radioactivity present in the organic phase at the completion of the extraction procedure which was 76,500 ± 4,961 and 69,624 ± 3,205 cpm in control and PHA-stimulated cells, respectively.

‡ The total radioactivity in the pellet at the completion of the incubation is given.

§ Although phosphatidylinositol and phosphatidylserine comigrate in the above solvent system, in two of four experiments, two-dimensional thin layer chromatography was performed and 90% of the counts in these two phospholipids and all of the change in radioactivity with PHA was shown to be in phosphatidylinositol.

‖ Statistically different from control ($P < 0.01$).

TABLE VII

Gas Liquid Chromatographic Analysis of Fatty Acid Release

Fatty acid	Fatty acid (mean ± SEM)	
	Control	PHA
	ng/10^7 cells	
C16:0, C16:1	28 ± 8	48 ± 4
C18:0	28 ± 5	34 ± 3
C18:1	40 ± 10	130 ± 11
C20:4	28 ± 10	220 ± 10

Unfiltered lymphocytes were labeled with [^{14}C]AA, washed, incubated 30 min at 37°C in Eagle's 2% AB, washed again, suspended in Gey's medium with lipid-poor bovine serum albumin (2.5 mg/ml) at 2 × 10^7 cells/ml and incubated in triplicate in a total vol of 7.0 ml with and without P-PHA (20 μg/ml) for 10 min at 37°C. Fatty acids were extracted, esterified, partially purified by thin layer chromatography, and analyzed by liquid chromatography in the presence and absence of 8,11,14-eicosatrienoic acid as described in the text. C16:0 and C16:1 were not completely resolved on our columns and are therefore expressed as a total. However, results with internal and external standards indicate that at least 90% of the signal in this region of the chromatograph was from C16:0.

columns, but at least 90% of the signal appeared to be from C16:0.) Thus, the release reaction was partially, but by no means completely, selective by AA. To further evaluate the selectivity of release, cells were prelabeled with [^{3}H]oleic acid together with [^{14}C]AA, and stimulated with PHA. While a small increase in release of ^{3}H (oleate) radioactivity was observed in two of three experiments, it was considerably smaller than that seen with the [^{14}C]AA (not shown).

Discussion

From the results of these studies, it is clear that preparations of human peripheral blood lymphocytes release AA and, to a lesser extent, other fatty acids in response to PHA or Con A, and that a substantial part of the response is in lymphocytes themselves, rather than in contaminating cells. Much of the release of [^{14}C]AA was from phosphatidylinositol which lost up to 40% of its AA label. Because the amount of [^{14}C]arachidonate released is small relative to the total cell [^{14}C]arachidonate content, other sources, particularly phosphatidylcholine and phosphatidylethanolamine, cannot be excluded. Because AA is selectively incorporated into the 2 position of phospholipids, the rapid release of AA could be, at least in part, through the activation of phospholipase A_2. However, quantitative measurements of phospholipids and a direct demonstration that lysophospholipids are being generated would be needed to establish this mechanism. Because the role of phospholipase is uncertain, it is not clear how our findings relate to the earlier work of Resch and his colleagues (8) in a different assay system in which plasma membranes from lectin-stimulated cells underwent small increases in phospholipase activity for phosphatidyl-choline in erythrocyte ghosts, but not for free phosphatidylcholine. An equally, or more, attractive explanation for our observations is that phospholipids are being degraded by phospholipase C to diacylglycerol which is subsequently deacylated to provide free fatty acid. Phospholipase C has been demonstrated in pig lymph node lymphocytes by Allan and Michell (31). Studies in immunologically and nonimmunologically stimulated rat mast cells by D. Kennerly in our laboratory strongly support this mechanism as an important source of released fatty acid.[2] The diacylglycerol pathway could better explain the partial selectivity of the release reaction for AA. Although it is apparent that other fatty acids are also being released, a major contribution by other lipases appears less likely. While mono-, di-, and triacylglycerides as well as esterified cholesterol contain AA, based on quantitative thin layer chromatography studies (presented for triglyceride), they do not appear to be quantitatively important sources of released radioactive AA, at least under the relatively short incubation conditions used in our experiments. Because PHA and Con A bind to the cell surface, it seems likely that much of the fatty acid release is from the plasma membrane. However, if this is true and phosphatidylinositol is the major source, the magnitude of the phosphatidylinositol-cleavage reaction is somewhat surprising considering that phosphatidylinositol has been reported to be present in sizable quantities in the Golgi apparatus, endoplasmic reticulum, mitochondria, and nuclear membranes, as well as in plasma membranes (3, 5).

One consistent observation in this study was that release of fatty acid into the medium was considerably greater in serum-containing medium (usually 2% AB serum

[2] Kennerly, D., T. J. Sullivan, T. Sylvester, and C. W. Parker. Submitted for publication.

in Eagle's medium) than in media without added serum. Even though unextracted serum contains AA, and unlabeled AA augmented the release of fatty acid, 1 μM or higher concentrations were required, whereas the concentration of AA in 2% serum is probably <20 nM (32). Moreover, dialysed lipid-poor albumin was also stimulatory, suggesting that much of the effect of serum may involve the serum proteins themselves, presumably through their ability to bind released AA and prevent its reuptake by cells. A similar mechanism has been suggested for albumin effects on fatty acid release by thrombin in human platelets (20).

As far as the basis for the stimulation of release by AA itself is concerned, because lectins stimulate lymphocyte-acyl-CoA-lysophospholipid acyltransferase (acyltransferase) (33), an enzyme promoting the reacylation of lysophospholipids, perhaps the simplest explanation is that the unlabeled AA is competing with released [^{14}C]AA for reuptake by cells. If this is the explanation, the inability of AA CoA, the presumed intermediate in reacylation of lysophospholipids, to promote the accumulation of AA in the medium is somewhat surprising. But instability or failure to penetrate into appropriate areas of the cell may be a problem. A more difficult question is why other unlabeled fatty acids are so much less effective in promoting the release of ^{14}C radioactivity than AA because they are also substrates for the acyltransferase. However, juding from previous studies in broken lymphocyte preparations (33), AA CoA has a 20-fold higher affinity for the acyltransferase than oleyl CoA. If similar differences exist for the other fatty acids, AA should have considerable selectivity for the enzyme.

In view of the recently demonstrated importance of AA as a precursor for a variety of biologically active lipids, one might speculate as to the importance of the early AA release reaction in the activation process. As will be presented in detail elsewhere,[3] other mitogens for human lymphocytes including ionophore A-23187, sodium metaperiodate, trypsin, $HgCl_2$, and pokeweed also stimulate the release of AA from human lymphocytes, strengthening the argument that AA release is involved in some way in the activation process. Much of the metabolism of AA in mammalian cells is initiated through one of two enzyme systems: fatty-acid lipoxygenase or a cyclooxygenase (4). Indomethacin and aspirin which inhibit the cyclooxygenase are moderately effective inhibitors of mitogenesis in human lymphocytes, whereas 5,8,11,14-eicosatetraenoic acid, a triple-unsaturated analogue of AA, which inhibits both the lipoxygenase and the cyclooxygenase, is a potent inhibitor (inhibition approaching 100% at low μM concentrations) (34). Moreover, low concentrations (0.13–10 μM) of exogenous AA considerably enhance mitogenesis both in lipid-poor media and in media containing 10% fetal calf serum. Finally, while previous studies suggest that increases in prostaglandin synthesis are delayed for many hours in lectin-stimulated human mononuclear cells (35), recent work in our laboratory indicates that metabolites of AA are present in increased quantities very soon after exposure to lectin. Because AA availability is rate-limiting in prostaglandin synthesis in many tissues, the initial site of stimulation may be the release of free fatty acid. If AA metabolizing enzymes exist in close anatomic relationship to enzymes involved in AA release, local concentrations of AA much higher than those in the cells as a whole might be generated. Because activated lymphocytes also exhibit increased acyltransferase activity, and the affinity of the

[3] Parker, C. W. In preparation.

enzyme for AA is high (33), resynthesis of phospholipid may be occurring, providing additional substrate for AA release and metabolism.

In addition to the possible utilization of released AA as a substrate, the removal and reuptake of fatty acids provides a mechanism for changing or redistributing the acyl chains in phospholipids as may be important in the control of membrane function (33, 36). Moreover, if increased phospholipase A activity is occurring, it could also be important in helping to establish communicative contacts between cells. The data of Hulser and Peters (37) indicates that free ionic flow is established between lymphocytes within a few minutes after exposure to PHA. They have speculated that this communication may be critical in the mitogenic response. While the mechanism by which these communications are established is uncertain, increases in phospholipase activity have been observed in acanthamoeba castellanii cells when intercellular communications are being established (38), raising the possibility of a similar role for this enzyme in lectin-stimulated lymphocytes. Thus, there are a number of possible reasons why increases in phospholipase, or other lipolytic enzyme, activity might be important in lymphocyte activation.

Summary

After exposure to mitogenic lectins in vitro, human mononuclear cells (95% lymphocytes) that had been prelabeled with [^{14}C]arachidonic acid rapidly released a portion of their radioactivity in the medium. Most of the released radioactivity was demonstrated to be free arachidonic acid. Although other sources are not excluded, the most important source of cell-bound radioactivity in the release reaction appeared to be phosphatidylinositol, suggesting that at least part of the response is occurring through an increase in phospholipase A_2 activity. By gas liquid chromatography, other fatty acids were also shown to be released, but there was considerable selectivity in the response for arachidonic acid. The response was dependent on the availability of free Ca^{++} in the medium and was enhanced by serum proteins and unlabeled arachidonic acid. Most of the response appeared to be from the lymphocytes themselves rather than from contaminating cells.

The rapid generation of free arachidonic acid in response to mitogenic lectins suggests a possible role for arachidonic acid metabolites in the activation process.

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