The phosphatidylinositol response and proliferation of oxidative enzyme-activated human T lymphocytes: suppression by plasma lipoproteins

Ann L. Akeson,* David W. Scupham,† and Judith A. K. Harmony**

Chemistry Department, Indiana University, Bloomington, IN 47405† and the Departments of Biological Chemistry* and Anatomy and Cell Biology,** University of Cincinnati College of Medicine, Cincinnati, OH 45267

Abstract The phosphatidylinositol (PI) response and DNA synthesis of neuraminidase and galactose oxidase (NAGO)-stimulated human T lymphocytes are suppressed by low density lipoproteins (LDL). To understand the mechanism of lymphocyte activation more fully, the PI response and DNA synthesis and suppression of these events by LDL in NAGO-stimulated T lymphocytes were characterized. Between 30 min and 6 hr after NAGO stimulation, there was an increase of ^{32}Pi incorporation into PI without increased incorporation into the phosphorylated forms of PI or into other phospholipids. DNA synthesis as determined by [^{3}H]thymidine incorporation depended on the lymphocyte-accessory monocyte ratio and total cell density. Optimal stimulation of the PI response and DNA synthesis occurred at the same concentration of neuraminidase and galactose oxidase. While the PI response was only partially suppressed by LDL with optimal suppression at 10 to 20 μg of protein/ml, DNA synthesis was completely suppressed although at much higher LDL concentrations, >100 μg protein/ml. As monocyte numbers are increased, LDL suppression of DNA synthesis is decreased. The ability of NAGO to stimulate the PI response and DNA synthesis in a similar way, and the suppression of both events by LDL, suggests the PI response is important for lymphocyte activation and proliferation.■ Stimulation of human T lymphocytes by oxidative mitogens, neuraminidase, and galactose oxidase caused increased phosphatidylinositol metabolism and increased DNA synthesis. Both responses were suppressed by low density lipoproteins.—**Akeson, A. L., D. W. Scupham, and J. A. K. Harmony.** The phosphatidylinositol response and proliferation of oxidative enzyme-activated human T lymphocytes: suppression by plasma lipoproteins. *J. Lipid Res.* 1984. **25:** 1195–1205.

Supplementary key words DNA synthesis • neuraminidase-galactose oxidase • low density lipoproteins

The response of human T lymphocytes to lectin stimulation is suppressed by human plasma low density lipoproteins (LDL) (1). Both early activation events such as accelerated phosphatidylinositol (PI) metabolism (1) as well as DNA replication and cell division (2) are suppressed by LDL. This suggests that LDL suppression may be used as a tool for exploring the relationship between the early activation events and lymphocyte proliferation.

Current interest in cell activation in a variety of systems has focused on accelerated PI metabolism (3). PI metabolism involves several components: *1*) phospholipase C-catalyzed hydrolysis of PI, phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIPP) to diacylglycerol (DG) and the inositol phosphates with subsequent synthesis of phosphatidic acid (PA) and resynthesis of PI; *2*) further phosphorylation of PI to PIP and PIPP catalysed by kinases; and *3*) de novo synthesis of PI (3). In this work PI metabolism, termed the PI response, is followed by monitoring incorporation of radiolabeled inorganic phosphate, ^{32}Pi, into PI, PIP, PIPP as compared to other phospholipids (PL). The PI response in lymphocytes accompanies lectin receptor-stimulated calcium ion mobilization (4) and activation-specific protein phosphorylation (5). It has been suggested that it is the inositol phosphate products of the breakdown of PIP, and PIPP, rather than PI, which controls the opening of membrane Ca^{2+} pools (6, 7). Phosphatidic acid, synthesized from diacylglycerol, has also been implicated in this role as a Ca^{2+} ionophore (8). Diacylglycerol regulates protein kinase C, which appears to play a role in transmembrane control of protein phosphorylation (5). However, the

Abreviations: cGMP, cyclic guanosine 3,5 monophosphate; conA, concanavalin A; DG, diacylglycerol; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HDL, high density lipoproteins; HPTLC, high performance thin-layer chromatography; IDL, intermediate density lipoproteins; LPDS, lipoprotein-deficient serum; LDL, low density lipoproteins; MO, monocytes; NAGO, neuraminidase-galactose oxidase; PA, phosphatidic acid; PBM, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PHA, phytohemagglutinin; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIPP, phosphatidylinositol-4,5-bisphosphate; PL, phospholipid; PS, phosphatidylserine; [^{3}H]TdR, [methyl-^{3}H]-thymidine; TLC, thin-layer chromatography; VLDL, very low density lipoproteins.

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role of the PI response in the molecular mechanism of activation is not understood and the question remains: Is the PI response a necessary event for receptor-stimulated activation of lymphocytes?

Mitogenic stimulation and suppression of stimulation by LDL is best studied using a system of relatively homogeneous cells which does not require the continued presence of mitogens, which requires only short incubation times with mitogen, and which yields chemically definable changes on the cell surface. Oxidative enzyme-induced mitogenesis (9) by neuraminidase (NA) followed by galactose oxidase (GO) creates a well-defined chemical modification on the cell surface, the formation of aldehydes on terminal or penultimate galactose or N-acetyl galactosamine residues (10). Different cell types involved in the lymphocyte proliferative response, e.g., lymphocytes and monocytes, can be treated with the enzymes separately and then cell-cell interactions can be studied. The oxidative mitogens are easily removed before the initiation of proliferation assays, thus eliminating the interaction of mitogen with other proliferation mediators, specifically plasma lipoproteins.

These studies demonstrate that, at optimal conditions for NAGO stimulation of the PI response and DNA synthesis, LDL suppress both the PI response and DNA synthesis. The PI response in NAGO-stimulated lymphocytes is, however, only partially suppressed by LDL with maximum suppression at 10 to 20 μg of LDL protein/ml. In contrast, DNA synthesis is completely suppressed, although at much higher concentrations of LDL (100 μg of LDL protein/ml). Therefore, the use of NAGO for stimulation and lipoproteins for suppression may aid in understanding the multi-stepped sequence of events required for both activation and proliferation of lymphocytes.

METHODS

Materials

Galactose oxidase (E.C.1.1.3.9.), Type IV from *Dactylium dendroides*, mitomycin C, and phospholipids were obtained from Sigma Chemical Co. Neuraminidase (E.C.3.2.1.18.), from *Vibrio cholerae*, was purchased from Grand Island Biological Co., as was RPMI 1640 medium (supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.3 μg/ml L-glutamine). Isolymph was obtained from Gallard-Schlesinger Chemical Mfg. Corp. Fetal bovine serum (FBS) was purchased from KC Biologicals. [Methyl-^{3}H]thymidine (6.7 Ci/mmole, [^{3}H]TdR) and ^{32}Pi as inorganic orthophosphate were obtained from either New England Nuclear or ICN. Sheep erythrocytes were purchased from Cordis Laboratories.

Lipoprotein isolation

Human plasma lipoproteins were isolated by sequential ultracentrifugation of heparinized plasma obtained from healthy, fasting donors (11) or from patients with Type IIa hyperlipoproteinemia. Very low density lipoproteins (VLDL) were isolated at a buoyant density of less than 1.006 g/ml, whereas LDL were isolated at a buoyant density of 1.019–1.063 g/ml, and high density lipoproteins (HDL) were isolated at 1.063–1.21 g/ml. The purity of each fraction was determined by 1% agarose gel electrophoresis in tricine buffer, pH 8.6. Immediately prior to use, lipoproteins were dialyzed against 10 mM HEPES buffer, pH 7.4, then filter sterilized using a 0.45 micron Millex-HA filter (Millipore Corp.).

Cell isolation

Human peripheral blood lymphocytes were prepared using a modification of the procedure of Böyum (12). Heparinized blood from healthy donors was diluted 1:3 with sterile phosphate-buffered saline (PBS), then centrifuged on an Isolymph gradient at 400*g* for 30 min at room temperature. The PBM layer was collected by aspiration from the interface, and the cells were washed three times with saline. The cells were resuspended in RPMI 1640 containing 10% FBS. PBM were then mixed at 4°C with neuraminidase-treated sheep erythrocytes (13). Rosette-positive cells were eluted from a column packed with nylon wool to remove contaminating adherent cells. Rosette-negative cells were further purified by adherence to glass and designated monocytes (MO). The resultant MO-enriched pool was treated with 40 μg/ml of mitomycin C at a cell density of 5×10^6 cells/ml, washed, and resuspended in fresh medium.

Cell evaluation

Cell morphology was assessed by light microscopy. Smears were prepared by cytocentrifugation and were stained with Diff-Quik (Dade Diagnostics, Inc.). Cytological analysis showed that the isolated lymphocytes were >95% lymphocytes, <1 to 4% monocytes, and <1% neutrophils or platelets. In some experiments, cell populations were evaluated by flow cytometry (FACS III, Becton Dickinson), using monoclonal antibodies raised against T lymphocyte surface antigens (OKT3, OKT4, OKT8) and a monocyte surface antigen (63D3). By FACS analysis, T lymphocytes isolated by procedures outlined above were greater than 95% T lymphocytes with less than 5% B lymphocytes and 63D3$^+$ monocytes. Cell viability was determined by Trypan blue exclusion or by staining with fluorescein diacetate and ethidium bromide (14) and was always greater than 98%.

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Mitogenic treatment

T lymphocytes were treated simultaneously with neuraminidase and galactose oxidase. Typically, 8×10^6 cells/ml were treated with 50 units of NA and 0.5 units of GO. The mixture was then incubated at 37°C for 30 min. The reaction was terminated by washing the cells three times with RPMI 1640. The cells were resuspended in medium, and enumerated using a hemocytometer or a Coulter Counter. NAGO-treated lymphocytes were analyzed for DNA synthesis and/or ^{32}Pi incorporation into PL.

DNA synthesis assay

Lymphocytes and monocytes were cultured separately or at the T:MO ratios indicated in RPMI 1640 medium at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cultures were established in triplicate in sterile 96-well flat-bottomed tissue culture cluster plates (COSTAR). The total volume of the culture varied from 0.21 ml/well to 0.24 ml/well, and total cell number varied from 69×10^3 to 210×10^3 as indicated depending upon the conditions of the individual experiment. Additions to cultures are as noted in the figure legends. Two hr prior to termination, 1 μCi of [^{3}H]TdR was added per well. Cells were harvested onto glass fiber filters, and DNA synthesis was assayed by the incorporation of [^{3}H]TdR. The amount of [^{3}H]TdR uptake by the cells was determined by liquid scintillation spectrometry on the glass fiber filters. The percent suppression of [^{3}H]TdR incorporation by LDL was calculated as follows:

$$\frac{[\text{cpm }(-\text{LDL})] - [\text{cpm }(+\text{LDL})]}{[\text{cpm }(-\text{LDL})]} \times 100 = \%\ \text{suppression.}$$

^{32}Pi incorporation into phospholipids (PL)

After termination of NAGO treatment, the lymphocytes were washed two times with phosphate-free Hanks' balanced salt solution (HBSS). NAGO-treated T lymphocytes or control lymphocytes (2×10^6) were incubated with 100 μCi/ml of ^{32}Pi in a total volume of 1 ml of HBBS or phosphate-free serum-free RPMI 1640 containing 10 μg/ml of transferrin, 15 μg/ml of insulin, 5 μg/ml of catalase, 10 μM β-mercaptoethanol, and 0.5 μg/ml of linoleic acid. At various times the lymphocytes were cooled in an ice bath, and washed twice in 1 ml of ice-cold PBS. Phospholipids were extracted by addition of 2 ml of 0.1 N HCl and 2 ml chloroform–methanol 2:1 (15). The chloroform layer was removed and dried under nitrogen; the phospholipids were separated by two-dimensional thin-layer chromatography (TLC), with a first dimension solvent system of chloroform–methanol–acetic acid 65:25:10 (v/v) and the second dimension solvent system of chloroform–methanol–formic acid 65:25:10 (16). Alternatively, phospholipids were analyzed by high performance thin-layer chromatography (HPTLC) using Silica Gel 60 plates (Merck) predeveloped in 1% potassium oxalate in methanol–water 3:2. The solvent system was chloroform–acetone–methanol–acetic acid–water 40:15:13:12:8 (17). The use of HPTLC provides a reproducible system for separation and identification of ^{32}Pi-labeled phospholipids from lymphocytes. As indicated by the R_f values in **Table 1,** this system separates PI and PIP and PIPP from each other and from the other major phospholipids. The PL, visualized with iodine vapor and radioautography, were identified by reference to authentic standards chromatographed on the same plate. The areas containing the individual PL were scraped from the plates and counted by liquid scintillation spectrometry using 2 ml of scintillation cocktail 4a20 (Research Products International).

It should be noted that the PI response and DNA synthesis were measured under slightly different conditions. In each case the conditions selected were optimal for the response measured. A similar relative PI response, comparing NAGO-stimulated and control lymphocytes, was obtained in phosphate-containing RPMI-2% FBS. Furthermore, the relative PI response of 5×10^5 T lymphocytes, a cell number comparable to that used for analysis of DNA synthesis, was the same as for 2×10^6 T lymphocytes. However, total incorporation of ^{32}Pi into all PL per assay was lower.

Analytical procedures

Protein concentrations were determined by the method of Lowry et al. (18), modified by the inclusion of 1% sodium dodecyl sulfate to clarify the solutions. Bovine serum albumin was used as the standard.

RESULTS

Treatment of human T lymphocytes with oxidative enzymes NAGO caused immediate stimulation of the PI response, and a few hr later increased ^{32}P-labeling of other PL. NAGO-stimulated lymphocytes incorporated 2.5 times more label into PI than control lymphocytes at 2 hr (Table 1). PI represented a majority (71%) of ^{32}Pi incorporated into cellular PL 2 hr after stimulation. In control lymphocytes PI represented only 36% of ^{32}Pi-labeled PL. At 24 hr, PI incorporation was 7 times higher in NAGO-stimulated lymphocytes compared to control lymphocytes and 19 times higher than 2 hr after NAGO stimulation. Yet, due to increased incorporation of ^{32}Pi into all PL, PI represented only 23% of total ^{32}Pi-labeled PL. The relative level of PI was the same (24%) for control lymphocytes after 24 hr of

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TABLE 1. Incorporation of ^{32}Pi into lymphocyte phospholipids after NAGO stimulation[a]

		2 Hours				24 Hours			
		Control		NAGO		Control		NAGO	
	R_f	cpm	%	cpm	%	cpm	%	cpm	%
PI	0.50	843 ± 27	36	2066 ± 1037	71	5489 ± 450	24	39,400 ± 2990	23
PIPP	0.29	32 ± 3	1	21 ± 2	<1	67 ± 25	<1	379 ± 67	<1
PIP	0.33	98 ± 4	4	48 ± 5	2	166 ± 15	<1	1266 ± 135	<1
PS	0.62	131 ± 45	5	20 ± 10	<1	437 ± 74	2	3840 ± 734	2
PC-PE	0.69	488 ± 216	14	84 ± 1	3	5933 ± 3080	26	88,143 ± 17,000	51
PG	0.73	285 ± 118	9	211 ± 117	7	8182 ± 3028	36	16,930 ± 8827	10
PA	0.82	278 ± 130	10	234 ± 95	9	2027 ± 282	9	18,073 ± 2311	10
Unidentified lipids		333 ± 130	10	171 ± 21	6	562 ± 73	2	4094 ± 209	2

[a] Lymphocytes were incubated with or without NAGO. The enzymes were removed and 2×10^6 cells, 100 μCi ^{32}Pi, and phosphate-free serum-free RPMI 1640 in a total volume of 1.0 ml were incubated at 37°C in 5% CO_2/air. At the indicated times, total PL were extracted and analyzed by HPTLC as indicated in Methods. Data represent mean cpm ± SD for triplicate determinations or percent of total phospholipid cpm in each phospholipid.

labeling. These results are typical of lymphocytes obtained from a number of different donors.

The PI metabolism of unstimulated lymphocytes was relatively active compared to other PL as demonstrated in Table 1. Two hr after initiation of labeling, 36% of total ^{32}Pi-labeled PL in control lymphocytes was PI, while the major PL constituents, PC-PE and PG, account for only 14% and 8%, respectively. The incorporation of ^{32}Pi into PI after 24 hr of labeling was 6.5 times higher than at 2 hr, but PI represented only 24% of total ^{32}P-labeled PL. Levels of ^{32}Pi incorporation into all PL at 24 hr by control lymphocytes indicate equilibrium labeling, giving the relative mass of each PL as %. With the exception of higher PI levels, values are similar to those reported by Gottfried for unfractionated polymorphonuclear leukocytes (19).

While there was a dramatic increase of ^{32}Pi incorporation into PI 2 hr after stimulation, incorporation into other PL was relatively low. The data in Table 1 indicate that there was a decrease in the incorporation of label into PIP, PS, and PC-PE 2 hr after NAGO stimulation. This decrease was not detected in subsequent experiments, suggesting lymphocyte donor variability. Another source of variability is the contaminating cells (e.g., non-lymphocytes). Incorporation into other PL was low and equivalent for NAGO-stimulated and control lymphocytes. NAGO-stimulated lymphocytes incorporated 2- to 16-fold more ^{32}Pi into each PL than control lymphocytes after 24 hr of labeling. Yet, with the exception of PC-PE and PG, the relative levels of PL as indicated by % values in Table 1 were not different from those of control lymphocytes. There was a relatively modest 2-fold increase of labeled PG in NAGO-stimulated as compared to control lymphocytes with the relative level of PG only 10% of total ^{32}P-labeled PL in stimulated compared to 36% in control lymphocytes. In contrast, PC-PE was 51% of the ^{32}P-labeled PL in NAGO-stimulated lymphocytes compared to 36% in control lymphocytes. This may reflect both increased PC-PE turnover and increased synthesis due to NAGO stimulation.

Fig. 1 depicts the time course of ^{32}Pi incorporation into PI and PC-PE following NAGO treatment. NAGO-stimulated lymphocytes rapidly incorporated ^{32}Pi into PI throughout the incubation period. Control lymphocytes incorporated ^{32}Pi into PI much more slowly. Measurement of ^{32}Pi incorporation beyond 24 hr indicates control lymphocytes reached labeling equilibrium by 24 hr. As early as 30 min after addition of ^{32}Pi, NAGO-stimulated lymphocytes incorporated 36% more label into PI than did control lymphocytes (inset Fig. 1A). Optimal differential stimulation of the PI response occurred between 1 and 4 hr following NAGO treatment. At 10 hr, NAGO-stimulated lymphocytes incorporated 10 times more ^{32}Pi into PI than control lymphocytes. Yet, because incorporation of ^{32}Pi into all PL was increasing at this time in both stimulated and control lymphocytes, 35% of the total ^{32}Pi in PL was in PI in both cases. The differential PI response within 4 hr after NAGO stimulation indicates accelerated turnover or increased de novo synthesis of PI or a combination of both. Lymphocytes did not readily take up exogenous [^{3}H]inositol, making analysis of de novo PI sythesis difficult. Methods for microanalysis of changes in PI mass are currently being developed. With this analysis, distinction between synthesis and turnover should be possible.

Increased synthesis and turnover of PC also occurred in phytohemagglutinin (PHA)-activated PBM (20). NAGO stimulated change in PC-PE, but the time course of incorporation of ^{32}Pi into PC-PE was clearly different from that of PI (Fig. 1B). Through 4 hr, stimulated and

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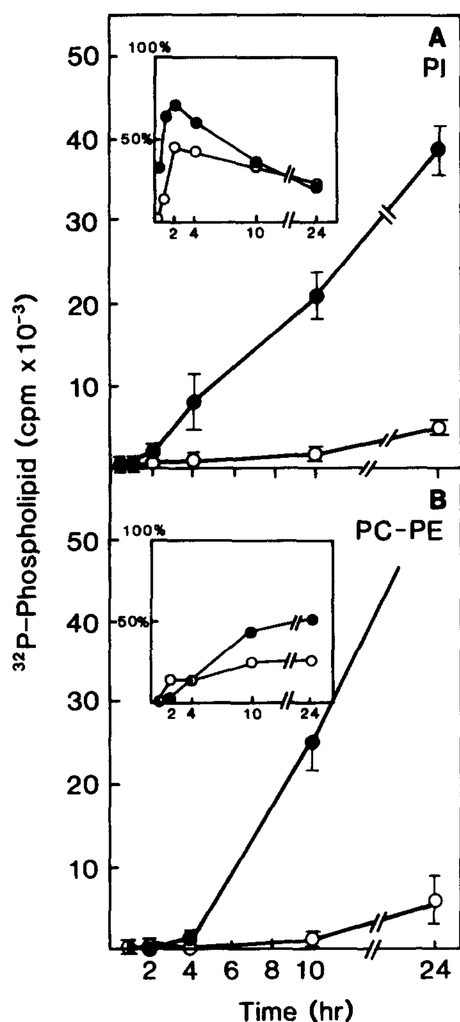

Fig. 1. Time course of ^{32}Pi incorporation into PI and PC-PE by NAGO-stimulated lymphocytes. NAGO-stimulated lymphocytes (●) and control lymphocytes (○) (2×10^6 cells) were incubated with ^{32}Pi (100 μCi) in 1.0 ml of phosphate-free serum-free RPMI 1640. At the times indicated total PL were extracted and analyzed by HPTLC as described in Methods. Data are presented as cpm ± SD in PI (A) and PC-PE (B) for triplicate determinations. At 24 hr NAGO-stimulated lymphocytes incorporated 88, 140 ± 17,000 cpm into PC-PE (point not shown). The inserts represent the percent of total ^{32}P-labeled PL in PI (A) and PC-PE (B).

control lymphocytes incorporated little ^{32}Pi into PC-PE. Only about 15% of the total label in PL was in PC-PE at 4 hr. By 10 hr NAGO-stimulated lymphocytes incorporated 20 times more ^{32}Pi into PC-PE than control lymphocytes. Also, a higher percentage of the total labeled PL was in PC-PE at 10 hr in stimulated lymphocytes. This contrasts sharply with the percentage of total labeled PL in PI at 10 hr, which is equivalent for stimulated and control lymphocytes. Twenty-four hr after NAGO stimulation lymphocytes incorporated 88,143 ± 17,000 cpm into PC-PE, a 15-fold higher incorporation than in control lymphocytes.

Optimal conditions for the mitogenic response of lymphocytes to NAGO were determined by independently varying NA and GO concentrations. In the analysis of the early PI response, isolated lymphocytes were treated with 150 units/ml of NA and 0 to 1.5 units/ml of GO (**Fig. 2A**). Conversely, lymphocytes were treated with 1.5 units of GO and 0 to 150 units of NA (Fig. 2B). When measured at the optimal time for the PI response, 2 hr, the maximum incorporation of ^{32}Pi into PI was achieved with 0.50 units GO and 25 units NA. A typical [^{3}H]TdR response, a measure of DNA synthesis, by control and stimulated lymphocytes at 72 and 96 hr is shown in **Fig. 3.** With a fixed NA concentration of 50 units/ml, GO concentrations saturated at 0.25 units/ml (Fig. 3A). In the parallel experiment in which GO was constant at 0.5 units/ml, the response to NA saturated at 25 units/ml (Fig. 3B). For the experiment illustrated by Fig. 3, the ratio of T cells

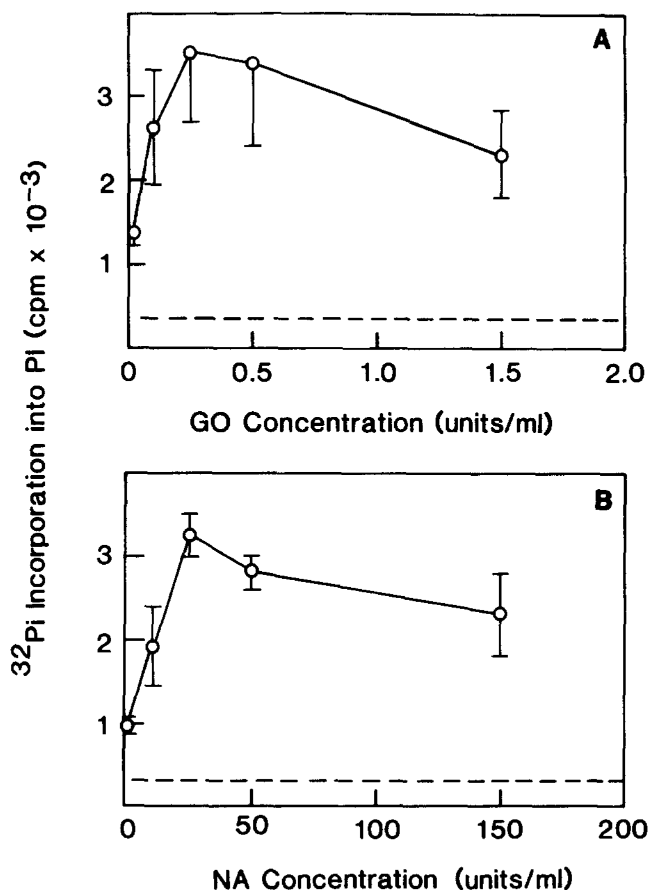

Fig. 2. NAGO stimulates lymphocyte PI response in a dose-dependent manner. T lymphocytes were incubated as described in *Methods* for 30 min with 150 units/ml NA and 0 to 1.5 units/ml GO (A) or with 1.5 units/ml GO and 0 to 150 units/ml NA (B). The lymphocytes (2×10^6) were pulsed with 100 μCi ^{32}Pi. After 2 hr the PL were extracted and analyzed by two-dimensional TLC as described in *Methods.* Data are presented as cpm ± SD in PI for triplicate determination. The dashed line (– – –) represents mean cpm in PI for untreated control lymphocytes.

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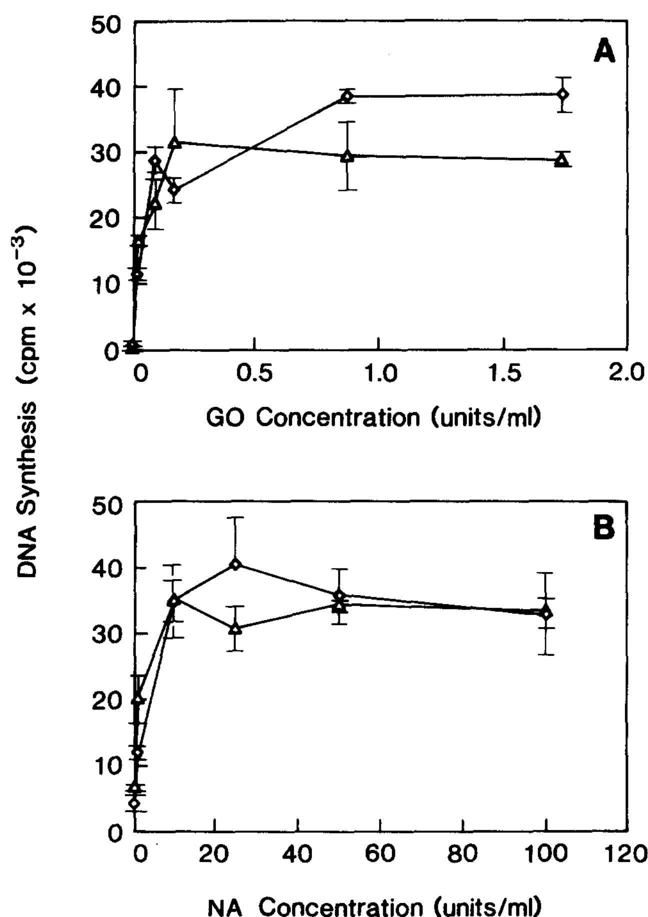

Fig. 3. NAGO stimulates DNA synthesis in a concentration-dependent manner. A, Eight × 10^6 T lymphocytes were treated with 50 units/ml NA, with various concentrations of GO as indicated. NAGO-treated T lymphocytes (100,000) were co-cultured with 25,000 MO, and harvested either at 70 hr (◇) or 94 hr (△). B, Eight × 10^6 T cells were treated with various concentrations of NA, and with 0.5 units/ml GO. NAGO-treated T lymphocytes (100,000) were co-cultured with 25,000 MO. Two hr prior to harvesting, 1 μCi of [^{3}H]TdR was added to each culture. The cells were harvested at either 72 hr (◇) or 96 hr (△). The data represent the mean value of triplicate determinations ± SD.

to MO was 4:1. When unfractionated PBM or T lymphocytes plus varying numbers of MO were assayed, there was no change in the saturation points of these curves (data not shown), indicating that the amount of NAGO needed to stimulate T lymphocytes was independent of MO number in culture. While the optimal dose response for the mitogenic enzymes was the same for the PI response and DNA synthesis, events separated by 70 hr, interesting differences exist. Dixon, Parker, and O'Brien (21) reported that GO alone causes a modest increase in DNA synthesis while NA alone has no effect on human lymphocytes. Our data are in good agreement. However, lymphocytes treated independently with NA as well as independently with GO have an increased PI response, as can be seen in Fig. 2. NA alone (150 units) stimulated a 4-fold higher PI response compared to control lymphocytes. GO alone (1.5 units) stimulated a 3-fold higher PI response. In subsequent experiments, T lymphocytes were stimulated with 0.50 units GO and 25 units NA.

Although the optimal concentration of oxidative enzymes needed to elicit a T lymphocyte response was not affected by co-culturing with accessory MO, the amount of DNA synthesis was influenced greatly by the total cell number and by the ratio of lymphocytes to MO in culture. In the absence of exogenously added MO, NAGO-stimulated lymphocytes do not synthesize DNA. **Fig. 4** illustrates that at each T cell number, as the MO number per culture increased, the level of DNA synthesis increased. Each culture was labeled for 2 hr with 1 μCi of [^{3}H]TdR before harvesting. This labeling period was well within the limits established wherein [^{3}H]TdR incorporation is proportional to the dividing cell mass (22). Hence, the value of [^{3}H]TdR incorporation provides an indication as to the number of responding cells in an individual culture. When the number of NAGO-stimulated T lymphocytes cultured was low (69,000), the highest level of proliferation occurred when the ratio of T lymphocytes to MO was approximately 1:1 (curve a). At higher cell densities (curves b and c), the response of the cultures was uniformly lower on a potential responding T lymphocyte basis as compared to that in the low density culture (curve a). Although two parameters were varied simultaneously in this ex-

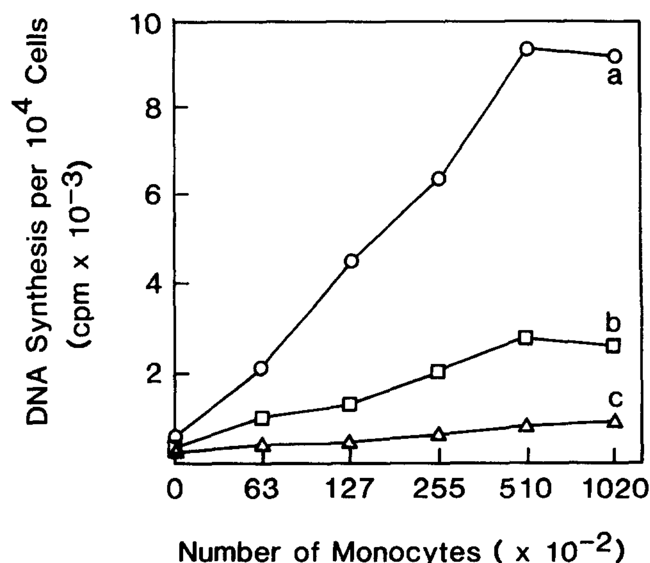

Fig. 4. Effect of monocyte number on NAGO-induced DNA synthesis. Lymphocytes were treated with 50 units/ml NA and 0.87 units/ml GO. Various numbers of MO were added to a fixed number of NAGO-treated T lymphocytes; 69,000 (○), 138,000 (□), or 277,000 (△). A 2-hr [^{3}H]TdR pulse was used. Each point represents the mean of triplicate assays harvested 4 days after stimulation and normalized to 10^4 potentially responding cells.

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periment, the number of T lymphocytes per culture and the ratio of T:MO, the data clearly indicate that the best response is achieved with a low total cell number and a T:MO ratio ranging from 1:1 to 10:1. Therefore, proliferation assays were routinely established with approximately 10^5 cells per well, and T:MO ratios ranging from 1:1 to 10:1.

By contrast, the PI response 2 hr after NAGO stimulation occurred in the absence of detectable accessory cells. When isolated by procedures outlined in Methods, "pure" T lymphocytes stimulated with NAGO had a 2.5- to 6-fold higher PI response than "pure" control lymphocytes. The same "pure" lymphocytes did not enter the S phase of the cell cycle in response to NAGO as determined by [^{3}H]TdR incorporation. These NAGO-stimulated lymphocytes replicated their DNA only when monocytes were present. The PI response of NAGO-stimulated lymphocytes was, however, modulated by adherent monocytes (T:MO ratios of 10:1, 5:1, 1:1; A. L. Akeson and J. A. K. Harmony, unpublished data). When monocytes were added to NAGO-treated T lymphocytes immediately after stimulation, the PI response of the cultures was higher than the sum of the response for NAGO-stimulated T lymphocytes and monocytes alone. However, complicating the analysis of the data is the fact that incorporation of ^{32}Pi into other PL by both control and NAGO-stimulated lymphocyte-monocyte cultures was higher than for lymphocytes alone, reflecting active membrane metabolism of the monocytes. Further analysis of the effect of accessory cells on the PI response of T lymphocytes is currently in progress.

Human lipoproteins inhibited both the early PI response and DNA synthesis in NAGO-stimulated lymphocytes (**Fig. 5**). Optimal suppression of the PI response in NAGO-stimulated lymphocytes occurred at 10 μg of LDL protein/ml (Fig. 5A). The PI response increased by NAGO was never fully suppressed by LDL. Maximal suppression with this LDL was 60%. LDL concentrations above 20 μg protein/ml did not cause further suppression. This contrasts to the situation for PHA-stimulated nonadherent lymphocytes where complete suppression is achieved by LDL at concentrations over 100 μg protein/ml (1).

To determine if suppression of the PI response to NAGO was due to this particular LDL preparation, other LDL were tested. **Fig. 6** shows optimal suppression of the PI response of NAGO-stimulated T lymphocytes from one donor by LDL isolated from a normolipemic donor (D-S) and from patients with Type IIa hyperlipoproteinemia (P-1 and J-9). Optimal suppressive concentrations of LDL were between 7.6 and 13.0 μg protein/ml. Maximal suppression of the PI response was variable with the LDL sample and was never greater than 45% for these lymphocytes. As is evident in Fig.

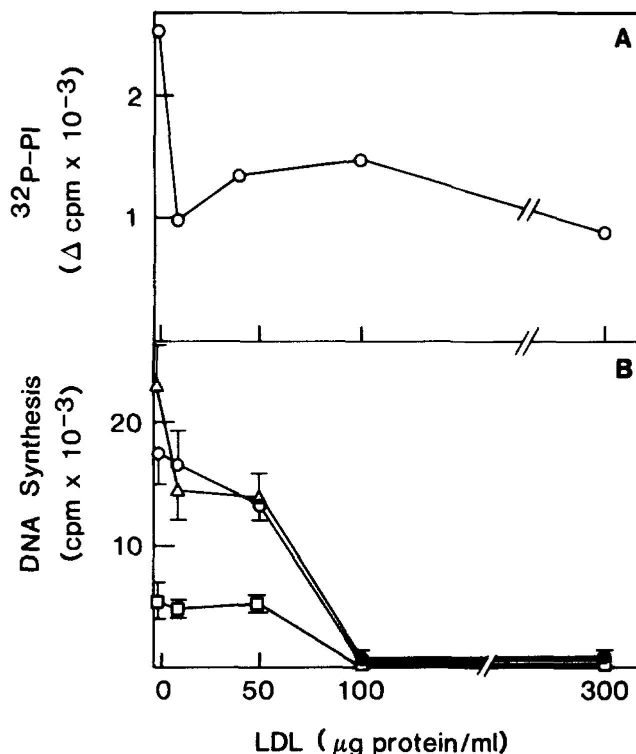

Fig. 5. Concentration-dependent suppression by LDL of early PI response and DNA synthesis of NAGO-stimulated T lymphocytes. For the PI response (A), 2×10^6 NAGO-stimulated or control lymphocytes were incubated with increasing concentrations of LDL and 100 μCi ^{32}Pi. LDL was from a patient with Type IIa hyperlipoproteinemia. After 2 hr the PL were extracted and analyzed by two-dimensional TLC as described in Methods. Data are presented as Δcpm (cpm in PI NAGO-lymphocytes − cpm in PI control lymphocytes) for triplicate samples. In the DNA synthesis assay (B) 1×10^5 NAGO-stimulated T lymphocytes plus 1×10^4 MO were incubated with increasing concentrations of LDL as described in Methods. Following a 6-hr pulse with 1.0 μCi of [^{3}H]TdR, cells were harvested at 48 (□——□), 72 (Δ——Δ), and 96 (O——O) hr. Data represent the mean of triplicate samples ± SD. Control lymphocytes did not incorporate significant [^{3}H]TdR.

6, the PI response of control lymphocytes was suppressed 20 to 30% by certain LDL preparations. This did not occur in T lymphocytes isolated from some donors. The occasional inhibition of control lymphocyte PI metabolism may therefore reflect variations in the responses of T lymphocytes from different donors to isolation procedures used. A small portion of T lymphocytes may be activated by rosetting or by passage over nylon wool, and it may be the response of these cells that is suppressed by LDL in control cultures. Oxidized sterols in some preparations of LDL may also inhibit the control lymphocyte PI response. However, oxidized sterols cannot entirely account for the inhibitory affect of LDL, as apoB isolated from LDL also suppresses the PI response of NAGO-stimulated T lymphocytes (23).

DNA synthesis by the same NAGO-stimulated lymphocytes was also suppressed by LDL in a concentration-

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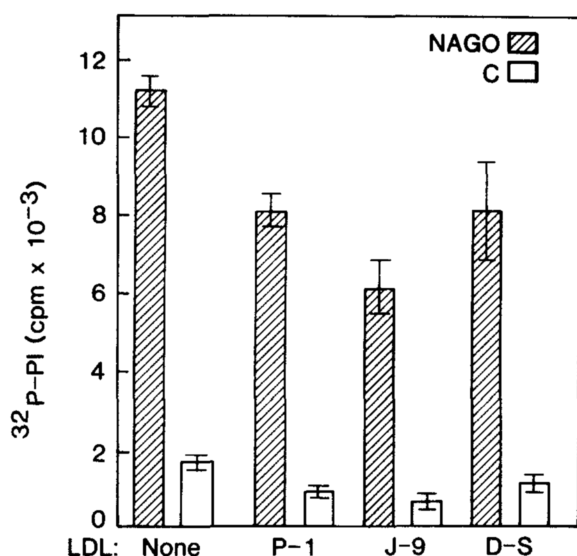

Fig. 6. The PI response of NAGO-stimulated lymphocytes is suppressed by LDL. NAGO-stimulated and control lymphocytes (2×10^6 cells) were incubated in the presence of LDL (P-1, 13.0 μg protein/ml; J-9 11.6 μg protein/ml; D-S, 7.6 μg protein/ml), 100 μCi ^{32}Pi in phosphate-free, serum-free RPMI 1640 in a total volume of 1 ml for 2 hr. LDL (P-1 and J-9) were isolated from patients with Type II hyperlipoproteinemia or (D-S) from a normal donor. The PL were extracted and analyzed by HPTLC as described in Methods. Data are presented as cpm ± SEM in PI. Incorporation of ^{32}Pi into other PL was not influenced by LDL.

dependent manner (Fig. 5B). The degree of suppression of DNA synthesis was greater when cells were harvested after 3–4 days as compared to 2 days. Low concentrations of LDL (<50 μg of protein/ml) did not significantly suppress [^{3}H]TdR incorporation until 4 days after stimulation. Maximal suppression by LDL of the PI response and of DNA synthesis occurred at different LDL concentrations, 10–20 μg LDL protein/ml and >100 μg LDL protein/ml, respectively.

As noted, the extent to which T/MO cultures were stimulated to incorporate [^{3}H]TdR by NAGO was dependent upon the ratio at which the cells are cultured. It was therefore not unexpected that the extent of suppression by LDL was also dependent upon the ratio of T lymphocytes to MO. For three different T:MO ratios (1:1, 5:1, 10:1), the incorporation of [^{3}H]TdR in cultures with identical LDL concentrations showed differing amounts of suppression. At low LDL, no apparent effect of cell ratio on suppression was noted. However, at high LDL concentrations, T lymphocyte:MO cultures of cell ratios 10:1 or 5:1 were more sensitive to LDL suppression by 40% compared to the culture in which the cell ratio was 1:1 (**Table 2**). The trend of these data is in agreement with that of Okano, Macy, and Harmony (24) who found that, as the MO number per culture increased, the extent of lipoprotein suppression of PHA-induced lymphocyte activation was reduced.

The suppressive potency of three different lipoprotein classes from different donors was tested (**Table 3**). The results clearly indicate the effect of lipoproteins with hydrated densities less than 1.063 g/ml (VLDL and LDL) is suppression. The addition of high density lipoproteins (HDL) to the cultures had no effect on the amount of stimulated DNA synthesis relative to control values. Moreover, the data reported in Table 3 also illustrate that the suppressive effect of lipoproteins on DNA synthesis is not dependent on the type of serum used to maintain the cultures. For the experiment in Table 3, 5% lipoprotein-deficient human serum (LPDS) was used, whereas 5% FBS was used for the experiment reported in Table 2. This suggests that the suppressive mechanism of lipoproteins is not due to a serum component. Since LPDS is used to generate the high affinity LDL receptor in both fibroblasts and lymphocytes (25), it is interesting that suppression in lymphocytes can be achieved in LPDS. This result is consistent with other data that indicate that the classic high-affinity LDL receptor is not involved in the suppression of DNA synthesis by lipoproteins (26).

DISCUSSION

This is the first report that the oxidative mitogens, NAGO, induce an early PI response in human T lym-

TABLE 2. Percent suppression of DNA synthesis by a given lipoprotein is dependent on the T:MO ratio[a]

	T:MO Ratio					
	1:1		5:1		10:1	
LDL	cpm ± SD	% Suppression	cpm ± SD	% Suppression	cpm ± SD	% Suppression
0 μg/ml	19203 ± 2895		10252 ± 1260		5507 ± 1278	
75 μg/ml	16458 ± 1190	14	8416 ± 1276	18	4317 ± 2071	21
300 μg/ml	10904 ± 851	43	1490 ± 441	85	1098 ± 559	80

[a] DNA synthesis was assessed after a 2-hr pulse of [^{3}H]TdR. NAGO-treated T lymphocytes were cultured at the ratios given. LDL was from a normal donor. The total number of cells initially cultured was 10^5. Data represent either the mean of triplicate determinations ± SD or % suppression as calculated in Methods.

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TABLE 3. Lipoproteins inhibit NAGO-induced DNA synthesis[a]

Lipoprotein[b]	DNA Synthesis	
	cpm ± SD	% Suppression
None	12780 ± 540	
VLDL + IDL_D[c]	2386 ± 490	81
LDL_D	3485 ± 405	73
$VLDL_H$	491 ± 42	96
IDL_H	1349 ± 76	89
LDL_H	1473 ± 172	88
HDL_H	13930 ± 414	(−9)[d]

[a] DNA synthesis at 72 hr was assessed after a 2-hr pulse of [^{3}H]TdR. NAGO-treated T lymphocytes were cultured at a ratio of 1:1 with MO in 5% LPDS. Data represent either the mean of triplicate determinations or the % suppression.

[b] The protein concentration of VLDL was 76 μg/ml. All other lipoprotein concentrations were 170 μg/ml. The subscripts represent lipoproteins isolated from different donors, D and H.

[c] A mixture of VLDL and intermediate density lipoproteins (IDL).

[d] A negative number indicates that the amount of [^{3}H]TdR incorporated by cells incubated in the presence of lipoproteins was greater than that in the absence.

phocytes. The accelerated PI response, detected as early as 30 min after stimulation, is sustained for up to 6 hr. The metabolism of other PL is not greatly altered during this time. Increased metabolism of other PL begins after 10 hr and probably reflects proliferation-associated requirements for increased membrane synthesis (20). The importance of the PI response in activation is supported by the data presented in this study. Optimal stimulation of the PI response and DNA synthesis is reached at the same oxidative enzyme concentrations, 0.50 units GO and 25 units of NA per 8 × 10^6 T lymphocytes per ml, implying that those cells with an increased PI response are the same cells within the population that are stimulated to synthesize DNA.

The PI response apparently does not require accessory cells. Any residual monocytes capable of acting as accessory cells comprise less than 5% of the isolated T lymphocyte population. This level of accessory cells is not sufficient for significant NAGO-stimulated T lymphocyte proliferation. While NAGO stimulates DNA synthesis by T lymphocytes, the presence of additional accessory cells is an absolute requirement for a positive response. It may be that two signals are required for the induction of T cell proliferation by NAGO. One signal is dependent upon the formation of oxidized products on the surface of the stimulated cell and is either independent of accessory cells or requires very few accessory cells. The second signal is clearly monocyte-dependent, requiring T:MO of at least 10:1, and probably involves the release of soluble mediators into the medium by the monocytes and possibly cell-cell contact. Only the first signal is required for the PI response, but both signals are essential for lymphocyte DNA synthesis and proliferation. Lipkowitz et al. (27) have shown that NAGO-treated macrophages are potent indirect stimulators of T lymphocytes but that minimal proliferation occurs in the absence of viable macrophages or macrophage-dependent soluble mediators, adding support to the concept that two signals are required for NAGO-triggered lymphocyte proliferation.

Since the mitogenic challenge involves covalent modification of the lymphocyte surface, this result definitively establishes that lipoprotein suppression is not due to interference with the primary mitogenic signal. The NAGO-induced PI response of purified human T lymphocytes was suppressed by LDL. When the PI response was measured between 2 and 4 hr after initiation of NAGO treatment, optimal suppression by LDL occurred at 10 μg of LDL protein/ml. LDL preparations isolated from normal donors and from donors with hyperlipoproteinemia varied in the degree to which they suppressed the PI response. However, the pattern of inhibition, with optimal suppression at LDL concentrations of 10–20 μg of LDL protein/ml and maximal suppression never greater than 60%, was qualitatively similar. The level of ^{32}Pi incorporation into the other major PL was not affected by LDL when measured 2–4 hr after stimulation.

Why is the PI response of NAGO-stimulated lymphocytes only partially susceptible to LDL suppression? One explanation is that there are two pools of cellular PI. Upon mitogen stimulation, one pool is rapidly labeled by ^{32}Pi due to the immediate breakdown and resynthesis of PI. A second pool, while more rapidly labeled by ^{32}Pi than other PL, is not altered by mitogen action at the cell surface. This second pool of PI is labeled in unstimulated as well as NAGO-stimulated lymphocytes. Monaco (28) has suggested the existence of two pools of cellular PI in a vasopression-sensitive rat mammary tumor cell line WRK-1. In lymphocytes, cycling of the first pool may be suppressed by LDL while the second is unaffected in NAGO-stimulated lymphocytes. If this were the case, LDL should suppress the PI response of NAGO-stimulated lymphocytes to the levels seen with control lymphocytes. Yet complete suppression does not occur. However, mitogen-stimulated PI breakdown and resynthesis cannot be considered a closed cycle: DG and PA, major components of the cycle, can be derived from sources other than PI. Thus, while one portion of the cycle, i.e., breakdown of PI to DG, may be suppressed by LDL as suggested by Hui and Harmony (1), DG and PA derived from other sources can be utilized for resynthesis of PI. This portion of the cycle may not be affected by LDL, accounting for the fact that total inhibition of the PI response cannot be achieved.

The DNA synthesis of lymphocytes from the same donor was also suppressed by the LDL in a dose-

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dependent manner. Yet there are distinct differences between lipoprotein suppression of the PI response and DNA synthesis. Optimal suppression of the DNA synthesis occurs at a higher LDL concentration, 100 μg of LDL protein/ml, than for suppression of the PI response. Further, as discussed previously, LDL suppression of the early event was never greater than 60%, while DNA synthesis is suppressed by nearly 100%. The difference in LDL dose required for suppression of these cellular responses may reflect in part the requirement for accessory cells in proliferation. The ratio of T lymphocytes to MO has been shown to affect the degree to which LDL suppress the proliferative response of lymphocytes to NAGO and PHA (24).

LDL suppressed in a dose-dependent manner the PI response and subsequent DNA synthesis caused by the oxidative enzymes NAGO. Hui and Harmony have also found that LDL suppress a series of early proliferation-associated events, Ca^{2+} and cyclic guanosine 3',5' monophosphate (cGMP) accumulation (29, 30), as well as PI turnover (1) and DNA synthesis (29). However, not all proliferation-associated events are affected by LDL. Blast formation (31), RNA and protein synthesis (32), and the appearance of receptors for T cell growth factor and transferrin (31) are not suppressed by LDL. Since LDL selectively suppressed the PI response and other early activation-associated events as well as DNA synthesis, these early events appear necessary for lymphocyte proliferation. Perhaps LDL suppress the cellular response to the primary mitogenic signal in such a way that, even though the secondary signals are available, DNA synthesis and proliferation cannot occur.

Is the PI response in activated lymphocytes the same as that in other activated cell types where cellular messengers bind cell surface receptors? In many systems proliferation is not required for functional activation. However, proliferation of lymphocytes is necessary for effective response of the immune system. Many questions need to be answered before the relationship between this early biochemical event and later DNA synthesis and proliferation is understood. Which PI metabolites are essential for signal transmission? Our work indicates that PI rather than PIP, PIPP, or PA is the important metabolite in lymphocyte activation. We cannot, however, exclude inositol phosphate or DG as essential. Is an early PI response obligatory for lymphocyte activation and subsequent proliferation? Or, is the PI response a parallel but nonessential event of activation? The ability of NAGO to stimulate and LDL to suppress both the PI response and DNA synthesis indicates that the PI response is important for lymphocyte activation and proliferation.

Stimulation of early PI response and DNA synthesis of human T lymphocytes by the oxidative mitogens, NAGO, provides a useful system for elucidation of the relationship between these events. The suppression of these events by lipoproteins allows closer study of the molecular and biochemical basis of lipoprotein-cell and cell-cell interactions. Using this system, we have begun to characterize the role of plasma lipoproteins in the regulation of T lymphocyte proliferation.

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