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Immunology

Phospholipid methylation and phospholipase A_2 activation in cytotoxicity by human natural killer cells

(3-deazaadenosine/large granular lymphocytes/antibody-dependent cell-mediated cytotoxicity)

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ABSTRACT **The role of phospholipid methylation and phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) in natural killer (NK) function by human peripheral blood mononuclear cells was studied. Pretreatment of effector cells with a methyltransferase inhibitor, 3-deazaadenosine, in the presence of homocysteine thiolactone, reduced cytotoxicity in a dose-dependent fashion. This effect was closely associated with inhibition of methylation of lipids but not of nucleic acids or proteins. The suggestion for a role of phospholipid methylation was supported by the observation that the interaction between NK-susceptible tumor targets and peripheral blood mononuclear cells caused increased phospholipid methylation only when susceptible target cells were used. Phospholipase A_2 was also implicated in human NK activity. Inhibitors of the enzyme such as tetracaine, mepacrine, Rosenthal's inhibitor, and corticosteroids impaired NK function. Rosenthal's inhibitor was also shown to exert an inhibitory effect on a purified NK-cell population obtained by the isolation of large granular lymphocytes on Percoll gradients. Peripheral blood mononuclear cells were also directly shown to display phospholipase A_2-like activity, as measured by the decrease in radioactive arachidonate from prelabeled phospholipids, specifically phosphatidylcholine, in effector cells. These data suggest that enhanced phospholipid methylation occurs during the recognition function of NK cells. Consequent activation of phospholipase A_2 might be involved in the mechanisms leading to lytic events within the target cell.**

Transmethylation, the donation of methyl groups to species of RNA, DNA, proteins, carbohydrates, or lipids is necessary for a wide variety of important cellular functions. Recently, two methyl transferases located on the cell membrane have been identified that participate in the synthesis of phosphatidylcholine from phosphatidylethanolamine, with attendant influences on membrane fluidity and receptor functions (1–3). In the immune system, chemotaxis (4–6), histamine release by mast cells and basophils (7, 8), mitogen stimulation (9), and cell-mediated lympholysis (10) are among the actions affected by this pathway.

The metabolism of glycerophospholipids through the action of phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4), with the resulting liberation of the corresponding lyso- compounds and free fatty acids, has also been implicated in regulatory and effector function in cells of the immune system (4, 9–11). Particular interest has focused on the accumulation of lysolecithin, a detergent and fusogen, in tumors, which could potentially mediate their lysis.

Natural killer (NK) cells are a subpopulation of lymphoid cells, present in most normal individuals, that have substantial cytotoxic activity against a variety of tumor target cells (12). Little clear information exists as to how these effector cells recognize susceptible targets or mediate their destruction. These considerations prompted the present study of a possible role for synthesis of phospholipids via the transmethylation pathway and of their catabolism by phospholipase A_2 in human NK function.

We now report the stimulation of phospholipid methylation and phospholipase A_2 activity during the interaction between NK cells and susceptible targets.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear (PBMN) cells were isolated from heparinized venous blood by Ficoll/Hypaque density gradient separation (13). In experiments in which phospholipid metabolism was measured, three to six washes of cells harvested from the interface were done to achieve fewer than two platelets per mononuclear cell. Large granular lymphocytes (LGL) were isolated by Percoll density gradient separation as described (14).

Long-term cell lines were grown in suspension culture in medium containing fetal calf serum. Human cells used included K562, derived from a patient having chronic myelogenous leukemia, and F265, derived from Epstein–Barr virus-transformed B cells. MBL-2 and RL♂1 represent cultures of murine lymphomas. Cell-line cells were washed three times before use, and any dead cells were removed by density gradient centrifugation to ensure viability >95% in all assays.

Cytotoxicity Assays. NK activity was measured by a ^{51}Cr release assay using labeled K562 cells as described (15). Antibody-dependent cell cytotoxicity (ADCC) was measured by a similar technique using RL♂1 cells that had been sensitized with a 1:5000 dilution of rabbit anti-mouse brain antiserum.

Assay of Phospholipid Methylation. Phospholipid methylation was assayed by using intact PBMN cells and measuring incorporation of L-[*methyl*-^{3}H]methionine into the phospholipid fraction. Cells ($0.5–1 \times 10^6$) were incubated in a total volume of 0.5 ml with 20 μCi of L-[*methyl*-^{3}H]methionine (2 μCi/nmol; 1 Ci = 3.7×10^{10} becquerels). After incubation for 30 min, the cells were washed twice and suspended in Dulbecco's modified Eagle's medium/1 mM unlabeled methionine. The reaction was stopped by the addition of 0.5 ml of 10% (wt/vol) trichloroacetic acid/10 mM methionine. After centrifugation at $27,000 \times g$ for 15 min and subsequent wash of the precipitate, the pellets were extracted with 3 ml of chloroform/methanol, 2:1 (vol/vol). The chloroform/methanol extract was washed twice with methanol/0.1 M KCl, 1:1 (vol/vol), and methylated lipids were identified by thin-layer chromatography on silica gel

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Abbreviations: NK, natural killer; PBMN, peripheral blood mononuclear (cells); LGL, large granular lymphocytes; ADCC, antibody-dependent cell-mediated cytotoxicity; DZAdo, 3-deazaadenosine.

G, using a solvent system of chloroform/propionic acid/*n*-propyl alcohol/water, 2:2:3:1 (vol/vol) (2).

In experiments in which methylation of protein and nucleotides was measured, the residues after chloroform/methanol extraction were obtained by filtration on Whatman 3-mm filter paper and dissolved in 1.0 ml (total volume) of $NaDodSO_4$. One-half milliliter of this solution was extracted with 2 ml of 80% phenol. Nucleotides were precipitated by addition of 2 ml of ethanol to 1 ml of phenol extract. The protein fraction was precipitated by addition of 3 ml of 10% trichloroacetic acid to 0.1 ml of $NaDodSO_4$ solution.

To measure phospholipase A_2 activity, 20×10^6 PBMN or tumor cell-line cells were labeled in 4 ml of medium with 3 μCi of [1-^{14}C]arachidonic acid (55 μCi/μmol). After incubation at 37°C for 30 min, the cells were washed three times with fresh medium and suspended to 1×10^6/ml in medium containing unlabeled arachidonate (10 μg/ml). After the addition of an equal number of unlabeled cells and centrifugation (1000 rpm, 5 min), the reaction was initiated by placing the tubes in a 37°C water bath. At the end of the incubation, 0.5-ml aliquots of supernatant were assayed for radioactivity. The cell pellet was solubilized with 1% $NaDodSO_4$, and radioactivity incorporated into lipids was measured after chloroform/methanol extraction and thin-layer chromatography with chloroform/methanol/water, 65:25:4, (vol/vol). The plates were scanned for radioactivity with a thin layer chromatography scanner (Bioscan).

Chemicals. Dulbecco's modified Eagle's medium was purchased from Microbiological Associates (Walkersville, MD). Fetal calf serum was purchased from Biofluids (Rockville, MD). L-[*Methyl*-^{3}H]methionine was obtained from New England Nuclear. [1-^{14}C]Arachidonic acid was a product of Amersham/Searle. Deazaadenosine was obtained from Southern Research (Birmingham, AL). Homocysteine thiolactone, hydrocortisone, prednisolone, estradiol, tetracaine, mepacrine (quinacrine), and Rosenthal's inhibitor [D,L-(2,3-distearoyloxy)propyldimethyl-2-hydroxy)ethylammonium acetate] were purchased from Sigma.

RESULTS

Effect of Inhibitors of Phospholipid Methylation on NK Activity. Pretreatment of PBMN cells with 3-deazaadenosine (DZAdo), an inhibitor of transmethylation reactions (16), in the presence of 100 μM homocysteine thiolactone resulted in impaired cytotoxicity against ^{51}Cr-labeled K562 target cells in a dose-dependent manner (Fig. 1). Inhibition of cytotoxicity was observed for 100–10 μM DZAdo, with 50% inhibition at 20 μM. When drug was present throughout the course of the 4-hr Cr-release assay, slightly greater inhibition was observed. DZAdo or homocysteine thiolactone alone at the highest concentration tested (100 μM) inhibited cytotoxicity slightly (30% and 10%, respectively) when present throughout the experiment, while pretreatment had no effect. Cell number and viability were unaffected by pretreatment with either drug, alone or combined.

The ADCC of RL♂1 cells, measured simultaneously with NK activity, was less susceptible to inhibition by the same agents. Pretreatment with 100 μM DZAdo resulted in 50–60% inhibition of lysis (Fig. 1), whereas NK-activity inhibition was > 90%. No significant inhibition of ADCC was seen for DZAdo at 10 μM or less.

Adenosine and its analogs and homocysteine thiolactone effectively inhibit a variety of methylation reactions, including those involved in phospholipid metabolism, by causing the accumulation of *S*-adenosylhomocysteine. Previous studies (17) have shown that the treatment of various cells with these drugs produces greater inhibition of phospholipid methylation as compared with other methylation reactions such as that of nucleotides or protein. To verify that impairment of phospholipid

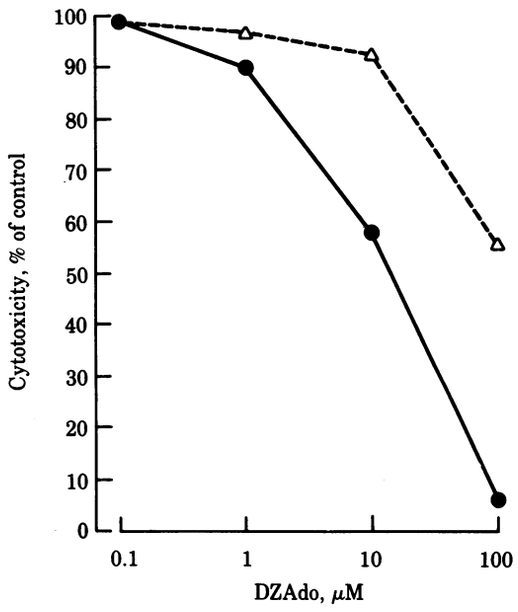

FIG. 1. Inhibition of NK cytotoxicity by DZAdo. PBMN cells (5×10^6) were incubated for 60 min at 37°C with drug as indicated plus 100 μM homocysteine thiolactone. After three washes with medium, effectors were incubated with ^{51}Cr-labeled K562 (●) or RL♂1 (△) cells plus antibody at an effector/target ratio of 100:1. Cytotoxicity (percent ^{51}Cr release) in the absence of drug was 43.6% for NK and 28.7% for ADCC. Data are representative of four experiments.

methylation mainly accounted for the decrease in NK activity, direct measurement of methylation of proteins and nucleic acids, as well as lipids, was undertaken. The incorporation of tritiated methionine (a precursor of the methyl donor S-adenosylmethionine) into these three fractions of whole PBMN cells in the presence or absence of DZAdo (100 μM) or homocysteine thiolactone (100 μM) in combination was measured (Fig. 2). Such treatment resulted in 10% inhibition of protein methylation, 50% inhibition of lipid methylation, and negligible inhibition of nucleotide methylation.

Association Between Increased Phospholipid Methylation and NK Activity. To further establish the role of phospholipid methylation in NK activity, changes in phospholipid methyla-

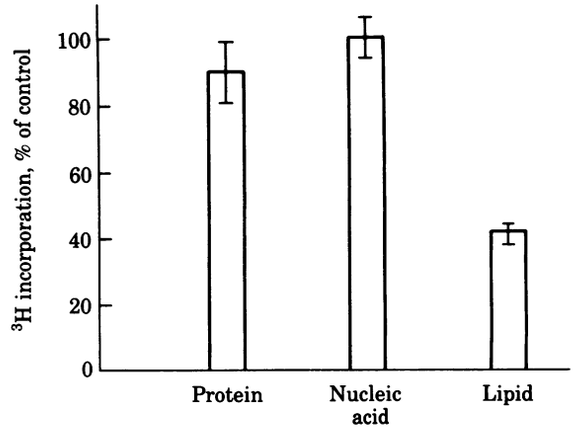

FIG. 2. Inhibition of methylation by DZAdo (100 μM) and homocysteine thiolactone (100 μM). Methylation was measured in 1×10^6 PBMN cells incubated for 60 min with 20 μCi [^{3}H]methionine. Lipid, protein, and nucleotide fractions were isolated as described in *Materials and Methods*. Total incorporation in untreated controls was 2000, 900, and 600 cpm for *H* lipid, protein, and nucleic acid, respectively. Data are mean and SD of two experiments.

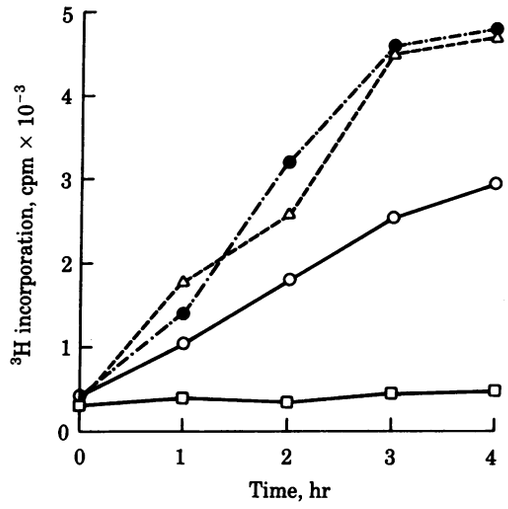

FIG. 3. Increase of phospholipid methylation on coincubation of PBMN cells and tumor cells. [^{3}H]Methionine (20 μCi) and 1×10^4 tumor cells were added simultaneously to 1×10^6 PBMN cells. Incorporation of radioactivity into phospholipids was measured for either cell line alone (□), PBMN cells (○), PBMN cells plus K562 cells (●), and PBMN cells plus F265 cells (△). Data describe one representative experiment of three performed with this protocol.

tion were examined directly. Peripheral blood cells were incubated with [^{3}H]methionine, with or without the addition of target cells. As tumor cell lines have high rates of phospholipid methylation, small numbers were added to an excess of PBMN cells at the highest ratio of effector cells to targets used in cytotoxic assays (100:1).

The incorporation of radioactivity into PBMN cell phospholipids on incubation with two tumor cell lines, K562, exquisitely sensitive to NK activity, or F265, less sensitive to NK activity, is shown in Fig. 3. Both cell lines stimulated phospholipid methylation compared with that of peripheral blood cells alone (in a more than additive fashion), with demonstrable increases apparent as early as 30 min after the admixture of effector and target. Despite the differences in susceptibility to cytotoxicity, the increase of phospholipid methylation proceeded over a similar time course for the two lines. When two cell lines derived from mouse lymphomas, shown to be not susceptible to NK activity by standard Cr-release assays, were tested in the same

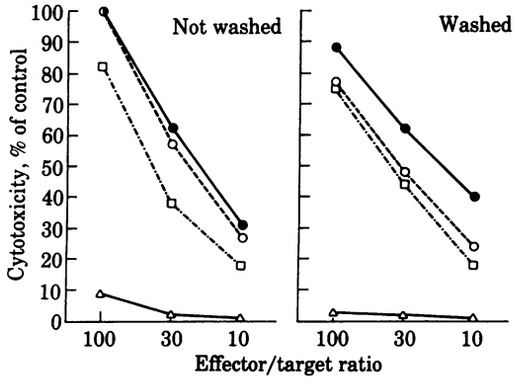

FIG. 4. Inhibition of NK cytotoxicity by tetracaine. Treatment of PBMN cell effectors was performed at the indicated concentrations for 60 min at 37°C. Concentrations of cells were adjusted to the effector/target ratios indicated either before or after three further washes. Drug doses were 0 mM (●), 1 mM (△), 100 μM (□), and 10 μM (○). Untreated control cytotoxicities were 87.3%, 57.2%, and 34.6%. Data are representative of two separate experiments.

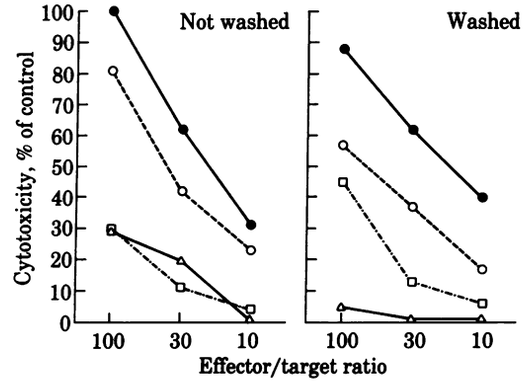

FIG. 5. Inhibition of NK cytotoxicity by mepacrine (quinacrine). The protocol is similar to that described in the legend to Fig. 4.

way, no apparent increase in phospholipid methylation was observed (data not shown).

Role of Phospholipase A_2 in NK Activity. As phospholipid methylation appears to play a role in NK activity, other pathways of phospholipid metabolism were also examined. PBMN cells were treated with tetracaine, a local anesthetic known to inhibit phospholipase A_2 activity (18). The drug (1 mM) strongly inhibited (>90%) cytotoxic function at all three effector/target ratios tested (Fig. 4). At 100 μM, but not at 10 μM, partial inhibition was observed that was slightly greater when the drug was present throughout the reaction.

Similar data were obtained when effectors were treated with mepacrine, another phospholipase A_2 inhibitor (19) (Fig. 5). Inhibition of cytotoxicity was dose dependent and was not readily reversible.

Either drug at the highest concentration tested showed no effect on spontaneous ^{51}Cr release and caused no loss of viability or decrease in cell number.

Rosenthal's inhibitor was used as another type of inhibitor of phospholipase A_2 activity, as previous studies had shown its ability to inhibit ADCC (20). In experiments using partially purified LGL, highly enriched in NK activity, obtained by Percoll density gradient centrifugation, Rosenthal's inhibitor was found to inhibit cytotoxicity (Table 1).

Corticosteroids are also known to inhibit phospholipase A_2 activity by inducing the synthesis of an inhibitor of the enzyme (21). However, because of the long duration of action of the steroid and its rapid reversibility, longer pretreatment and persistence in the assay were necessary. Hydrocortisone (10 μM) and prednisolone (1 μM) inhibited NK activity >90% (Table 2). At lower doses, drug treatment did not inhibit NK activity but treatment with estradiol and, paradoxically, prednisolone caused slight increases in cytotoxicity.

Increased Phospholipase A_2 Activity After Incubation of Peripheral Blood Cells with Tumor Cells. As experiments using inhibitors of phospholipase A_2 suggested a role for this enzyme in natural killing, an attempt was made to determine whether phospholipase A_2 activity was manifest during the interaction

Table 1. Effect of treatment of LGL with Rosenthal's inhibitor on NK activity

	Cytotoxicity*	Lytic units†/ 10^6
Cells + medium	59.5	509
Cells + inhibitor at 1 mM	−3.5	<1
Cells + inhibitor at 0.1 mM	46.8	207

* As percent of ^{51}Cr release at an effector/target ratio of 100:1.
† For 20% ^{51}Cr release.

Table 2. Effect of corticosteroids on NK activity

Treatment	Cytotoxicity*	Lytic units†/ 10^6
Medium	55.8	250
Hydrocortisone		
10 μM	15.2	15
1 μM	53.3	221
0.1 μM	50.7	310
Prednisolone		
10 μM	16.2	9
1 μM	35.9	211
0.1 μM	66.0	527
Estradiol		
10 μM	36.2	223
1 μM	57.6	275
0.1 μM	71.8	396

* As percent of ^{51}Cr release at an effector/target ratio of 100:1.
† For 20% ^{51}Cr release.

between effector cells and targets. Cells were labeled with [1-^{14}C]arachidonic acid, which is incorporated principally into position 2 of phospholipids. Under the conditions used, 80% of arachidonate incorporated into PBMN cells could be extracted with chloroform/methanol. The identity of the arachidonic acid-containing phospholipids was examined by thin-layer chromatography. The arachidonic acid was incorporated principally into phosphatidylcholine (55–65%) and an unidentified nonpolar fraction (25–33%). A small proportion was found in phosphatidylethanolanine and its methylated forms, but little or none was found in phosphatidylinositol.

When PBMN cells prelabeled with [^{14}C]arachidonate were incubated with unlabeled autologous cells or tumor cell lines, less label remained incorporated in the phospholipid fraction when NK-susceptible cells (K562) were added, compared with the addition of cells from a nonsusceptible tumor line (RL♂1) (Fig. 6). This effect was perceptible at 30 min and apparent throughout the incubation period, resulting in 30% less radioactivity remaining in the phospholipid fraction at 2 hr. By using a similar experimental protocol to that illustrated in Fig. 6, 50% and 70% decreases in retained radioactivity at 1 and 2 hr, respectively, were observed in additional experiments with

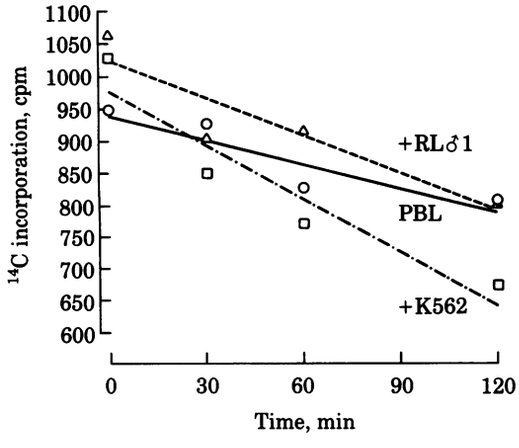

FIG. 6. Phospholipase A_2-like activity in PBMN cells incubated with tumor cell lines. PBMN effector cells (1×10^6) prelabled with arachidonate were incubated at 37°C alone (○) or with K562 at 0.6×10^6 (□) or RL♂1 at 0.3×10^6 (△) in 1 ml of serum-free medium. Cell pellets were extracted with chloroform/methanol, and radioactivity in the phospholipid fraction was assayed. Data represent the mean of duplicate determinations ± 5%.

PBMN cells. Identical results were also obtained in experiments using LGL in place of PBMN cells. When the phospholipids of either tumor cell line were labeled with [^{14}C]-arachidonate and then incubated with PBMN cells, incorporated radioactivity remained unchanged in each experiment (data not shown). When phospholipids from peripheral blood effector cells were analyzed by thin-layer chromatography, the decrease in radioactively labeled arachidonic acid was found to occur solely in the phosphatidylcholine fraction (Table 3). As in experiments in which total radioactivity was measured, incubation of effector cells with K562, but not RL♂1, cells led to decreased incorporation of label into phosphatidylcholine but no change in other fractions.

DISCUSSION

This study demonstrates a role for phospholipid metabolism in cytotoxicity by human NK cells. By the use of specific enzyme inhibitors, as well as direct measurement of the reactions involved, both transmethylation and phospholipase A_2 were identified as components of the lytic interaction between NK cells and target cells.

Although phosphatidylcholine is synthesized in the cell mainly by the cytidine diphosphatidylcholine pathway, the methyl transferases in the cell membrane appear to play an important role in membrane fluidity and receptor function. Inhibition of methyltransferase reactions by DZAdo, at concentrations that inhibit phospholipid methylation but not nucleic acid or protein methylation, resulted in loss of NK activity. Furthermore, phospholipase A_2, which acts on phospholipids arising from the transmethylation pathway, was shown to be involved in NK activity; inhibition of the enzyme in effector phospholipid cell populations blocked NK activity. Inhibition of cytotoxicity observed when purified LGL were treated with a phospholipase A_2 inhibitor (Rosenthal's) suggests that these events are important for the function of NK cells themselves, rather than for accessory cells present in PBMN cell preparations.

Alterations in phospholipid methylation have been shown to affect coupling of receptors to adenylate cyclase (4), Ca^{2+}-dependent ATPase activity (22), and unmasking of cryptic β-adrenergic receptors (23), mainly as a consequence of the change in membrane fluidity due to accumulation of phosphatidyl-*N*-monomethylethanolamine. The demonstration of increased phospholipid methylation on incubation of PBMN cells with susceptible cell-line targets supports the hypothesis that phospholipid methylation is associated with a recognition process or signal mediated by receptors on NK cells. The relatively minor effect of phospholipid methylation inhibition on ADCC indicates that IgG Fc-receptor-mediated recognition events are not as strongly coupled to the transmethylating enzymes. Alternatively, antibody–target interaction leads directly to lysis, bypassing methylation-associated pathways.

Phospholipase A_2 activity has been suggested to cause the lysis of cells in a number of systems, based principally on the possibility that generation of the detergent-like compound lysophosphatidylcholine could have a destructive effect on membranes. The previous evidence for this is the effect of Rosenthal's inhibitor on antibody-dependent lysis of chicken erythrocytes by mouse splenocytes (20), a cytotoxic system known to be mediated by macrophages (24). Inhibition of NK activity was seen with three inhibitors of phospholipase A_2 (quinacrine, tetracaine, and Rosenthal's inhibitor), as well as with glucocorticoids, which increase the production of a normally occuring inhibitor of phospholipase A_2, lipomodulin (21, 22).

The decreased NK activity observed with each type of inhibitor might be explained by actions other than on phospho-

Table 3. Selective loss of arachidonic acid from phosphatidylcholine after interaction between PBMN and NK-susceptible cells

	Medium		K562		RL♂1	
	0 min	90 min	0 min	90 min	0 min	90 min
Phosphatidylcholine	5400	4600 (−15)	4750	3610 (−24)	4480	4270 (−5)
Phosphatidylethanolamine	635	580 (−9)	570	575 (1)	570	630 (10)
Nonpolar fraction	2300	2180 (−5)	2450	2490 (2)	2150	2030 (−6)

PBMN cells (50×10^6) were labeled with 6 μCi of [^{14}C]arachidonic acid for 45 min at 37°C and then washed twice. Aliquots (2.5×10^6) were incubated with medium alone or with K562 or RL♂1 cells at an effector:target ratio of 2:1 for 30 min and analyzed as described in *Materials and Methods*. Numbers in parentheses represent percent change.

lipase A_2. However, the common action of this group of compounds with diverse chemical structures is inhibition of both phospholipase A_2 and cytotoxicity.

Activation of a phospholipase A_2-like activity in PBMN cells was demonstrated by directly measuring the amount of arachidonate remaining in effector cell phospholipids in the presence of NK-susceptible target cells. The decreased incorporation of arachidonic acid was shown to occur from the phosphatidylcholine fraction of the effector cell phospholipids. In some experiments, not reported in this study, this effect was accompanied by release of arachidonate but, in others, arachidonate released into the supernatant was less in the presence of tumor, despite evidence of phospholipase A_2 activity. Free arachidonate liberated from phosphatidylcholine apparently remained in the effector cell or may have entered other metabolic pathways. Products of phospholipase A_2 or their metabolites might eventually result in cytotoxicity, but elucidation of the multiple possibilities awaits direct measurement.

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