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Biochemical Analysis of Initial Triggering Events of Mediator Release from Activated Mast Cells

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Introduction

Similarities in the response of guinea pig and human airways to a variety of contractile and relaxant substances[1], and the putative involvement of similar primary mediators(histamine and leukotrienes)in antigen-induced pulmonary smooth muscle contraction in the two species[2-6]have contributed to the long time use of the sensitized guinea pig as a model of allergic bronchoconstriction.

While the role of IgG_1 antibody in immediate hypersensitivity reactions in the guinea pig has been extentively studied, the characterization of a similar role for IgE antibody has been less well investigated[6-11]. In 1984, the pulmonary smooth muscle contractile activity mediated by IgE antibody was studied and compared to the activity of IgG_1 in the same organ. These results obtained that the concept of separate receptors for IgG_1 and IgE in pulmonary tissue and stimulation of each antibody coated receptor leads to smooth muscle contraction[1,12-13]. Human and guinea pig pulmonary mast cell have been isolated from each lung tissue[14-16], and it has been found that isolated mast cells can release significant amounts of mediators on stimulation with an-

Abbrebiations used in this paper : AA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PLD, phospholipase D; DAG, diacylglycerol; MT, methyltansferase; Hist, histamine; LT, peptidoleukotrienes; PAPase, phosphatidic acid phosphohydrolase; PLC, phospholipase C; PLA_2, phospholipase A_2; AdoMet, S-adenosylmethionine; DZA, 3-deazaadenosine; Asc, Ascaris; Ox, Oxazolone; PlA, palmitic acid; HSA, human serum albumin.

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tigen and anti-IgE antibody at all stages of the purification procedure[6-17]. This suggests that a secondary cell is not necessary for the release of these mediators. We first demonstrated substantial differences in measurable Hist and LT released during similar levels of specific antigen-induced contraction in guinea pig pulmonary tissues after passively sensitization of the animal with IgG_1 versus IgE antibody. These studies suggested that the substantial differences might be related to the possibility of differential regulation of release by activation of each antibody receptor[18,19]. We also observed substantial differences in measurable Hist and LT released by isolated mast cell activation with IgG_1 versus IgE antibody (preliminary experiment).

Inflammatory mediator release (Hist and LT)form mast cells and basophils is generally initiated when antigen binds and cross-links to IgE occupying high affinity FcεRI receptors on the surface of these cells[20]. Subsequent activation of complex intracellular biochemical process ultimately results in the release of two categories of mediators : preformed mediators, such as Hist and serotonin, and newly formed mediators such as LT and prostaglandin that are synthesized de novo as a result of cell activation. It has been suggested that the signal transduction pathways leading to the release of both categories of mediators may be similar, at least in the initial stages.

Current evidence indicates that events linking cross-bridging of IgE receptor to degranulation of mast cells and basophils are mediated via G proteins[21-24] in the initiation of a number of specific signal-transduction events, including PLC-catalysed PI hydrolysis[25], PLD-dependent PC hydrolysis[26-29], the elevation of intracellular free Ca^{++} levels[30-31], the activation of src-related tyrosine kinases[32-33], and an increase in intracellular DAG levels[28-29,34] with resulting activation of protein kinase C[29,35-37]. However, recent evidence suggests an important role for PLD in IgE-dependent degranulation of mast cells[27]. This enzyme acts at the terminal phosphodiester groups of phospholipids, primarily PC, to yield PA. The PA produced by the action of PLD can be subsequently dephosphorylated by the action of PAPase to form DAG. This increase in cellular DAG results in the activation of protein Kinase C and ultimately exocytosis[34]. Free AA can also be liberated from AA-containing DAG by the action of DAG lipase. It has recently been reported that PLD-dependent PC hydrolysis in the signal transduction cascade leading to the production of DAG following IgG_1-dependent degranulation of guinea pig lung mast cells plays an important role[38].

It has also been proposed that the increased N-methylation of PE is an important part of the transduction mechanism linking IgE-receptor activation to mast cell and basophil release of preformed and newly generated mediators[39-40]. It has been demonstrated that in rat and mouse peritoneal mast cells[41-43], rat basophil leukemia cells[44] and human lung mast cells[45], the perturbation of IgE-receptors is rapidly followed by increased transfer of

radiolabel from [^{3}H]-methyl-AdoMet into cell lipids, and ultimately precedes mediator secretion. Studies with methylation inhibitors such as DZA and homocysteine might support an important role of PE methylation in IgE-dependent secretion as these compounds inhibit IgE-dependent increases in PE methylation, c-AMP and Ca^{++} accumulation and mediator secretion.

The aim of this study was therefore to examine whether substantial differences of mediator release from lung mast cell activation after passively sensitization with IgG_1 vs IgE antibody are from the differences of PLD activity in the signal transduction cascade by the production fo DAG or from the differences of methyltransferase activity catalyzing phospholipid methylation.

Material and Method

Preparation of hapten-protein conjugates

Preparation of hapten-protein conjugates was performed as detailed previously[19,46]. Briefly, in the preparation of the hapten-protein conjugate Ox-Asc, 10ml of an Asc protein extract(10mg/ml)was adjusted to pH 9.0 at 25℃ by addition of 5% Na_2CO_3. Then, 0.5 ml of 10 % Ox in dioxane was added dropwise, whereas the pH was maintained at 9.0 by addition of 5 % Na_2CO_3. After 2hr of stirring, the mixture was dialyzed extensively against PBS, pH 7.4. The Ox-Asc conjugate protein was concentrated and determined. Ox was conjugated to HSA and GPA in a similar manner to that described above. Ox-Asc and Ox-HSA were used for the production fo IgE and IgG_1 anti-Ox antibody, respectively(see below). The hapten-protein conjugate Ox-GPA was used as antigen to detect anti-Ox Ab responses.

Immunization procedure

Serum rich in IgE antibody to the hapten-protein conjugate, Ox-Asc, was obtained with techniques described previously[46]. Briefly, ten outbred female Hartley albino guinea pigs, weighing approximately 300g, received i.p.injections of 250mg/kg of cyclophosphamide 2 days before primary i.p. immunization with 1μg of conjugate(Ox-Asc) adsorbed to lmg of Al $(OH)_3$(Alum) in lml of similar dose of antigen in alum was administered i.p. At the end of 5 months, the animals were exanguinated. The sera obtained from the bleedings were stored in aliquots at −90℃ until time of use.

Serum rich in IgG_1 antibody to Ox−HSA was obtained with the immune-deviation procedure as described previously[19,46] Briefly, ten guinea pig were administered with 10μg of the antigen in alum (1 mg) in a primary i.p. immunization. Two weeks later, 50 μg of antigen emulsified within complete Freund's adjuvant was injected into the four footpads with care not to cause excessive pain of reaction. Every 2 weeks for 2 months thereafter, 50μg of antigen in incomplete Freund's adjuvant was injected subcutaneously in multiple sites on the animals.

On seven days after the last injection, ani-

mals were exanguinated. Serum obtained from the bleedings were stored at - 90℃ until time of use.

Affinity column chromatography IgG_1

Serum rich in IgG_1 or IgE antibody was separated by passage through a column of protein A - Sepharose CL - 4B with a modification according to the method of Martin[47]

Quantitation of guinea pig IgG_1 and IgE

A quantitative estimation of IgG_1 and IgE anti - Ox antibody in guinea pig serum was obtained with PCA. PCA antibody titrations were performed as described by Ovary[48]. We have also previously demonstrated by ELISA that $<$20 ng/ml of IgG_1 antibody remains in affinity column - adsorbed IgE rich serum.

Guinea pig lung mast cell preparation

Guinea pig lung mast cells were isolated and purified using techniques similar to the method previously reported[49]. Briefly described here, lungs obtained from 8 to 10 unsensitized guinea pigs were each perfused with 50ml of the modified Tyrode buffer (TGCM) consisting of(mollimolar):NaCl, 137;NaH_2PO_4, 0.36;KCl, 2.6;$CaCl_2$, 1;$MgCl_2$, 0.5;$NaHCO_3$,119; glucose, 5.5;gelatin, 1g/L, pH 7.4. After removing large airways and blood vessels, the lungs were minced with a McIlwain tissue chopper. Pooled tissue was treated three times with 125 U/g tissue and 5U/g tissue of collagenase and elastase, respecively.

Times(min) of each consecutive exposure of lung fragments to the enzymes were separated from residual tissue by filtration through mesh and Nytex mesh(100μm). Cell suspensions(total of 4.0×10^8)obtained after the enzyme digestion were layered over gradients consisting of 10ml of roagh Percoll(density, 1.045g/ml), and centrifuged at 1400 rpm for 20min.

Pelleted cells(containing mast cells) were resuspended in modified Tyrode buffer and fractionated by count current elutriation[6]. Cell fractionation was accomplished by decreasing rotor speed. At each rotor speed, 90ml of cell suspension were collected and designated by number.

Fraction 6 and 7 were used alone for further purification utilizing a discontinuous Percoll density gradient(consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10g/ml). This gradient was centrifuged at 1400 rpm for 20min (3.5×10^7 cells/gradient). The cell band obtained between the 1.09 and 1.10 g/ml densities contained the highest purity and number ($1.0-2.0\times10^7$) of mast cell. This gradient band was removed, washed with modified Tyrode buffer and designated the "highly purity" mast cell preparation. Mast cell counts were obtained using alcian blue staining and cell viability was determined using trypan blue exclusion. Cell viability was consistently greater than 98%.

Prelabelling mast cells

In order to label mast cell phospholipid, puri-

fied cells were prelabeled with [^{3}H]-PlA. Purified cells (10 to 20×10^6) were susperded in a final volumn of 1 ml TGCM and [^{3}H]-PlA(at final concentration of 3.3 μ-M;200 μ Ci/ml), and incubated at 37℃ for 1hr. Cells were washed twice and resuspended in TGCM before use in cell activation.

Sensitization, stimulation, and lipid extraction in mast cells

Prelabeled cells(0.75 to 1.25x10^6)were sensitized by IgG_1 or IgE antibody(anti-Ox antibody, 1ml antibody/10^6 cells) at 37℃ for 45min, and washed and resuspended in TGCM. Sensitized cells were stimulated at 37 ℃ for 10min by each specific antigen(0.1μg/10 μg Ox-antigen and added PS(15 μg/ml) or PS alone in a final 200 μl volume in 5ml polypropylene tubes. Ethanol(0.5% v/v) was added at various time before stimulation. Reactions were stopped by adding 2ml cold TGCM and centrifuged for 10min at 1400rpm. [Cellular lipids 2ml cold TGCM and centrifuged for 10min at 1400rpm.] Cellular lipids were extracted from the cell pellet by using a modification[27]of the Bligh and Dyer procedure[50].

Separation of phospholipids by TLC

A double one-dimensional TLC(precoated silica gel G plates, 10x20cm) was used to separate phospholipids of interest from extracted lipids and from neutral lipids. A series of samples were spotted 12cm from the bottom of the plate and developed in hexane/ether/acetic acid(30:70:1) to 20cm in order to resolve labeled neutral lipids from phospholipids that remained at the origin. Plates were then at 0.8cm above the origin, rotated 180 ° and developed to the top with chloroform/methanol/ammonium hydoxide(65:35:5). After they had dried, plates were sprayed with En^{3}Hance, and autoradiography perfomed by using Kodak XAR film for 1 week. Radioactive bands were removed from the silica plates by gentle scraping, placed and counted in scintillation vials containing 5ml of scintillation cocktail. The identities of labeled band on Rf values obtained for authentic neutral lipids and phospholipids visualized by iodine staining.

Preparation of synthetic standards

Authentic [^{32}P]-PEt were synthetized by using [^{32}P]-PC obtained from mast cells by prolonged prelabeling by suing ^{32}Pi. Labeled PEt was then formed in vitro by PLD-mediated transphospatidylation of labeled PC in the presence of ethanol.

Lipid methylation

Purified mast cells were incubated at 37 ℃ for 25min with L-[^{3}H-methyl] methionine (4 μM, 12 Ci/mmol). Duplicate aliquots of 45 μl of radiolabeled cells were transferred to 5ml polypropylene tubes, and sensitized with each antibody(IgG_1 or IgE). Sensitized cells were washed twice and resuspended with TGCM. The cells were challenged with Ox-antigen, reactions werre quenched at various times by addition of 900 μl of ice-cold TCA

(10 % w/v) containing 10 mM methionime. After centrifugation, lipid extracts were extracted for 10min with 3ml of chloroform/methanol(2/1, v/v)[51]. The lower chloroform phase(1ml) was removed to glass vials and evaporated(3hr, 80℃) before addition of scintillation cocktail. The radioactivity was measured.

TLC of methylated lipids

The methylated lipid extracts prepared as described above. Aliquots(1 ml) of extract were evaporated under N_2 an the residue redissolved in 50 μl of chloroform/methanol(2/1) containing 5μg of authentic stantards of PE, PC, lyso-PC, and neutral lipid(mixed fatty acid) were chromatographed on LK5DF silica gel plates by using a solvent of n-propanol/propionic acid/chloroform/water(3/2/2/1. v/v.). Lipid spots were identified by iodine staining and corresponding with Rf values. Each spot was transferred to scintillation vials and counted.

Mediator release from mast cell

Purified mast cells($0.4x10^6$)were passively sensitized with each anti-Ox antibody(IgG_1 or IgE) in a shaking water bath(60min at 37℃) . After this incubation period, the cells were washed resuspended in TGCM and challenged with Ox-antigen(0.1 and 1.0μg/ml) at 37℃ for 15min. The reaction was terminated by placing the tubes in an ice bath. Supermatants obtained after centrifugation were taken for determination of histamine and LT[19].

Histamine assay

Histamine was analyzed by the automated fluorometric method(with dialyzer) described by Siraganian[52]. Total histamine content of each cell suspension was determined after treating cell pellets with 0.4% Triton X-100. Histamine release was expressed as a percentage of the total histamine content and corrected for spontaneous release, which ranged from 2 to 4% as determined in paired samples.

Pepitidoleukotriene(LT) radioimmunoassay

Aliquots of 100μl of cell supenatants were taken for the determination of LT release. The LT antibody(#332) was diluted in buffered saline(5 mM MES, HEPES adjusted to pH 7.4 with 1 N NaOH) containing 0.1% gelatin. Each assay tube contained 100 μl of supernatant, antibody(50μl of a 1 : 1000 dilution), and 50μl of ^3H-LTD_4(2500 to 3000 cpm)in buffered saline. Incubations were for 2 hr at 4℃ and the reaction was terminated by addition of 0.5ml dextran coated charcoal (200mg charcoal and 20mg dextran mixed with 100ml buffered saline). After a 5min incubaton the mixture was centrifuged at 3000rpm at 4℃ and 0.4ml of the supernatant was added to Aquasol (NEN Research Products) for counting by liquid scintillation spectrometry(Packard, Model 3225). Standard curves were constucted in the presence of an-

tigen using LTD_4.

The detection limit of the assay is 0.045pmol LTD_4[53]. LT release is expressed as pmol/ml

Materials

The following substances were used: Oxazolone, human serum albumin, guinea pig albumin, complete Freund's Adjuvant, histamine free base, collagenase(type I), elastase (type I, porcine pancreatic), alcian blue, trypan blue, DZA(Sigma Chemical Co., Phillipsburg, NY);gelatin, agar(Difco Laborotrories, Ditroit, MI); Percoll(Pharmacia Fine Chemicals AB, Uppsala, Swden);protein A-sepharose CL-4B(Pharmacia Fine Chemicals, Piscataway, NJ); Triton X(Research Products International, EIK Grove IL);cycolphosphamide(Mead Johnson Co., Evansville, Ind), Precoated silica gel G plates (Instruments westbury, NY;LK5DF silica gel plates (whaterman, Maidstone, Kent, U.K); [^{3}H]-palmitic acid(s.a; 10-30 Ci/mmol), [^{3}H-methye]-methionine (s.a; 12Ci/mmol), En^{3}Hance,[^{3}H]-LTD_4(s.a; 39Ci/mmol) (new England Nuclear, Boston, Ma); Scintillation cocktail(Amersham); Kodah AR film (Eastman Kodak, Rochester, NY); Ascaris (VW-Madison, WI, USA); leukotriene antibody and standard LT(Stuart Pharmaceuticals, Divison of ICI American, Inc., Wilmington, Del); Several chemicals used in these studies and other reagents were used best grade.

Analyses

Statistics analysis was performed by student's t-test for unpaired samples.

Results

Mediator release from mast cell sensitized with IgG_1 vs IgE antibody

In order to confirm whether the differences of mediators released by tissue pulmonary responses sensitized with IgG_1 vs IgE antibody were evoked in the purified mast cells, purified mast cells were passively sensitized with IgG_1 vs IgE antibody, challenged with varying concentrations or Ox-antigen(for 15min) and Hist and LT measured. The results are shown in table 1. We observed that both Hist & LT released by IgG_1 antibody cross-linking significantly differed from those released by IgE antibody cross-kinking in the purified mast cells. Ox-antigen included release of LT as well as Hist was obtained in a dose-dependent manner from high purity mast cell(>90 % purity) sensitized with IgG_1 vs IgE antibody. When the mast cells sensitized with IgG_1 vs IgE antibody were challenged by 10.0μg/ml Ox-antigen, the amounts of Hist and LT released were similar to those released from mast cells challenged by 1.0μg/ml Ox-antigen(data in table not shown). Peak of Hist release(approx 30-40% with IgG_1 antibody; 7-15% with IgE antibody) and LT release (approx 10-30 pmole/10^6 cells with IgG_1 antibody; 0-8 pmole/10^6 cells) were indicated utilizing Ox-antigen concentrations between

0.1 to 1.0 μg/ml.

Formation of a PLD product(PEt) in the mast cells sensitized with IgG_1 vs IgE antibody

In order to assess the differences of mediator release caused by both antibodies, the production of labeled PEt by PLD-mediated transphosphatidylation was examined in prelabeled guinea pig mast cells stimulated by IgG_1 vs IgE receptor cross-linking in the presence and absence of ethanol. TLC chromatographic analysis of phospholipids extracted from[^{3}H]-PlA prelabeled-mast cell activation exposed to ethanol revealed the presence of a unique spot that was not found in cells unsimulated in the absence of ethanol(Fig 1). Identification of this spot as PEt was made based upon the apperance of this spot in stimulated cells in proportion to the cocentration of ethanol and cochromatography with authentic methylated PEt(data in Figure not shown; see column 2 in Figure 1 of reference 38).

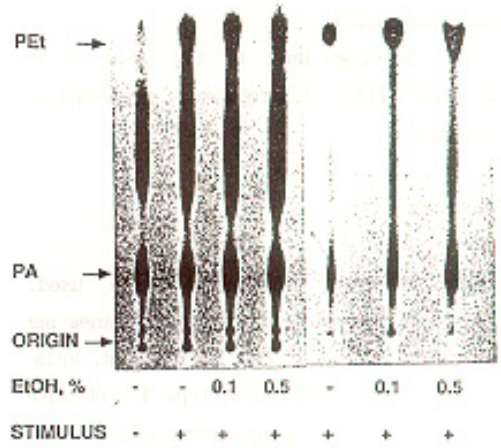

Fig 1. Ethanol-dependent PEt formation in the activation of purified mast cells sensitized with IgG_1 vs IgE antibody. Purifed mast cells(1 to 2×10^7) were prelabeled with [^{3}H]-PlA, and washed extensively, cells were sensitized with anti-Ox-HSA or anti-Ox-Asc antibody (1 ml/10^6 cells) at 37℃ for 45min. Five minutes before Ox-HSA or Ox-Asc challege, each concentration on ethanol indicated or medium was added. Ten minutes after stimulation, lipids were extracted and chromatographed by TLC. Autoradiography was performed. The abbrebiations used in this are; PEt, phosphatidylethanol; PA, phosphatidic acid; EtOH, ethanol.

Effect of IgG_1 vs IgE sensitization and ethanol on phospholipids

The phospholipid labeling pattern obtained when mast cells were preleabeled with [^{3}H]-PlA precursor, activated with two different antibodies, and subjected to stimulation in the presence or absence of ethanol revealed in table 2. Labeling PEt and PA varied significantly depending upon stimulation and the presence of ethanol. Labeled PA increased dramatically in IgG_1 receptor-mediated stimulation in the absence of ethanol, compared to IgE receptor-mediated stimulation in the absence of ethanol. Although a variety of pathways could account for increased accumulation of labeled PA, this finding is at least consistent with a receptor-mediated increase in PLD activity. In order to more specifically assess mast cell PLD activity, we examined the PEt production in both receptor-stimulated mast cells because ethanol dependent pro-

Table 1. Antigen-induced responses of highly purified guinea pig lung mast cells after passive sensitization with IgG_1 vs IgE antibody[a].

Abs	Antigen			
	0.1 mg/ml		1.0 mg/ml	
	Hist(%)[b]	LT(pmole/ml)[c]	Hist(%)	LT(pmole/ml)
IgG_1	31.5±1.8*	10.5±3.2*	41.9±3.5*	28.5±5.1*
IgE	8.4±0.7	—	15.2±2.4	7.6±2.1

a. Guinea pig lung mast cells were isolated and purified by digestion and perconn density gcadient, count current elutriation method. Mast cells(0.25×10^6 cells) were passively sensitized by anti-Ox-HSA or anti-Ox-Asc antibody and challenged by varying concentration of antigen.
b. Values represent total histamine found in all cells (0.25×10^6 cells) after antigen challenge.
c. Values represent total leukotrienes found in all cells (0.25×10^6 cells) after antigen challenge.
* A value that is statistically decreased ($p<0.05$) from the value obtained with the highly purified mast cell sensitized with IgE antibody.

duction of PEt can only occur as a result of PLD-mediated transphosphatidylation. But the modest labeling found in the PEt zone of the TLC in the absence of ethanol can repersent count carried-over from other lipids. However, when cells are stimulated in the presence of ethanol, there was a 2-to 5-fold rise in amount incorporated into PEt.

PEt formation was markedly increased by both receptor-mediated activation at 0.1 and 0.5% ethanol. PEt accumulation in mast cells stimulated by both antibodies is proportional to the concentration of ethanol(Fig1, Table2). These dramatic increase in labeled PEt occurring as a result of stimulating mast cells strongly suggests that PLD activity is activated as a result of both antibody receptor cross-linking. Howefer, the degree of PLD activity in IgG_1 receptor-stimulated mast cells. The both receptor-dependent rise in labeled PA is almost not attenuated by the presence of 0.5% ethanol(Table 2). It will be discussed in more detail.

Effect of IgG_1 and IgE on methylation of mast cell phospholipids

We have previously observed that IgG_1-dependent activation of purified guinea pig mast cells by using[^{3}H-methyl] methionine was associated with increased ^{3}H-methyl incorproation in the preliminary studies. In order to confirm whether there are the differences of ^{3}H-methyl incorporation between IgG_1-and IgE-receptor stimulated mast cell phospholipids, the purified mast cell were prelabeled with [^{3}H-methyl] methionine, activated with both antigen-antibody cross-linking reaction(Fig 2). The incorporation of the ^{3}H-methyl moiety into the phospholipids reached a maximum at 15 sec after the addition of antigen in both antibody receptor-stimulated mast cells. It has been observed that the phospholipid methylations are similar to each other in both antibody receptor-stimulated mast cells.

TLC separation of lung cell lipids was performed to analyze more closely the effects of both antibody receptor stimulation on PE methylation(Table 3). In unstimulated cells, only a little portion of the total radiolabel in lipid extracts was recovered in PE derivatives, such as PC and Lyso-PC(PMME and PDME in PE derivatives were not studied because those materials could not be got). The major fraction(>35% of the label) was associated with neutral lipids migrating near the solvent

Table 2. Phospholipid labeling in the activation of mast cells passively sensitized with IgG_1 vs IgE antibody[a].

phospholipid	Ab	unstim[b] (−)	unstim (+)	stim (−)	stim (+)
PA	IgG_1	9150±320*[c]	8100±250	11550±433*	9558±205*
	IgE	3781±178	2735±105	4743±215	5940±381
PEt	IgG_1	980±153	3891±121*	4418±373*	8990±347*
	IgE	574±187	1339± 95	1925±189	5435±281

a. Mast cells(10 to 20×10^6) were prelabeled with [^{3}H]-PIA, and washed extensively. Five minutes before anti-Ox-HSA or anti-Ox-Asc antibody stimulation, ethanol (0.5%) or medium was added. Five minutes after stimulation, lipids were extracted and chromatographically separated by TLC. Autoradiography was performed & radioactivity incorporated into individual lipids determined by lipid scintillation spectrophotometry.

b. Four conditions examined are indicated in the table, unstim=no antigenic stimulation; stim=stimulation by anti-Ox-HSA or anti-Ox-Asc receptor cross-linking: (−)=without ethanol; (+)=with 0.5% ethanol.

c. Data represent mean±SEM of 6 observations.

* $p<0.05$

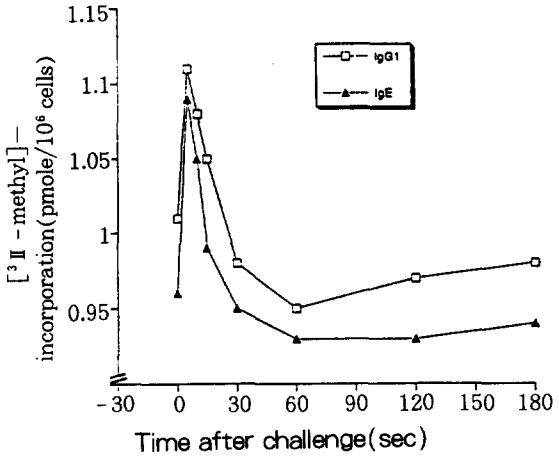

Fig 2. Effect of IgG_1 vs IgE antibody on [^{3}H-methyl] incorporation into lipid extraction of purified guinea pig lung mast cells. Purified mast cells(1×10^6) were incubated at 37℃ for 30min with [^{3}H-methyl] methionine before activation with IgG_1 vs IgE receptor cross-linking. Prelabeled cells were sensitized with anti-Ox-HSA or anti-Ox-Asc antibody (1 ml/10^6 cells), stimulated with Ox-HSA or Ox-Asc for 10min. The reactions were stopped by adding of TLC, the lipids extracted, and evaporated brfore the addition of scintillation cocktoil for radioactivity.

Table 3. Effect of IgG_1 vs IgE antibody on [^{3}H-methyl] incorporation into phospholipids of purified guinea pig lung mast cells*.

Antibody	Preparation	^{3}H-methyl incorporation(fmol/10^6cells) 0 sec	15 sec	30 sec
IgG_1	PC	15.1	19.4	18.4
	Lyso-PC	3.0	3.9	3.1
	Neutral lipid	132.4	136.1	126.4
	Total applied	357.5	343.3	338.7
IgE	PC	16.2	18.9	17.3
	Lyso-PC	3.2	3.8	3.5
	Neutral lipid	127.6	128.9	121.5
	Total applied	347.4	337.6	355.8

a. Highly purified mast cells(1×10^6 cells) were incubated for 30min with [^{3}H-methyl] methionine before activation with igG_1 vs IgE receptor cross-linking. After mast cell activation, cell lipids were extracted, and chromatographed on TLC. Results are mean of dupulicate determinations in three experiments in which spontaneous and net antigen-antibody-induced histamine release were 3.4%(IgG_1), 3.6%(IgE) and 40.1%(IgG_1), 14.5%(IgE), respectively.

front (Rf 0.95 to 1.00). Challenge with antigen-antibody reaction in both antibody stimulation released 40.1% and 14.5% histamine, respectively, but had minimal effects on radiolabeling of phospho-or neutral lipids at 15 and 30 sec after antigen challenge. We also could not find any differences in phospholipid methylation between both antibody stimulation. Methylated derivatives of PE prelabeled with [^{3}H-methyl]methionine were less than 20%.

Effect of methylation inhibitor(DZA) on antigen-antibody reaction-induced histamine secretion

Preincubation of purified guinea pig lung mast cells for 1 hr with methylation inhibitor, DZA, inhibited ^{3}H-methyl incorporation into total lipids(data not shown) and inhibited both antibody-stimulatied histamine release by 52.3%(IgG_1) and 39.7%(IgE) at a concentration of 1mM.

Discussion

We have previously demonstrated the substantial differences in measurable Hist and LT released during similar levels of antigen-induced contraction in guinea pig pulmonary tissues sensitized with IgG_1 versus IgE antibodies [1,19]. As mediators can be released from other cell types in guinea pig pulmonary tissues, the release of mediators was examined in the highly purified guinea pig lung mast cells. As a results,, we have obtained that the mediators are released by antigen-antibody reactions in highly purified guinea pig lung mast cells[38,54]. These studies also obtained substantial differences of Hist and LT released in purified guinea pig mast cells sensitized with IgG_1 versus IgE antibodies(Table 1). This differential degree of activation of mast cells might account for the differences between IgG_1 and IgE activation in AA formation, degradation, and granular-associated mediator release.

One important pathway activated after receptor cross-linking(cell activation) is recent-ly known that PC by PLD is a quantitatively more important source of increased DAG than PI hydrolysis in mast cells is. It also demonstrated that,,when DAG levels become maximal, no more than 25% is derived from PI, whereas as much as 75% could come from PC[28]. It has also been reported that increased N-methylation of PE is important part of the transduction mechanism linking cell surface receptor activation to mast cell mediator release[39-43].

Therefore, the purpose of this study was to investigate the role of PLD and MT activation in signal transduction cascade linking cross-bridging of cell surface receptors to the differences in the release of mediators (Hist and LT) in purified guinea pig lung mast cells sensitized with IgG_1 vs IgE antibodies.

In our study, when purified guinea pig mast cells were prelabeled with [^{3}H]-PIA, then sensitized with anti-Ox-HSA(IgG_1)or anti-Ox-Asc(IgE), and challenged with both Ox-antigen, respectively, there was an initial rapid increase in [^{3}H]-PA levels during both receptor cross-linking. Either direct hydrolytic formation by a PLD-mediated mechanism or phosphorylation of DAG by DAG Kinase can

lead to PA accumulation. Utilizing the transphosphatidylation under the presence of ethanol, PEt formation is detected after activation of mast cell sensitized with IgG_1 vs IgE antibodies. PEt is not detected by unstimulated cells in the absence of ethanol (Column I in Fig1). When mast cells are stimulated in the presence of ethanol, the formation of labeled PEt rises significantly in a dose-dependent manner after both receptor cross-linking in mast cells(Fig 1 and Table 2).

However, PEt formation from mast cells activated with IgG_1 antibody is increased much more than that activated with IgE antibody(Table 2). This observation suggests that PLD-mediated formation of PA in mast cells stimulated with IgG_1 antibody occurs stronger than in mast cells stimulated with IgE antibody.

Since the receptor-dependent increases in PA accumulation are caused by PLD activity, it is suggested by the finding of the reciprocally decreased PA fromation associated with increased PEt formation in the presence of ethanol. However, our results show that PLD activity, as detected by an increased PEt production, rises rapidly after both receptor cross-linking, but PA formation remains still increased level even 10min after both receptor-mediated stimulation(Fig 1 and Table 2). Also, PA formation from IgG_1 activated mast cells are significantly larger than that from IgE activated mast cells. This result may be implied that PA may be derived from DAG Kimase-mediated DAG which if formed by hydrolysis of PLC-mediated PI after receptor-mediated stimulation. It is also implied that DAG Kinase-mediated DAG formated by IgG_1 activation significantly is larger, compared to IgE activation of mast cells.

Thus, both the direct(PLC-mediated PI) and indirect(PLD-mediated PC)pathways of DAG fomation may be important. The elevation in DAG levels formed by PLC-mediated PI and the PLD-initiated indirect pathway after IgG_1 receptor-mediated stimulation plays the more important role in regulating PKC-mediated events associated with exocytosis and/or new mediator synthesis, compared to IgE receptor-mediated stimulation. Sato et al[55] have reported that PLA_2 is activated by PA endogenously formed through PLD action in rat peritoneal mast cell, and then PLA_2 activation may increase the liberation of AA and AA metabolidtes(LT and PG etc). Therefore more PA accumulation with IgG_1 stimulation may significantly increase LT release, compared to IgE stimulation.

With the use of the purified guinea pig lung mast cells, we examined whether ^{3}H-methyl incorporation into cellular phospholipids differs each other after challenge with concentrations of IgG_1 vs IgE-dependent stimulus which were effective in releasing histamine. An increase in both the incorportation of the ^{3}H-methyl moiety into phospholipids and histamine release was induced with both receptor-mediated stimulation(Fig 2). Maximal incorportion of the ^{3}H-methyl moiety into the lipid fraction of the cells was obserced with in 15sec after being challenged with both Ox-antigen. Howecer, the methylated phospholipid decreased rapidly and only a little portion of the total radiolabel in lipid extracts was recov-

ered in PE derivatives. We also examined that phospholipid methylations are similar to each other although there is the difference of mediator release between both receptor-stimulated mast cell(Fig 2).

In the TLC separation of phospholipid methylation, we could not observe any differences in ^{3}H-methyl moiety incorporation into PE derivatioves after IgG_1 vs IgE receptor-stimulated mast cells(Table 3).

In order to confirm whether phospholipid methylation is inhibited by methylation inhibitor(DZA), mast cells were incubated with DZA before mast cell activation. After both receptor-stimulated mast cell activation, phospholipid methylation was inhibited by DZA (Fig 3)

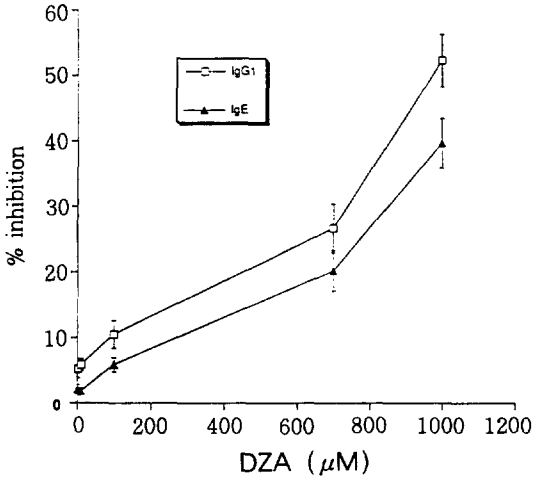

Fig 3. Inhibition of IgG_1 vs IgE-induced histamine release from purified guinea pig lung mast cells by varying concentration of 3deazaadenosine (DZA). Purified mast cells (1×10^6) were incubated for 1 hr with DZA. Cells were sensitized, challenged with IgG_1 or IgE antibody and Ox-antigen, respectively, at 37℃ for 10min. Supernatants obtained after centrifigation were taken for determination of Hist.

This observation in phospholipid methylation study show that IgG_1-or IgE-dependent actication of guinea pig lung mast cells is in part associated with enhanced phospholipid methylation by transmethylase, but there is no difference in phospholipid methylation between IgG_1 and IgE receptor-stimulated mast cell activation. Therefore, this results suggest that the differences in mediator release between IgG_1 and IgE activation is not associated with the differences in transmehylase activity stimulated by IgG_1 vs IgE antibodies.

Conclusion

These data suggest that a differential activation of PLD enzyme on the mast cell membrane receptors in the guinea pig pulmonary system might account for the differences in the release of mediators seen after activation of the IgG_1 vs IgE antibody receptors. The data also demonstrate that a differential activation of MT enzyme on mast cells is not associated with the differences in mediator release after both receptor activation, although methylation inhibitor(DZA) decreases histamine release by IgG_1 vs IgE antibody. This result implies that any particular transmethylase is involved with histamine release.

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국문초록

비반세포의 매체 유리기전에 관한 연구

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IgG_1 혹은 IgE항체에 의해 각각 피동적으로 감작된 해명 기도 및 폐조직을 항원으로 자극시 평활근 수축력은 두 항체에 의해 서로 유사하지만 유리되는 매개체인 histamine과 leukotriene 유리량은 현저한 차이를 나타낸다는 사실이 이미 보고되었다. 이런 해명 기도 및 폐조직에서 두항체에 의해 야기되는 매개체 유리량의 차이는 비반세포외 다른 세포의 관여에 의해서 일어날 수 있다는 가능성을 배제할 수 없으므로 본 저자들은 순수분리된 비반세포(>95%)를 이용하여 두 항체로 감작시 매개체 유리량을 조사하였다. 그 결과 IgG_1 혹은 IgG 항체로 활성화된 매개체(Hist과 LT) 유리량의 차이를 관찰하였다. 그러므로 본 연구는 IgG_1혹은 IgE 항체로 감작된 비반세포의 세포막 수용체가 항원과 교차결합으로 인해 활성화되면 여러 생화학적 신호변환이 일어나게 되는데 이 신호변환 과정중 PLD와 MT효소 활성의 역할을 조사하므로서 두 항체에 의해 나타나는 매개체 유리량의 차이를 규명하고자 하는데 그 목적이 있다.

해명 폐조직을 enzyme digestion, percoll density gradient, count current elutriation 방법에 의해 95% 이상의 순수한 비반세포를 분리 하였다. 순수분리된 비반세포를 [^{3}H]-palmitic acid 혹은 [^{3}H-methyl]-methionine으로 prelabeling 시키고, IgG_1혹은 IgG 항체로 감작시킨후 특수항원(Ox-antigen)으로 자극, 세포막 인지질을 추출하여 PLD 활성도와 MT 활성도를 측정하였다. 그리고 동시에 Hist 과 LT 유리량을 측정하였다. PLD효소활성도는 에타놀 존재하에서 PLD 효소매개로 인해 생성되는 PEt량을 TLC 분석법으로 직접 측정하였으며 MT 활성도는 PE 유도체에 전의된 ^{3}H-methyl moiety를 직접측정하였다.

항원-항체 교차결합(IgG_1 or IgE-cross linking)에 의한 해명 비반세포 활성화시 PLD활성도가 현저히 증가되었다. 그리고 PLD활성도는 IgE 항체 감작에 비해 IgG_1 항체감작의 경우에 의의 있게 높았다. 해명 폐조직 비반세포가 IgG_1 혹은 IgE 항체에 의해서 활성화되면 MT가 활성화되어 인지질의 methylation이 15초 이내에 증가되었다. 그러나 두항체로 인한 매개체 유리량의 차이에도 불구하고, 두항체로 감작된 비반세포 활성시에 MT활성도에 는 차이가 없었다. 두항체에 의해 활성화된 비반세포 막 인지질 methylation은 methylation억제제인 DZA에 의해 모두 억제되었다.

이상과 같은 결과는 해명 폐조직 비반세포막에 존재하는 IgG_1혹은 IgE항체 수용체들의 활성화시에 PLD효소활성화의 차이가 매개체들의 유리량의 차이를 나타날 수 있을 것으로 추측된다. 그러나 두 수용체 활성화시에 나타나는 매개체 유리량의 차이에도 불구하고 비반세포막의 MT 효소활성화의 차이는 나타나지 않았다. 또한 이런 인지질의 methylation은 DZA에 의해 억제되는 것으로 보아 어떤 특수한 transmethylase가 Hist 유리에 관여할 것으로 추정되어진다.

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