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ANTIGENIC STIMULATED RELEASE OF ARACHIDONIC ACID, LIPOXYGENASE ACTIVITY AND HISTAMINE RELEASE IN A CLONED MURINE MAST CELL MC9

Mark W. Musch and Marvin I. Siegel

Department of Allergy and Inflammation
Schering-Plough Corporation
Bloomfield, NJ 07003

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SUMMARY - A cloned murine mast cell MC9 expresses phospholipase and lipoxygenase activity when stimulated with IgE and hapten. Addition of DNP-BSA to sensitized MC9 cells causes release of 58% of the cell histamine and 127 pmoles $LTC_4/10^6$ cells. Prelabelling studies with [1-^{14}C]-arachidonic acid showed that LTC_4 production was proceeded by the release of arachidonic acid from membrane phospholipids. Approximately 8.7% of the cell arachidonic acid was released and half of this was converted to LTC_4. The remaining radioactivity was converted to diHETES including LTB_4 (15%), 5-HETE (10%), free arachidonic acid (10%), reesterified 5-HETE and arachidonic acid (8%) and prostaglandins (7%). This stimulation was dependent on hapten (DNP-BSA) and extracellular Ca^{++}. Under identical conditions the calcium ionophore A23187 stimulated the release of 10.3% of the total cell arachidonic acid, and 51% of this was metabolized to LTC_4. In addition the ionophore stimulated the release of 61% of the total cellular histamine.

Crosslinking of membrane bound receptors for IgE ($Fc_\epsilon R$) on mast cells and basophils has been demonstrated to activate secretory mechanisms responsible for acute allergic reactions (1). Preformed mediators (i.e. histamine, neutral proteases, hexosaminidase) contained in granules as well as newly synthesized arachidonic acid derived allergic mediators (such as LTC_4, LTB_4, and 5-HETE) are released when sensitized cells are exposed to the appropriate hapten, concanavalin A, or an anti-IgE antibody (1,2,3,4).

The abbreviations are: DNPBSA, dinitrophenyl bovine serum albumin; 5-HETE, 5(S)-6,8,11,14-eicosatetraenoic acid; 5,12-diHETEs, 5(S),12(R) and 5(S),12(S)-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid; LTB4, 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid; LTC_4, 5(S)-hydroxy-6(R)-sulfido-glutathionyl-7,9-cis-11,14-trans-eicosatetraenoic acid; HEPES, hydroxyethylpiperazine ethane sulfonic acid; HPLC, high performance liquid chromatography; TLC, thin layer chromatrography.

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Mast cells derived from peritoneal or pleural lavage of animals have IgE bound to their plasma membrane $Fc_\epsilon R$ receptors, however, using mast cells in culture such as MC9 cells, one must "passively sensitize" the cells with IgE. Utilization of murine hybridomas which produce IgE directed against DNP covalently bonded to albumin (5) provides specific IgE for addition to cultured cells. The binding of the IgE is of high affinity in these cells and can be performed in 60 to 90 minutes in the absence of extracellular calcium (6). MC9 cells express the $Fc_\epsilon R$ receptor early in their development and have numbers of receptors only slightly lower than that found on peritoneal mast cells (6). The affinity of the MC9 $Fc_\epsilon R$ receptor is comparable to that reported for peritoneal mast cells (6).

Common features of antigenic stimulation of mast cells are phospholipase activation (release of arachidonic acid) and a requirement for the influx of extracellular calcium (7,8). While calcium ionophore, somatostatin, and compound 48/80 do not require extracellular calcium, they do require intracellular sources (9). IgE-like secretagoges (hapten, concanavalin A, and anti-IgE) have an absolute requirement for extracellular calcium (9). These stimuli may also activate additional steps, i.e. phospholipid methylation and phosphatidyl inositol breakdown which non-IgE mediated secretagoges may not (10,11,12). The mechanism of calcium influx is not known, but phosphatidic acid, a phosphatidyl inositol metabolite via the PI cycle may act as an ionophore in this system (13,14,15).

In the present study the activation of MC9 cells via the $Fc_\epsilon R$ receptor was investigated particularly in regard to arachidonic acid release and metabolism of the released arachidonate.

METHODS - Cells - MC9 cells were grown and harvested as previously described (16). Cells were labelled with 0.2 uCi/ml [1-^{14}C]-arachidonic acid (approximately 2 uM) for 18 hours before sensitization with IgE. Initially MC9 cells were sensitized with varying amounts of IgE up to 1 ug/10^6 cells by resuspension at 5 X 10^7 cells/ml in Ca^{++}-free HEPES assay buffer (25 mM HEPES; 125 mM NaCl; 2.5 mM KCl; 0.7 mM $MgCl_2$; 0.5 mM EGTA; and 10 mM glucose, pH 7.4 with NaOH). Monoclonal mouse IgE anti-DNP-BSA

clone-26.82 was a gift from Dr. F. T. Liu (Medical Biology Institute; La Jolla, Ca.). In subsequent experiments 0.01 ug IgE was used per 10^6 cells. Upon stimulation, these sensitized cells formed approximately 60% of the maximal LTC_4 production and histamine release and were used in the calcium, hapten, and time dependence studies. The cells were incubated for 60 minutes at 37° C with the antibody and were then harvested by centrifugation (150g X 10 minutes) for assay.

Arachidonic Acid and Histamine Release - Release of arachidonic acid and its conversion to metabolites was performed as previously described (16). Briefly, sensitized cells were resuspended at 5 X 10^7 cells/ml in Ca^{++}-free HEPES assay buffer. Seven million cells (140 ul) were added to polypropylene tubes containing: 1) Ca^{++}-free assay buffer with 64 ug/ml phosphatidyl serine (final concentration in assay 50 ug/ml); 2) $CaCl_2$ solution added to achieve a final Ca^{++} concentration of 1 mM except in Ca^{++} dependence experiments; and 3) 700 ng DNP-BSA (16 molecules DNP per BSA) obtained from Dr. F.T. Liu (Medical Biology Institute, La Jolla, Ca.) except in hapten dependence experiments in a total volume of 560 ul. When added, ionophore A23187 was used at 1 uM after dilution from a stock of 10 mM in DMSO. For termination of the assay, 200 ul of the incubation volume (2 X 10^6 cells) was added to 200 ul Ca^{++}-free HEPES assay buffer plus 4 mM EDTA on ice. This aliquot was spun down and both histamine released and that remaining in the cells was determined on a Technicon autoanalyzer by the method of Shore (17). To the remaining 500 ul of the incubation mixture, 2 mls of ice-cold ethanol was added for lipid extraction. Quantitation of the LTC_4, released arachidonic acid and other metabolites was performed by silicic acid separations followed by thin layer chromatography (arachidonic acid, mono- and diHETEs, and di- and triglycerides) or by reverse phase high performance liquid chromatography (LTC_4) as described previously (16).

MATERIALS - All solvents used were either analytical or HPLC grade (Mallinkrodt; St. Louis, MO.; EM Industries; Gibbstown, NJ). Authentic LTC_4 standard was obtained from Dr. E.J. Corey (Harvard University; Boston, MA). Phosphatidyl serine was obtained from Serdary Research Laboratories (London, Ontario). [1-^{14}C]-arachidonic acid (58 mCi/mmol) and [^{3}H]-LTC_4 (45.7 Ci/mmol) were obtained from Amersham (Arlington Heights, IL).

RESULTS - The ability of MC9 cells to metabolize arachidonic acid has previously been determined using the calcium ionophore A23187 and a large excess of exogenous arachidonic acid (16). It was important to determine if MC9 cells would release endogenous arachidonic acid and metabolize it under the physiological stimulus, IgE plus antigen.

When grown with 0.2 uCi/ml [1-^{14}C]-arachidonic acid, MC9 cells incorporate greater than 98% of the label. Of the total cell label, triglycerides accounted for less than 0.5%. Of the label in phospholipids, phosphatidyl ethanolamine was the most heavily labeled followed by phosphatidyl choline, phosphatidyl inositol, and phosphatidyl serine (manuscript in preparation). All experiments were performed after 18 hours although label was constant in all phospholipids after 8 hours. Five

million cells incorporated 644,350 ± 90510 DPM. All results are expressed as percentage of total cell label except for certain LTC_4 measurements which are expressed as pmoles/10^6 cells. The specific activity of the LTC_4 produced was approximately 19 mCi/mmol, a three-fold dilution of the specific activity of arachidonic acid added to the cells. Assuming that there would be no enrichment or diminution of specific activity of released arachidonic acid or of its metabolites, expressing results as percentage of cell label would not bias the results.

Stimulation of histamine release and arachidonic acid release and metabolism depended on the amount of IgE in the sensitization period (Figure 1). Stimulation of MC9 cells requires only small amounts of IgE although the maximal effect occurs at 0.2 to 1.0 ug IgE/10^6 cells. Experiments were routinely performed with 0.01 ug IgE/10^6 cells. This IgE concentration gave approximately 60% of the maximal response in LTC_4 formation (127 ± 13 pmoles/ 10^6 cells) with the range being from 43 to 74%.

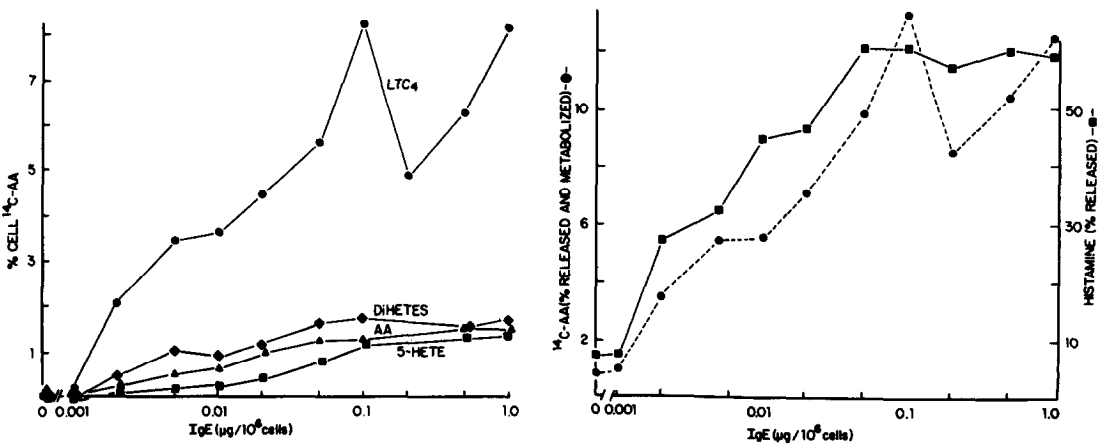

Figure 1. Antibody dependence of stimulation of MC9 cells. Seven million MC9 cells were incubated with various amounts of IgE for 60 minutes in Ca^{++}-free HEPES assay buffer. After centrifugation (150 g X 10 minutes) cells were resuspended in 140 ul and added to assay buffer with 700 ng DNP-BSA and 1 mm Ca^{++}. Cells were incubated with hapten plus Ca^{++} for 10 minutes at 37°C and the assay terminated as described in Methods. Data shown are values obtained in one experiment repeated with similar results on two occasions. Total ^{14}C released plus metabolized includes all four parameters in left panel, plus that arachidonic acid converted to prostaglandins. Histamine released is percent of total cellular content.

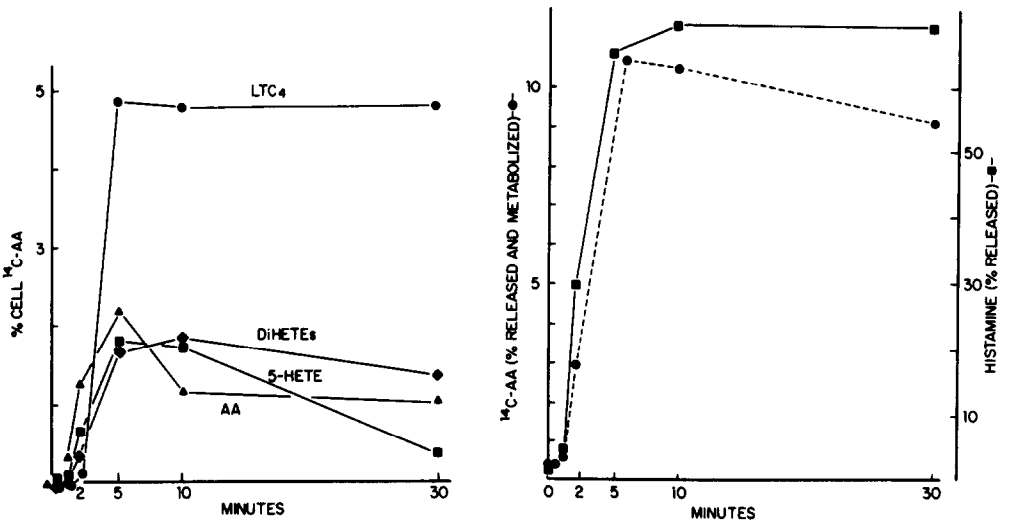

Figure 2. Time courses for the release of arachidonic acid and histamine and conversion of released arachidonic acid to its primary metabolites by MC9 cells. Cells were sensitized with 0.01 ug IgE/10^6 cells for 60 minutes in Ca^{++}-free HEPES assay buffer. After centrifugation (150 g X 10 minutes) and resuspension, assays were initiated by addition of cells to 700 ng DNP-BSA and 1 mM Ca^{++}. After 10 minute incubations at 37°C, assays were terminated as described in Methods. Data shown are values obtained in one experiment repeated with similar results on two occasions. Total ^{14}C released plus metabolized includes all four parameters in left panel plus that arachidonic acid converted to prostaglandins. Histamine released is percent of total cellular content.

Initially, assays were performed for 10 minutes since previous experiments with ionophore showed that arachidonic acid release was maximal at 10 minutes (data not shown). Arachidonic acid release was the first event stimulated by IgE (Figure 2). This was followed by production of LTC_4, the major metabolite, plus smaller amounts of 5,12 diHETES, including LTB_4, and 5-HETE. As in certain other systems, free arachidonic acid and 5-HETE decreased at 30 minutes accompanied by an increase in label in triglycerides (not shown). This most likely indicates reesterification of the free arachidonic acid and 5-HETE. Histamine release was significantly elevated at one minute and plateaued at five minutes.

Cell stimulation by IgE also depended on DNP-BSA concentration (Figure 3). Initially, 100 ng was used per 10^6 cells. This is a 20-fold excess over the amount required for maximal stimulation at this concentration of antibody.

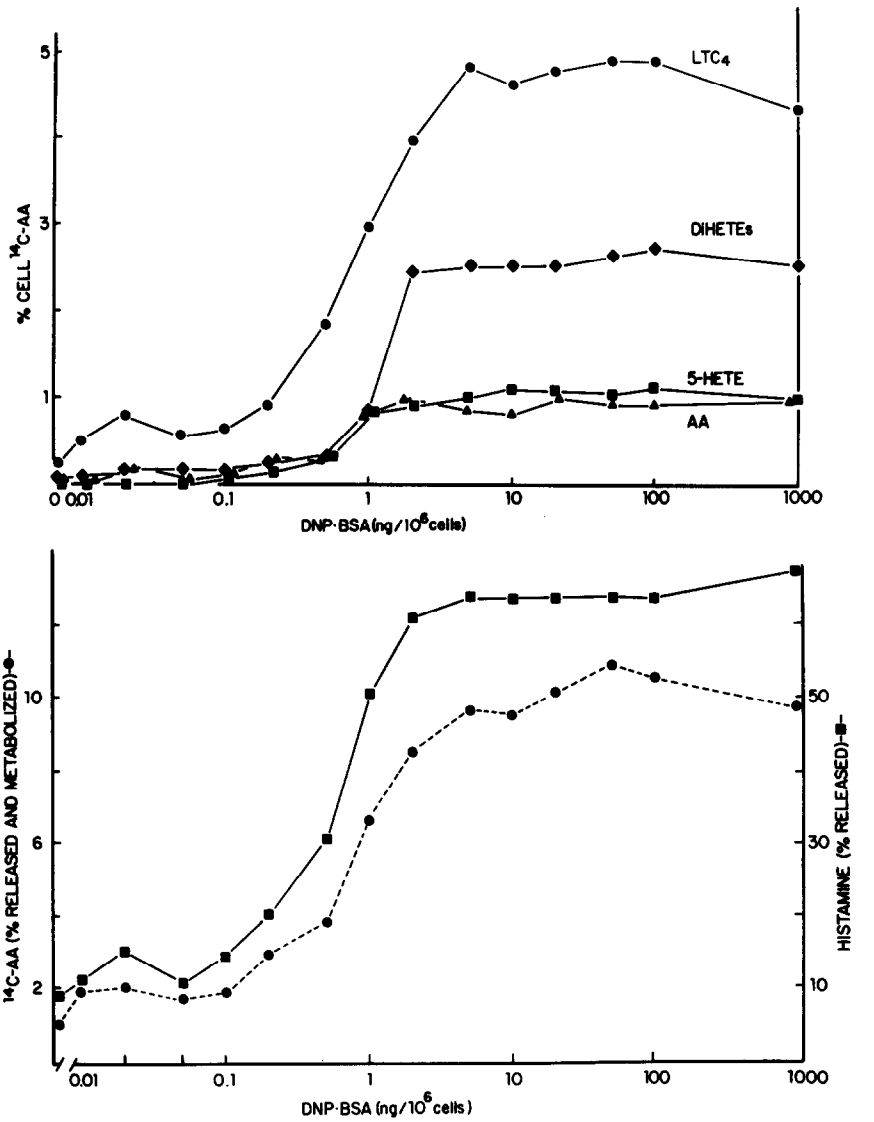

Figure 3. Calcium dependence of stimulation of sensitized MC9 cells. Cells were sensitized with 0.01 ug IgE/10^6 cells for 60 minutes in Ca^{++}-free HEPES assay buffer. After centrifugation (150 g X 10 minutes) and resuspension, assays were initiated by addition of cells to 700 ng DNP-BSA plus various amounts of extracellular Ca^{++} in the assay buffer. After 10 minutes at 37°C, assays were terminated as described in Methods. Data shown are values obtained in one experiment repeated on three other occasions. Total ^{14}C released plus metabolized includes all four parameters in top panel plus that arachidonic acid converted to prostaglandins. Histamine released is percent of total cellular content.

Antigenic stimulation required extracellular calcium (Figure 4); in fact only concentrations above 250 uM permitted activation of MC9 cells. Stimulation by the calcium ionophore A23187 did not require the addition of

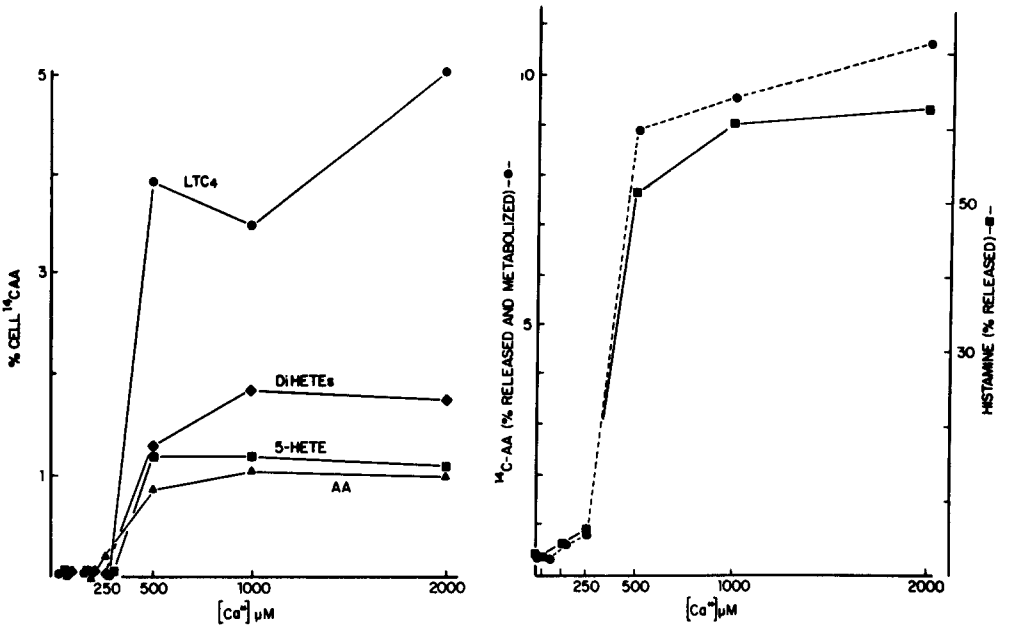

Figure 4. Hapten (DNP-BSA) dependence of stimulation of sensitized MC9 cells. Cells were sensitized with 0.01 ug IgE/10^6 cells for 60 minutes in Ca^{++}-free HEPES assay buffer. After centrifugation (150 g X 10 minutes) and resuspension, assays were initiated by addition of cells to 1 mM Ca^{++} in assay buffer plus various amount of DNP-BSA. After 10 minutes at 37°C, assays were terminated as described in Methods. Data shown are values obtained in one experiment repeated on two other occasions. Total ^{14}C released plus metabolized includes all four parameters in left panel plus that arachidonic acid converted to prostaglandins. Histamine released is percent of total cellular content.

extracellular calcium (16), indicating release from some intracellular source not accessible to antigenic stimulation.

Calcium ionophore A23187 also stimulated release of arachidonic acid and its metabolism primarily to LTC_4 along with histamine release (Table 1). Under these conditions ionophore stimulated all measured parameters slightly greater than did antigenic stimulation.

DISCUSSION - After passive sensitization with IgE and the addition of hapten, MC9 cells express phospholipase activity followed by metabolism of the released arachidonic acid via the 5-lipoxygenase pathway primarily to LTC_4. In addition, MC9 cells also release histamine and presumably other mediators found in their secretory granules upon antigenic stimulation. Antigenic stimulation was nearly as effective as ionophore. Neither stimulus, however, approached the maximal capacity of the cells to

TABLE 1

COMPARISON OF ANTIGENIC AND IONOPHORE STIMULATION OF MC9 CELLS

	% RELEASE					
	Total	LTC_4	AA	5-HETE	diHETEs	Histamine
A23187	10.3±0.3	5.2±0.7	1.5±0.2	1.0±0.1	1.1±0.1	60.3±3.4
IgE+DNP-BSA	8.7±1.2	4.4±0.5	0.8±0.2	0.8±0.3	1.1±0.2	57.6±5.7

Cells for antigenic stimulation were incubated with 0.01 ug IgE/10^6 cells for 60 minutes; those for ionophore stimulation were incubated at 37°C for 60 minutes without antibody. Assays were for 10 minutes at 37°C with appropriate stimulus (1 uM A23187 or 700 ng DNP-BSA) all assays were done in the presence of 1 mM Ca^{++}. Values are means ± SEM for three experiments. Total includes all four listed parameters, plus that arachidonic acid converted to prostaglandins. Histamine released is percent of total cellular content.

metabolize arachidonic acid as measured by ionophore stimulation using exogenous arachidonic acid.

How the crosslinking of membrane-bound $Fc_\epsilon R$ receptors stimulates phospholipase activity is unknown. Influx of extracellular Ca^{++} is required for $Fc_\epsilon R$ receptor mediated (but not ionophore or 48/80) stimulation of MC9 cells (16). Both phospholipase A_2 and C require Ca^{++}, although A_2 requires appreciably greater concentrations (18). It is possible that the initial activation step could be the opening of a Ca^{++} channel, liberation of membrane associated Ca^{++}, or generation of a lipid Ca^{++} ionophore such as phosphatidic acid by stimulation of phospholipase C activity such as occurs in platelets (18). Subsequent activation of phospholipase A_2 would occur concomitant with the rise in cytosolic Ca^{++}. These events are under investigation.

Synthesis of LTC_4 by antigenic stimulation in MC9 mast cells which contain chondroitin sulfate and are therefore of the mucosal type provides evidence that mast cells in the lung and nasal mucosa may be responible for the LTC_4 found in nasal washes of allergic patients and LTC_4 release from chopped lung fragments (19,20).

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