Binding of Monoclonal Antibody AA4 to Gangliosides on Rat Basophilic Leukemia Cells Produces Changes Similar to Those Seen with Fce Receptor Activation

Constance Oliver,* Noriyuki Sahara,* Seiichi Kitani,* April R. Robbins,§ Lawrence M. Mertz,‡ and Reuben P. Siraganian*  

*Laboratory of Immunology, ‡Clinical Investigation and Patient Care Branch, National Institute of Dental Research, and §Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Abstract. The mAb AA4 binds to novel derivatives of the ganglioside GD1α on rat basophilic leukemia (RBL-2H3) cells. Some of the gangliosides are located close to the high affinity IgE receptor (FceRI), and binding of mAb AA4 inhibits FceRI-mediated histamine release. In the present study, mAb AA4 was found to bind exclusively to mast cells in all rat tissues examined. In vitro, within 1 min of mAb AA4 binding, the cells underwent striking morphologic changes. They lost their normal spindle shaped appearance, increased their ruffling, and spread over the surface of the culture dish. These changes were accompanied by a redistribution of the cytoskeletal elements, actin, tubulin, and vimentin, but only the actin was associated with the membrane ruffles. Binding of mAb AA4 also induces a rise in intracellular calcium, stimulates phosphatidylinositol breakdown, and activates PKC.

However, the extent of these changes was less than that observed when the cells were stimulated with antigen or antibody directed against the FceRI. None of these changes associated with mAb AA4 binding were seen when the cells were exposed to nonspecific IgG, IgE, or four other anti-cell surface antibodies, nor were the changes induced by binding mAb AA4 at 4°C or in the absence of extracellular calcium. Although mAb AA4 does not stimulate histamine release, it enhances the effect of the calcium ionophore A23187 mediated release. The morphological and biochemical effects produced by mAb AA4 are similar to those seen following activation of the cell through the IgE receptor. Therefore, the surface gangliosides which bind mAb AA4 may function in modulating secretory events.

Mast cells and basophils contain a variety of inflammatory mediators which are released following cross-linking of high affinity IgE surface receptors (FceRI). The activation of the receptor results in a complex series of biochemical and morphologic events (48). Briefly, when FceRI are cross-linked, either by antigen on IgE-sensitized cells or by antibody directed against the receptor, there is stimulation of phospholipase C resulting in hydrolysis of phosphatidylinositol with the generation of 1,2 diacylglycerol and inositol 1,3,5 phosphate. Both the 1,2 diacylglycerol and the inositol 1,4,5 phosphate are thought to act as intracellular signals; the 1,2 diacylglycerol by activating protein kinase C (PKC), and the inositol 1,4,5 phos-

1. Abbreviations used in this paper: DNP44-HSA , 44 molecules of 2,4-dinitrophenyl conjugated to 1 molecule of human serum albumin; EMEM, Eagle’s minimum essential medium with Earl’s salts; FceRI, high affinity IgE receptor on basophils/mast cells; PKC, protein kinase C; NBD,N-[7 Nitrobenz-2-oxa-1,3 diazol-4-yl)]; RBL-2H3, rat basophilic leukemia 2H3 cloned cell line.
of IgE to its receptor, but bound IgE does not prevent mAb AA4 from binding. The proximity of the binding site for mAb AA4 to the FcεRI has been further documented by fluorescence resonance energy transfer experiments (16). Additionally, binding of mAb AA4 to the RBL-2H3 cells inhibits histamine release in a time- and concentration-dependent manner. Gangliosides are known to modulate receptor function in other cell types (19, 25, 54). Therefore, the present study was undertaken to further characterize the immediate effects of the binding of mAb AA4 to gangliosides on the surface of RBL-2H3 cells and to determine if there were any similarities between the effects produced by binding of mAb AA4 and activation of the FcεRI.

Materials and Methods

Tissue Culture

RBL-2H3 cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 15% FCS, penicillin, and streptomycin (4). For light microscopy, cells were plated at a density of 2 x 10⁶ cells per well in 8-chamber Lab-Tek Chamber Slides (Nunc Inc., Naperville, IL). For studies involving the interactive laser cytometer, cells were plated at a density of 1 x 10⁶ cells in 35-mm culture dishes and for scanning EM, 1 x 10⁶ cells were plated onto 13-mm-round glass coverslips. All cells were plated 48 h before use.

Antibody Preparation

plated 48 h before use. Antibody Preparation

The isolation and characterization of mAb AA4 has been previously described (5). Briefly, spleen cells from mice immunized with intact RBL-2H3 cells were fused with the X63-Ag8-653 myeloma cell line and the resulting culture supernatants were tested for their capacity to inhibit IgE binding. The immunoglobulins were precipitated from ascites fluid with 40% saturated ammonium sulfate. For mAb AA4, this step was repeated once using 50% saturated ammonium sulfate. The immunoglobulins were then purified by ion exchange chromatography on DE-52 (Whatman, Clifton, NJ). For some experiments, the mAb AA4 antibodies were further purified by HPLC using an ion exchange column (Sephorgel TSK DEAE 124; 60-mm inner diam x 15 cm) followed by a gel filtration column (TSK 3000 SWG; 21.5-mm inner diam x 60 cm). Several batches of ascites fluid were also affinity purified on Gamma-bind G Plus (Genes, Gaithersburg, MD) as recommended by the manufacturer. Final antibody concentrations were determined by the BCA protein assay (Pierce, Rockford, IL). Regardless of the method of purification, the antibodies appeared as a single band on Coomassie blue staining following SDS–polyacrylamide gel electrophoresis, and had identical effects when tested on RBL-2H3 cells. Fab fragments of mAb AA4 were prepared by papain digestion of purified antibody. The fragments were separated by HPLC. The other mAb were purified as described previously (5, 50). Antibodies were labeled with ¹²⁵I using iodobeads (Pierce, Rockford, IL).

Histamine Release

Histamine release from RBL-2H3 cells in monolayer cultures was performed as described previously (5). Briefly, cells were plated at 1 x 10⁶ cells per 16-mm-diam well and cultured for 18 h. The monolayers were then rinsed twice with EMEM containing 2% BSA and the appropriate secretagogue was added in the same medium. The reagents used and their concentrations are as follows: anti-FcεRI mAb, mAb BC4, 0.15 μg/ml; mAb AA4, 10 μg/ml; and the calcium ionophore A23187, 2.5 μg/ml (Sigma Chemical Company, St. Louis, MO). For IgE-mediated activation, the cells were cultured for 2–24 h with 0.3 μg/ml of mAb DNP 48, a monoclonal anti-dinitrophenol IgE (SO). The monolayers were then washed and stimulated with the corresponding antigen, dinitrophenol-human serum albumin (DNP48-HSA) at 10 μg/ml. At varying time intervals, the media were collected and the histamine content determined by the automated fluorometric technique. In experiments to assess the effects of calcium on histamine release and on the morphological changes, cells were cultured for 18 h, then rinsed twice in Hank's Balanced Salt Solution without Ca²⁺ and Mg²⁺ and stimulated in the same medium containing 40 μM EDTA.

Binding and Internalization of Antibodies

To determine cell surface binding, 2.2 x 10⁶ cells grown in suspension culture for 48 h were incubated with 25 μg of antibody for 90 min at 4°C, rinsed twice in EMEM, and incubated for an additional 60 min at 4°C with 5 x 10⁶ CPM of [¹²⁵I]goat anti-mouse IgG. Aliquots of the cell suspension were counted to determine total activity, and other aliquots were layered over (2-ethylhexyl) phthalate: dibutyl phthalate (2:3) (Kodak Chemicals, Rochester, NY) and centrifuged. The resulting cell pellets were then counted. Internalization of antibodies was determined by incubating the cells with 5 μg/ml of [¹²⁵I]mAb AA4 or 0.5 μg/ml [¹²⁵I]mAb BC4 for 30 min at 37°C. The cells were rinsed twice in PBS and once in PBS with 1 mM EDTA, and incubated with 0.5 mg/ml protease K (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 60 min at 4°C. Aliquots of the cells were then counted directly or centrifuged through bis (2-ethylhexyl) phthalate (2:3).

[¹²⁵I]Myo-inositol Labeling

[¹²⁵I]Myo-inositol labeling was done as previously described (24, 33). The cells were plated at 1 x 10⁶ cells per 22-mm well. After 6 h, the medium was replaced with fresh isoinositol-free EMEM containing 15% dialyzed FCS at 0.4 μCi/ml [¹²⁵I]myo-inositol. 18 h later, the cells were washed three times with Pipes ACM (191 mM NaCl, 5 mM KCl, 5.6 mM glucose, 25 mM Pipes, 0.4 mM MgCl₂, 1 mM CaCl₂, and 1.0 mg/ml BSA, pH 7.4) and preincubated for 10 min with Pipes ACM containing 5 mM LiCl. The cells were then stimulated with 0.1 μg/ml DNP₄-HSA, 0.15 μg/ml mAb BC4, or 10 μg/ml mAb AA4 for 5, 10, 15, 20, 30, or 60 min at 37°C. Lipids were extracted by adding 3 ml chloroform:methanol (1:2) directly to the medium in culture dishes. The liquid was removed, 1 ml chloroform added, and two phases separated by centrifugation. 1.5 ml of the aqueous phase was applied to a Dowex-6 column. The column was rinsed three times with 1 ml 5 mM myo-inositol and the inositol phosphates eluted with 1.5 ml 1 M sodium formate/0.1 M formic acid. Radioactivity of the inositol phosphates was determined by liquid scintillation spectrometry. Data are expressed as the mean ± SD. t test was performed to determine if significant differences exist between experimental groups.

Measurement of Cytosolic Calcium

For [Ca²⁺]i determinations, cell monolayers were detached from tissue culture flasks with trypsin-EDTA and placed in suspension culture 2–24 h before use. Cells were washed twice in EMEM without phenol red containing 1% BSA and incubated for 45 min at 30°C with Quin 2/AM (15 μM; Calbiochem, San Diego, CA). They were then washed twice with EMEM without phenol red containing 0.1% BSA and kept at 30°C. Just before use, the cells were washed in HBSS without phenol red containing 0.1% BSA, and 1.5 ml of cell suspension in this medium was placed in a cuvette and warmed to 37°C with stirring. Quin 2 fluorescence was measured using an SLM 8000 spectrofluorimeter (SLM/Aminco; SLM Instruments, Urbana, IL) with the slit width adjusted to 4 nm and the excitation and emission wavelengths set at 339 and 495 nm, respectively. The [Ca²⁺]i was calculated according to Tsien et al. (52). Fmax was obtained by lysing the cells with 0.07% Triton X-100, following which Fmin was obtained by stepwise addition of 10 mM EGTA and 1 M Tris to increase the pH to 8.3. Data are presented as the mean ± 1 SD. t test was performed to determine if significant differences exist between experimental groups.

Interactive Laser Cytometry

Monolayer cells in 35-mm culture dishes were rinsed twice in HBSS without phenol red containing 1% BSA. The mAb AA4, BC4, or the antigen DNP₄-HSA was added and the cells were returned to the incubator at 37°C. At varying time intervals (0–60 min), the samples were rinsed twice in PBS at 37°C and fixed at room temperature for 5 min in 4% formaldehyde (Ladd Research Industries, Burlington, VT) in PBS. They were then rinsed in PBS followed by PBS plus 0.1 M glycerol and stained for 1 h in 2 μM NBD-deoxycyclononyl-phospholipid-3-acetate (Meridian Instruments, Inc., Eugene, OR) (2, 38). After rinsing with cold PBS, 1 ml of cold PBS containing 0.167 M sucrose was added to each dish and the plates placed on ice. The staining of the cells with the fluorescent probe was reduced to 60 ± 3% when the cells were stained in the presence of a 10- to 20-fold excess of PMA. The cells were examined immediately with a Meridian ACAS 470 interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI). The amount of dye bound per cell was determined by measuring the total fluorescence of individual cells. A minimum of 35 cells from four experiments were examined.
Published February 1, 1992

for each treatment and the average of the cells' fluorescence calculated. Results are expressed as mean of percent change in average fluorescence intensity ± 1 SD.

Preparation of Cytoskeletal Extracts

Cytoskeletal extracts were prepared according to the method of Pfeiffer et al. (39). Following extractions, the samples were run on a 10% SDS-PAGE and the gels stained with Coomassie blue. The amount of actin was determined by scanning the gel with an LKB gel scanner and comparing the area under the actin peak in untreated and mAb AA4-treated cells. To determine the relative amounts of tubulin and vimentin, the proteins from the cytoskeletal extracts were separated by SDS-PAGE electrophoresis, transferred to Immobilon membranes (Millipore, Bedford, MA), and incubated with antibody to either tubulin or vimentin (Chemicon, Temecula, CA). Antibodies were then detected using biotinylated anti-mouse IgG and streptavidin alkaline phosphatase (Zymed, South San Francisco, CA).

Light Microscopy

The distribution of mAb AA4 on the surface of RBL-2H3 cells was assessed by adding 200 μl mAb AA4 (1–10 μg/ml) to culture medium at 37°C per well of 8-well chamber slides and then rotating the slides to the incubator. At varying time intervals, the cells were rinsed in PBS, fixed for 10 min in 2% formaldehyde, rinsed, and stained with goat anti-mouse FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). In some experiments the cells were fixed before the addition of mAb AA4. To stain for f-actin, cells were rinsed twice in PBS, fixed for 10 min in 2% formaldehyde, permeabilized with 0.1% Triton X-100, and stained for 30 min with FITC-phalloidin (Molecular Probes). In experiments involving inhibition with staurosporine (Kyowa Hakko USA, New York, NY), cells were incubated for 30 min in EMEM containing 1% BSA at 37°C in varying concentrations of staurosporine, then mAb AA4 was added and the cells incubated for an additional 30 min. For localization of vimentin, cells were fixed and permeabilized as above, then stained for 1 h at room temperature with a mAb to vimentin (Chemicon), rinsed in PBS and incubated with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). Tubulin was localized by fixing the cells for 2 min in 2% formaldehyde, followed by 5 min in absolute methanol at -20°C. The cells were further permeabilized with 0.1% Triton X-100 for 5 min and stained for 1 h at room temperature with a polyclonal antibody to tubulin (Polysciences, Inc., Warrington, PA). The cells were rinsed in PBS and incubated for 30 min in FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch). Controls consisted of cells incubated without primary antibody or cells incubated with normal mouse serum or normal mouse IgG instead of the primary antibody. Controls were negative. Changes in cell morphology were quantitated by scoring a minimum of 500 cells from each of three experiments. Changes were assessed on the basis of cell spreading and ruffling. Results are expressed as percent ± SD.

To localize mAb AA4 binding sites in tissue sections, tissues from 250 gm male Sprague-Dawley rats were fixed for 2 h in 4% formaldehyde, dehydrated in ethanol, and embedded in JB-4 plastic (Polysciences). 4-μm sections were mounted on glass slides and the sections treated with xylene for 15 min and rehydrated to water. The sections were blocked with 1% BSA in PBS and exposed to mAb AA4 for 2 h at 37°C. After rinsing in PBS, the sections were treated in AuroProbe LM gold-conjugated goat anti-mouse IgG (Janssen Life Sciences Products, Westbury, NY), and the gold silver enhanced (Intense M; Janssen). To identify mast cells, the sections were counterstained with antibody to either tubulin or vimentin (Chemicon, Temecula, CA). The sections were then dehydrated in ethanol, critically point dried in liquid CO2, and coated with gold-palladium. Samples were examined in a scanning electron microscope (35CF; JEOL USA, Peabody, MA).

Results

The mAb AA4 binds to novel α-galactosyl derivatives of the ganglioside GD₃ (18) which were originally isolated from RBL-2H3 cells, a cell with homology to mucosal mast cells. The gangliosides were present in the lipid extracts from several different rat tissues (18). Therefore an immunocytochemical survey with mAb AA4 was performed to determine the distribution of the gangliosides in rat tissues. With the exception of bone marrow in all 19 tissues examined, no cells other than mast cells stained with mAb AA4 (Fig. 1). In bone marrow, a population of large, poorly differentiated cells,
Figure 2. Scanning electron micrographs of activated RBL-2H3 cells. In unstimulated cells (A) the surface is covered with short microvilli. After 30-min stimulation with antigen (B), anti-receptor antibody, mAb BC4 (C), or mAb AA4 (D), the cells spread and their surface is ruffled and plicated.

most likely mast cell precursors, stained with mAb AA4. The morphology of these cells indicates that they are most likely immature mast cells. Thus mAb AA4 is mast cell specific and in tissue sections binds only to cells which can be identified as mast cells and the gangliosides recognized by mAb AA4 may be considered a marker for rat mast cells.

Initial experiments with RBL-2H3 cells documented the morphological changes produced by the binding of mAb AA4 to cell surface gangliosides on RBL-2H3 cells. These cells are normally spindle shaped, somewhat fibroblastic in appearance (Fig. 2) with small micro-villi on their surface. When stimulated to secrete with either antigen or with anti-FCeRI antibody (mAb BC4), their surface becomes plicated and the cells flatten and spread. When the RBL-2H3 cells are exposed to the mAb AA4, 10 μg/ml, the cells undergo similar morphological changes. The cell surface first becomes ruffled and then the cells begin to spread. The morphological changes depend on the concentration of mAb AA4 added and are less prominent at lower concentrations. When followed in time lapse video recordings, the effects are observed immediately upon addition of mAb AA4, with cell spreading reaching a maximum by 30 min. The cell shape slowly returns to normal over the next 1–2 h. These morphological changes were seen in 97 ± 0.01% of the cells examined, while only 4 ± 0.9% of untreated cells were morphologically similar to the mAb AA4–treated cells. Unlike the changes seen with antigen or mAb BC4, the mAb AA4 induced alterations are not accompanied by histamine release (5). Although Fab fragments of mAb AA4 bind to the cells as seen both in binding studies (5) and by immunofluorescence, no morphological changes were observed with Fab fragments of mAb AA4. In addition, four other mAb to non-receptor proteins on RBL-2H3 cells were also tested. These mAb all bind approximately the same number of sites as mAb BC4 (4 × 10^5). None of these antibodies had any effect on the morphology of the RBL-2H3 cells. No morphological changes were observed when the RBL-2H3 cells were treated with normal mouse IgG or IgE instead of mAb AA4, or when the experiments with mAb AA4 were done at 4°C or in the absence of calcium. In the present study, the morphological changes could be produced only by binding mAb AA4 or by activation of the FcεRI. Moreover, both mAb AA4 and FcεRI activation produced similar results.

The cross-linking of the FcεRI proteins with either antigen or anti-receptor antibodies often results in the clustering of these receptors and their internalization (27, 34, 35). It was, therefore, important to investigate whether the morphological changes accompanying mAb AA4 binding affects its dis-
distribution or results in its internalization. Following binding, the mAb AA4 is uniformly distributed on the cell surface (Fig. 3). Even as the cells undergo morphological changes, mAb AA4 retains its original distribution. Furthermore, unlike the results obtained with antigen or mAb BC4, binding of mAb AA4 to the cell surface does not stimulate endocytosis (Fig. 4) and mAb AA4 remains evenly distributed on the cell surface with little or no internalization. The apparent lack of internalization was confirmed using 125I-labeled mAb AA4 and mAb BC4. Following a 30-min incubation of RBL-2H3 cells with the 125I-labeled antibodies, 95.33 ± 0.94% of the mAb AA4 remained on the surface while only 38 ± 14.31% of the mAb BC4 was still on the surface. Although FceRI activation normally results in internalization of the receptor, under certain conditions mast cells will degranulate without receptor internalization (27, 34). Previously, it had been demonstrated that mAb AA4 binding to RBL-2H3 cells involves both of its Fab binding regions (5). Therefore, the lack of internalization is not because of the inability of mAb AA4 to cross-link gangliosides on the surface (5).

Figure 3. Surface distribution of mAb AA4 on RBL-2H3 cells. Cells which were fixed and then stained with mAb AA4 followed by rhodamine-anti-mouse IgG (A) are spindle shaped and the mAb AA4 is distributed evenly on the cell surface. When the cells were treated with 10 μg/ml AA4 for 30 min at 37°C and then fixed and stained with rhodamine-anti-mouse IgG (B) there is a dramatic change in cell shape, but the mAb AA4 is still evenly distributed over the cell surface.

Figure 4. mAb AA4 does not induce endocytosis. To measure endocytosis, cells were exposed to Lucifer yellow for 30 min either in the presence or absence of antigen or antibody before fixation. There is little uptake of the dye in control (A) or in mAb AA4 (D) treated cells when compared to antigen (B), or mAb BC4 (C) stimulated cells.
Figure 5. Changes in F actin staining induced by mAb AA4. Cells were treated with 10 μg/ml AA4 at 37°C and at varying time intervals fixed and stained with FITC-Phalloidin. At 0 min (A) actin is localized to a band just under the plasma membrane. A few cells are already beginning to show some ruffling. By 3 min (B) most of the cells now show some ruffling. Many of the cells are beginning to spread. At 5 min (C) all of the cells show a significant degree of ruffling and are spreading over the surface of the culture dish. By 30 min (D), the morphological changes are complete, and the cells are spread over the culture dish. Actin is localized in ruffles on the apical surface and in punctate areas on the basal surface. The response to mAb AA4 is concentration dependent. When the cells are treated with 5 μg/ml mAb AA4 for 30 min (E), the cells are not spread quite as much. At 1 μg/ml (F), the cell morphology is less affected by the antibody, and many cells are still spindle shaped.

Since mAb AA4 induced such striking morphological changes in the cell surface, the effects of mAb AA4 on the cytoskeleton were examined. Staining the cells for F actin (Fig. 5) showed that the redistribution of actin began as soon as the cells were exposed to the antibody. This redistribution continued for 20–30 min, by which time the morphological changes were complete with 97.7 ± 0.5% of the cells exhibiting a redistribution of their actin. The degree of change was dependent upon the concentration of mAb AA4 (Fig. 5). With time, the actin became concentrated in the apical sur-
The mAb induces redistribution of cytoskeletal elements. When untreated cells (A) are stained with FITC-phalloidin, the staining is primarily associated with the plasma membrane. In cells which have been treated with mAb AA4 for 30 min (B), the staining for actin is concentrated in the ruffles at the apical surface and in punctate areas at the basal surface. In untreated cells stained for tubulin (C), microtubules in the cell body and cytoplasmic processes stain. After exposure to mAb AA4 for 30 min (D), there is a dramatic rearrangement of the cytoskeleton and microtubules can be seen radiating from microtubule organizing centers to the cell periphery. In untreated cells (E), intermediate filaments in the cell body and cytoplasmic process are stained. After 30 min exposure to mAb AA4 (F), the intermediate filaments are seen in bundles throughout the cells.

face ruffles (Fig. 6 b) and in punctate contact points associated with the basal plasma membrane. When cytoskeletal extracts were subjected to SDS-PAGE and Coomassie blue staining, mAb AA4 binding resulted in a rapid polymerization of actin which was similar to that seen following antigen induced secretion (39). The time course of the actin polymerization paralleled the morphological changes. The intracellular distribution of two other cytoskeletal components, microtubules and intermediate filaments, was also examined (Fig. 6). The mAb AA4 binding resulted in a redistribution of both tubulin and vimentin; however unlike actin, there was no obvious association between these cytoskeletal elements.
The mAb AA4 induced the hydrolysis of inositol phospholipids. Following stimulation of RBL-2H3 cells with antigen (α) or anti-receptor antibody mAb BC4 (β), there is an immediate and sustained release of inositol phosphates into the aqueous phase as compared to controls (α). After exposure to mAb AA4 (γ) there is also a release of phosphoinositide breakdown products into the aqueous phase. However, the magnitude is not great as with activation of the FcεRI. Cells were labeled with [3H]myo-inositol and release of inositol phosphates into the aqueous phase was measured as outlined in Materials and Methods. Data are expressed as the mean of four experiments ± 1 SD. Antigen induced and mAb BC4 release of inositol phosphates was significant at p < .001 at 5 min and all later time points when compared to untreated cells or mAb AA4–treated cells. Release from mAb AA4–treated cells was also significant at p < .001.

Figure 7. The mAb AA4 induced the hydrolysis of inositol phospholipids. Following stimulation of RBL-2H3 cells with antigen (α) or anti-receptor antibody mAb BC4 (β), there is an immediate and sustained release of inositol phosphates into the aqueous phase as compared to controls (α). After exposure to mAb AA4 (γ) there is also a release of phosphoinositide breakdown products into the aqueous phase. However, the magnitude is not great as with activation of the FcεRI. Cells were labeled with [3H]myo-inositol and release of inositol phosphates into the aqueous phase was measured as outlined in Materials and Methods. Data are expressed as the mean of four experiments ± 1 SD. Antigen induced and mAb BC4 release of inositol phosphates was significant at p < .001 at 5 min and all later time points when compared to untreated cells or mAb AA4–treated cells. Release from mAb AA4–treated cells was also significant at p < .001.

The direct activation of PKC in RBL-2H3 cells with phorbol esters does not result in cell secretion but potentiates the release induced by calcium ionophores (6, 13). Because treatment of RBL-2H3 cells with mAb AA4 and phorbol esters results in similar morphological changes, we investigated the effect of mAb AA4 binding on calcium ionophore mediated histamine release. As has been observed previously, ionophore is an effective stimulus of histamine release in these cells (45), while the addition of mAb AA4 to the cells at concentrations from 1 to 100 μg/ml did not result in histamine release (5). However, mAb AA4 acted synergistically with calcium ionophore to significantly (p < .001) enhance histamine release from RBL-2H3 cells (Fig. 10). The enhancement was dose dependent and was not observed with...
several other mAb that bind to nonreceptor surface proteins on the RBL-2H3 cells. The effect of mAb AA4 was immediate as indicated by the fact that the mAb and the ionophore could be added together to the cells to produce this effect. In time course experiments, cells treated with mAb AA4, washed, and then challenged with calcium ionophore had enhanced release for up to 6 h after the addition of mAb AA4. Therefore, while mAb AA4 alone does not stimulate histamine release, it can augment the release induced by the calcium ionophore.

**Discussion**

The present study has demonstrated that binding of the mAb AA4 to RBL-2H3 cells produces changes similar to those seen with FcεRI activation. Morphological changes include ruffling of the cell surface and a polymerization and redistribution of cytoskeletal components. Biochemically, binding of mAb AA4 produces a slight, but significant, increase in intracellular calcium, phosphatidyl inositol hydrolysis, and redistribution of PKC. However, unlike FcεRI activation...
increased (p < .001) over ionophore alone. (0, no mAb AA4; ○, 1 µg/ml mAb AA4; ●, 10 µg/ml mAb AA4; ▽, 10 µg/ml mAb AA4).

Figure 10. The mAb AA4 enhances the calcium ionophore A23187-induced histamine release. mAb AA4 and the ionophore were added simultaneously to the RBL-2113 cells. The effect of mAb AA4 in potentiating ionophore induced histamine release is dependent upon the concentration of mAb AA4. Data are expressed as the mean of replicate samples from two experiments ± SD. Histamine release seen with either 1 µg/ml or 10 µg/ml was significantly increased (p < .001) over ionophore alone. (0, no mAb AA4; ●, 1 µg/ml mAb AA4; ▽, 10 µg/ml mAb AA4).

In the case of the RBL-2H3 cells, the cascade of biochemical events induced by mAb AA4 binding to cell surface gangliosides appears identical, though attenuated, to that produced with the FcERI is activated.

In the present study, the binding of mAb AA4 to RBL-2H3 cells had its most pronounced effect on the cytoskeleton. Although little is known about the effect of gangliosides on the cytoskeleton, the biochemical changes induced by mAb AA4 binding could all contribute to a rearrangement of the cytoskeleton. The breakdown of phosphatidyl inositol may be more important than the increase in intracellular calcium in producing changes in the cytoskeleton. The products of phosphatidyl inositol hydrolysis can act directly to exert their effect on the cytoskeleton. In platelets the phosphatidyl inositol cycle is an important factor in the assembly of actin filaments and their association with membranes (12). Likewise, direct activation of PKC by phorbol esters is known to alter cell morphology (8, 20, 28, 40, 43, 46). PKC phosphorylates many intracellular proteins, including a number of cytoskeletal components such as myosin light chain (32, 41, 42, 51), microtubule-associated protein-2 (1, 53), and vimentin (23). The response of RBL-2H3 cells to exposure to mAb AA4 suggests that the generation of inositol phosphates, the increase in intracellular calcium and the extent of PKC activation are sufficient to affect the cytoskeletal rearrangements which accompany secretion, but are not adequate to simulate exocytosis. Thus mAb AA4 may be useful in separating the early events of signal transduction from the later events of exocytosis.

The use of mAbs, such as mAb AA4, could help elucidate the role of these gangliosides in modulating signal transduction. Although the exact mechanism by which binding of mAb AA4 exerts its effects is not known, it is likely that the gangliosides have a physiological relationship to the FcERI similar to that seen for the epidermal growth factor receptor and GM3 (54). The FcERI receptor and the GM3-derived gangliosides may function by association with common intracellular components. These gangliosides are close to the FcERI (5, 16) and the intracellular signals induced by mAb AA4 have similarities to those produced by stimulation of the FcERI. Preliminary studies in our laboratory have shown that there are membrane proteins which are different from the...
known FcεRI components which are associated with the G

DB-derived gangliosides. These membrane proteins could be

important in transmembrane signaling by mAb A44 and

perhaps in linking the gangliosides to the FcεRI.

The authors wish to thank Mrs. Carolyn Tolbert, Mrs. Judy Waters, and Ms. Lynda Weedon for their excellent technical assistance and Mrs. Olevia Ambrose for her skillful photographic help.

Received for publication 3 April 1991 and in revised form 10 September 1991.

References


cent Tetraedroconolphorbol acetate: a noble probe of phorbol ester bind-


specific to the α-subunit of the mast cell’s Fcε receptor block IgE binding and trigger histamine release. J. Immunol. 138:2999–3004.


IgE-induced histamine release from rat basophilic leukemia cell lines:

isolation of releasing and nonreleasing clones. Eur. J. Immunol. 11:

317–323.


Monoclonal antibodies that inhibit IgE binding. J. Biol. Chem. 261:

11823–11831.


sible for the phorbol ester-induced formation of cytoplasmic processes:


USA. 87:1884–1888.


sociation of surface immunoglobulin with the detergent-insoluble cyto-


1984. Ganglioside-mediated modulation of cell growth, growth factor


leukemia (RBL-2H3) cell reveal that antigen-induced signals are not mimicked by the actions of phorbol myristate acetate and Ca2+ in-


sembly in electropermeabilized neutrophils: role of intracellular calcium.


16. Este, K. A. S. 1988. Studies of the interaction between the receptor for immunoglobulin E and other cellular components during signal transduc-


17. Fishman, P. H. 1986. Recent advances in identifying the functions of gan-


18. Guo, N., G. Her, V. N. Reinhold, M. J. Brennan, R. P. Siraganian, and

V. Gimbarg. 1989. Monoclonal antibody A44, which inhibits binding of

IgE to high affinity receptors on rat basophilic leukemia cells, binds to


265:18713–18716.


53. Tsuyama, S., G. T. Bramblett, K.-P. Huang, and M. Flavin. 1986. Calcium/phospholipid-dependent kinase recognizes sites in microtubule-associated protein 2 which are phosphorylated in living brain and are not accessible to other kinases. J. Biol. Chem. 261:4110-4116.
