Binding of Monoclonal Antibody AA4 to Gangliosides on Rat Basophilic Leukemia Cells Produces Changes Similar to Those Seen with Fcε 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 G_{D1b} on rat basophilic leukemia (RBL-2H3) cells. Some of the gangliosides are located close to the high affinity IgE receptor (FcεRI), and binding of mAb AA4 inhibits FcεRI-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 phosphatidyl inositol 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 FcεRI. 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 (FcεRI)[1]. The activation of the receptor results in a complex series of biochemical and morphological events (48). Briefly, when FcεRI 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 phosphatidyl inositol 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 phosphate by mobilizing intracellular calcium pools. Receptor activation also results in calcium influx, activation of phospholipase A2, the phosphorylation of a number of intracellular proteins (7), and the rearrangement of the cytoskeleton before mediator release (35, 37, 48).

The rat basophilic leukemia cell line, RBL-2H3, has been widely used as a model to study FcεRI-mediated events (4, 48, 49). A number of mAbs have been raised against surface molecules on these cells (3, 5, 29, 50). These mAb were selected for their ability to either stimulate or inhibit histamine release in the RBL-2H3 cells. The majority of the antibodies were directed against the FcεRI; however, two antibodies, mAb AD1 and AA4, bind other cell surface components. The mAb AD1 binds to a novel 50–60-kD protein on the surface of the RBL-2H3 cells and modulates histamine release (31). The other antibody, mAb AA4, binds to unique α-galactosyl derivatives of the ganglioside GD_{1b} (18). These gangliosides are closely related to the FcεRI, both spatially and functionally (5). Binding of both the intact mAb AA4 and its Fab fragments to cells inhibits the binding

1. *Abbreviations used in this paper*: DNP_{44}-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; FcεRI, 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 2×10^4 cells per well in plastic, 8-chamber Lab-Tek Chamber Slides (Nunc Inc., Naperville, IL). For studies involving the interactive laser cytometer, cells were plated at a density of 1×10^6 cells in 35-mm culture dishes and for scanning EM, 1×10^4 cells were plated onto 13-mm-round glass coverslips. All cells were 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 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 (Sepherogel TSK DEAE 124; 60-mm inner diam × 15 cm) followed by a gel filtration column (TSK 3000 SWG; 21.5-mm inner diam × 60 cm). Several batches of ascites fluid were also affinity purified on Gamma-bind G Plus (Genex, 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 ^{125}I 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 1×10^5 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, 0.01-0.3 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 (50). The monolayers were then washed and stimulated with the corresponding antigen, dinitrophenol-human serum albumin (DNP_{44}-HSA) at 1.0 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 Ca^{2+} and Mg^{2+} and stimulated in the same medium containing 40 μM EDTA.

Binding and Internalization of Antibodies

To determine cell surface binding, 2.2×10^6 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 5×10^5 CPM of ^{125}I goat anti-mouse IgG. Aliquots of the cell suspension were counted to determine total activity, and other aliquots were layered over bis (2-ethylhexyl) phtalate: dibutyl phtalate (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 ^{125}I-mAb AA4 or 0.5 μg/ml ^{125}I-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 proteinase 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) phtalate: dibutyl phtalate (2:3).

[^{3}H]Myo-inositol Labeling

[^{3}H]myo-inositol labeling was done as previously described (24, 33). The cells were plated at 1×10^5 cells per 22-mm well. After 6 h, the medium was replaced with fresh inositol-free EMEM containing 15% dialyzed FCS and 0.4 μCi/ml [^{3}H]myo-inositol. 18 h later, the cells were washed three times with Pipes ACM (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 25 mM Pipes, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 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 DNP_{44}-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 the 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 of four experiments ± 1 SD. *t* test was performed to determine if significant differences exist between experimental groups.

Measurement of Cytosolic Calcium

For $[Ca^{2+}]_i$ 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 8,000 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 $[Ca^{2+}]_i$ was calculated according to Tsien et al. (52). F_{max} was obtained by lysing the cells with 0.07% Triton X-100, following which F_{min} 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 DNP_{44}-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 glycine and stained for 1 h in 2 μM NBD-dodecanoyl phorbol-13-acetate (Molecular Probes, 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 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

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 returning 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 goat 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. All 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, 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 toluidine blue or alcian blue. Endocytosis was assessed by rinsing the cells twice in EMEM-BSA without phenol red and then adding EMEM-BSA without phenol red containing 1 mg/ml Lucifer yellow (Molecular Probes) for 30 min. The cells were rinsed in PBS at 37°C and fixed for 20 min in 2% formaldehyde at 37°C. All specimens were examined with a Nikon Microphot-FX microscope.

Scanning Electron Microscopy

Monolayer cells on 13-mm round glass coverslips were rinsed twice in PBS at 37°C and fixed for 2–4 h at room temperature in 2% glutaraldehyde (Ladd Research Industries Inc., Burlington, VT) in PBS, and then rinsed and stored at 4°C in 0.1 M cacodylate buffer, pH 7.4. The cells were postfixed for 2 h in 1% buffered osmium tetroxide, rinsed in distilled water, and treated for 10 min with 1% aqueous thiocarbohydrizide (EM Sciences, Fort Washington, PA). The cells were then treated with osmium again for 30 min. The last two steps were repeated once. Following this, the cells were dehydrated in ethanol, critically point dried in liquid CO_2, 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 GD_{1b} (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,

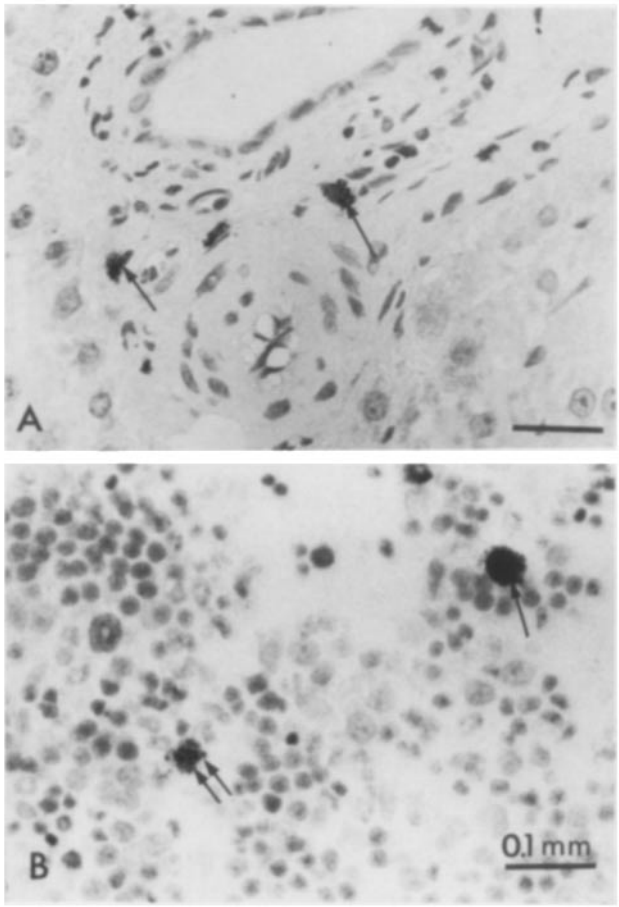


Figure 1. The mAb AA4 stains only mast cells in tissue sections. JB-4 plastic sections were treated with 10 μg/ml AA4 followed by anti-mouse IgG gold. The gold was silver enhanced and the sections counterstained with toluidine blue. In the liver (*A*), reaction product is localized only in mast cells associated with periportal blood vessels. No other cells are stained. However, in bone marrow (*B*) reaction product is associated with identifiable mast cells as well as a population of poorly differentiated cells. Only cells identifiable as mast cells by their staining with toluidine blue or alcian blue were positive with mAb AA4 in all other tissues examined (adrenal gland, brain, duodenum, epididymis, heart, kidney, lacrimal gland, lung, parotid gland, pancreas, skeletal muscle, skin, spleen, sublingual gland, submandibular gland, testis, and thymus).

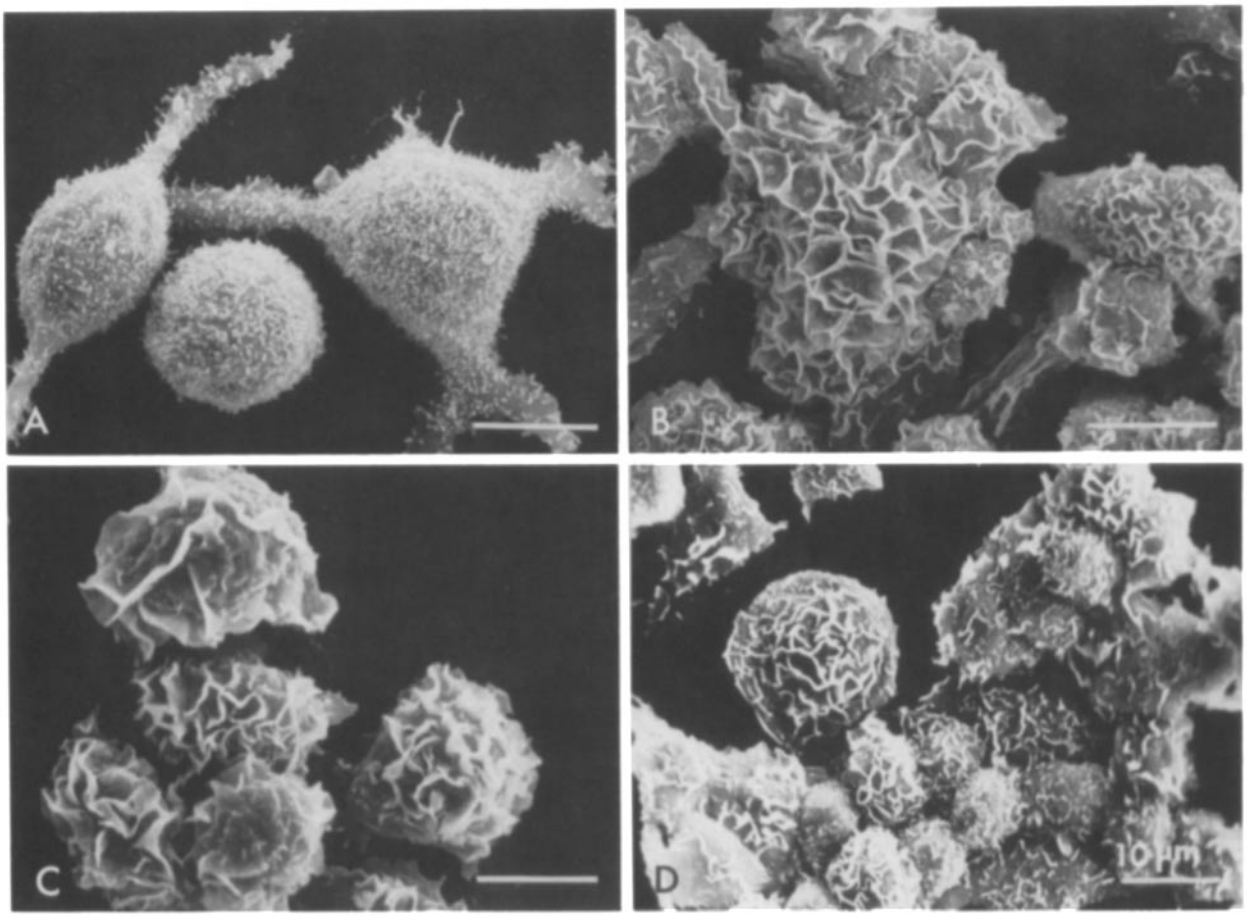
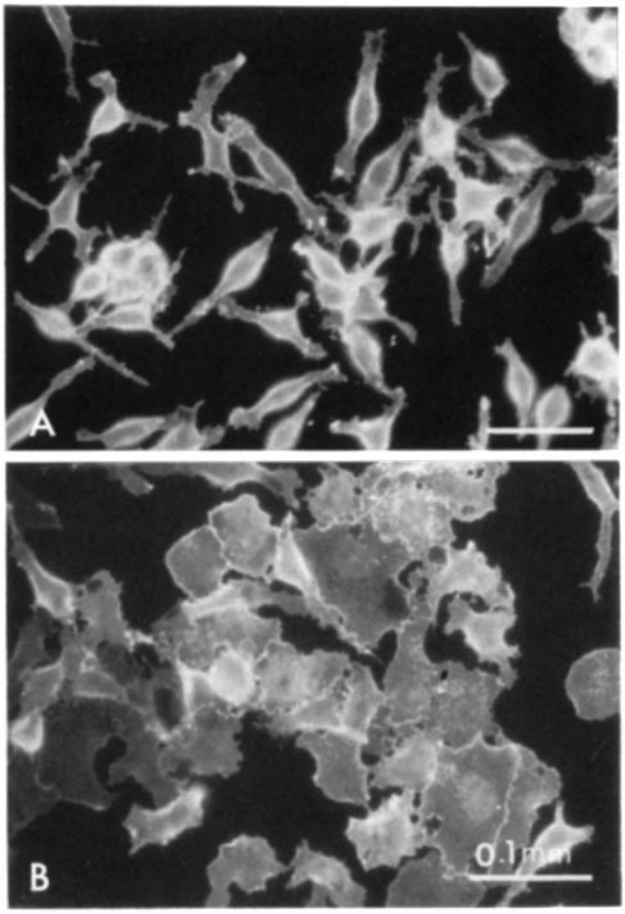


Figure 2. Scanning electron micrographs of activated RBL-2H3 cells. In unstimulated cells (*A*) the surface is covered with short microvilli. After 30-min stimulation with antigen (*B*), anti-receptor antibody, mAb BC4 (*C*), or mAb AA4 (*D*), the cells spread and their surface is ruffled and plicated.

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-FcεRI 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-



tribution 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 ^{125}I-labeled mAb AA4 and mAb BC4. Following a 30-min incubation of RBL-2H3 cells with the ^{125}I-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 FcεRI 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.

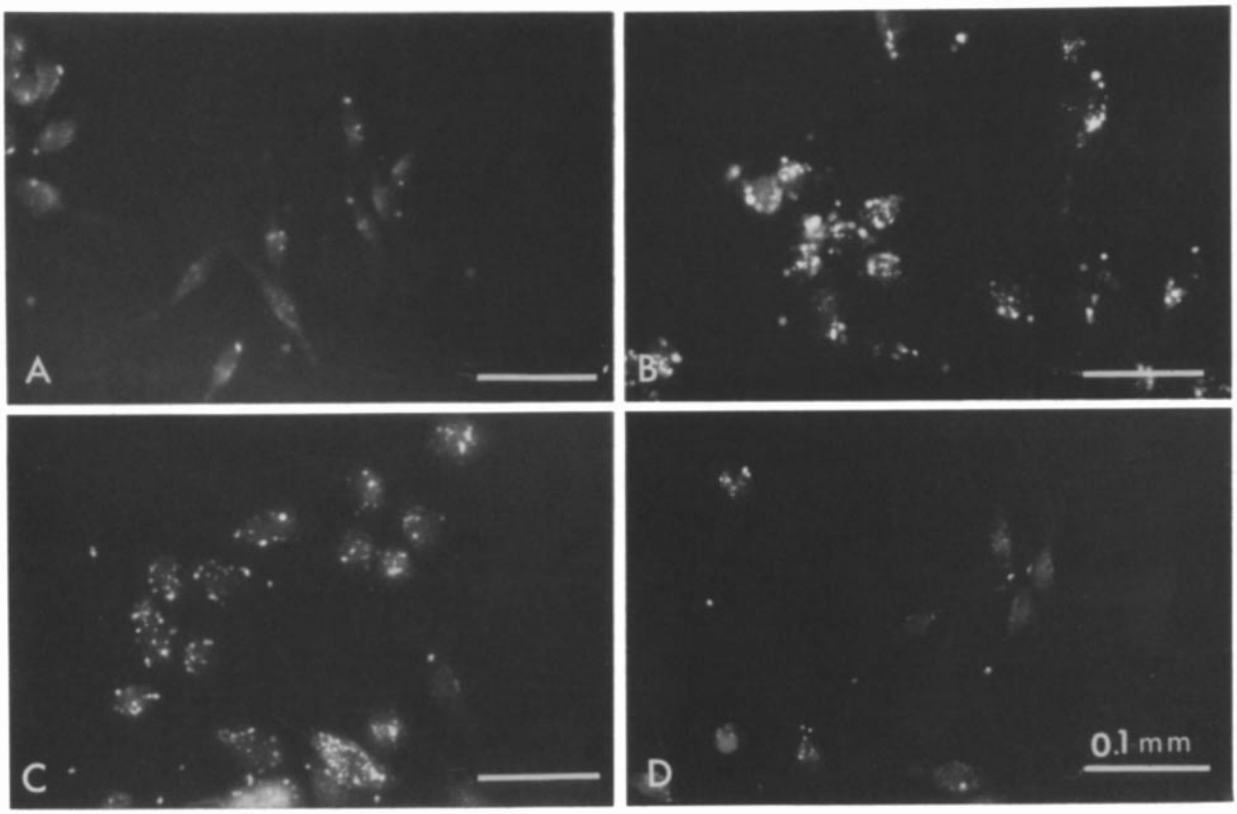


Figure 4. mAb AA4 does not induce endocytosis. To measure endocytosis, cells were exposed to Lucifer yellow for 30 min either in the presence or absence of antigen or antibody before fixation. There is little uptake of the dye in control (*A*) or in mAb AA4 (*D*) treated cells when compared to antigen (*B*), or mAb BC4 (*C*) stimulated cells.

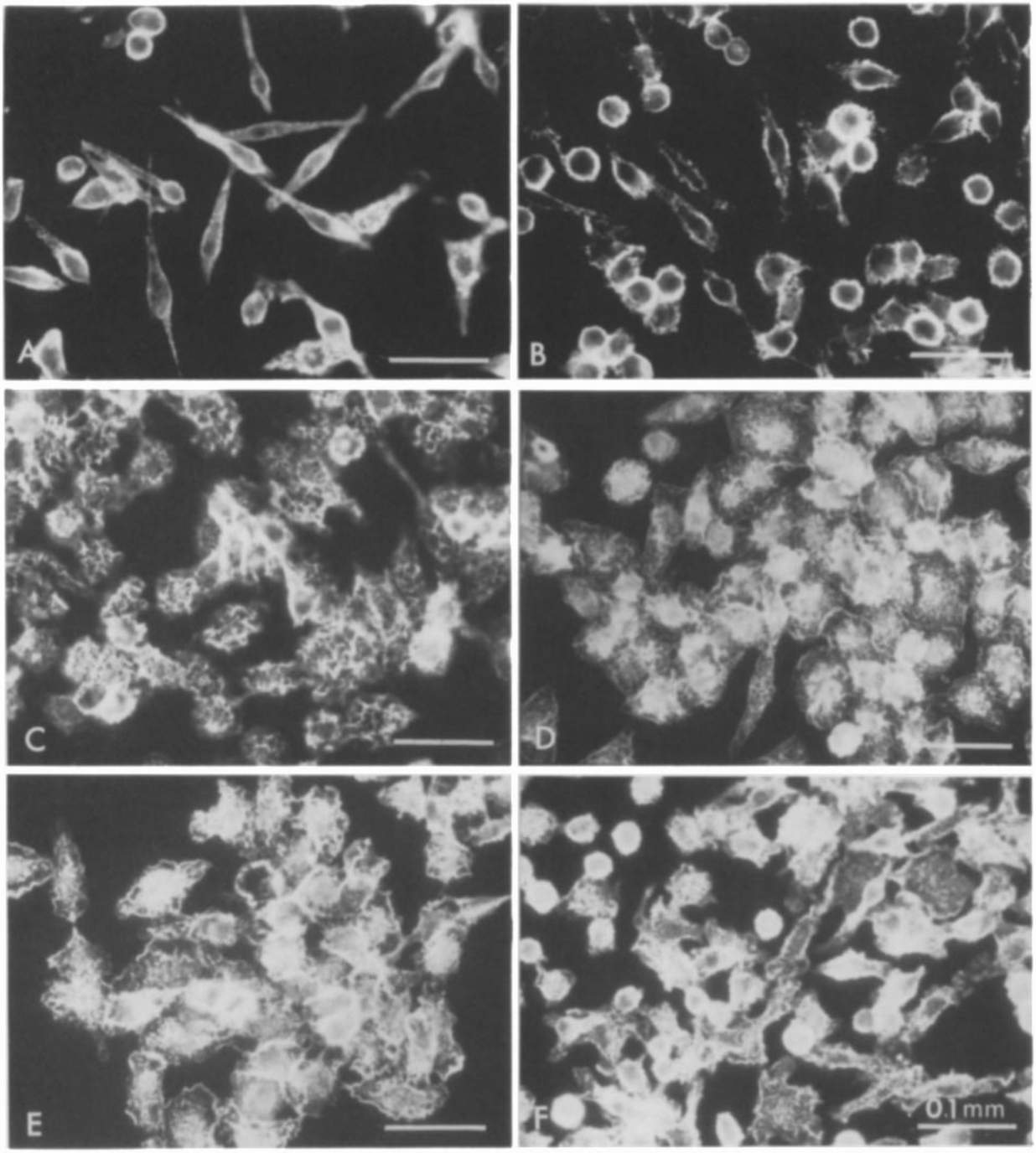


Figure 5. Changes in F actin staining induced by mAb AA4. Cells were treated with 10 μg/ml AA4 at 37°C and at varying time intervals fixed and stained with FITC-Phalloidin. At 0 min (*A*) actin is localized to a band just under the plasma membrane. A few cells are already beginning to show some ruffling. 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-

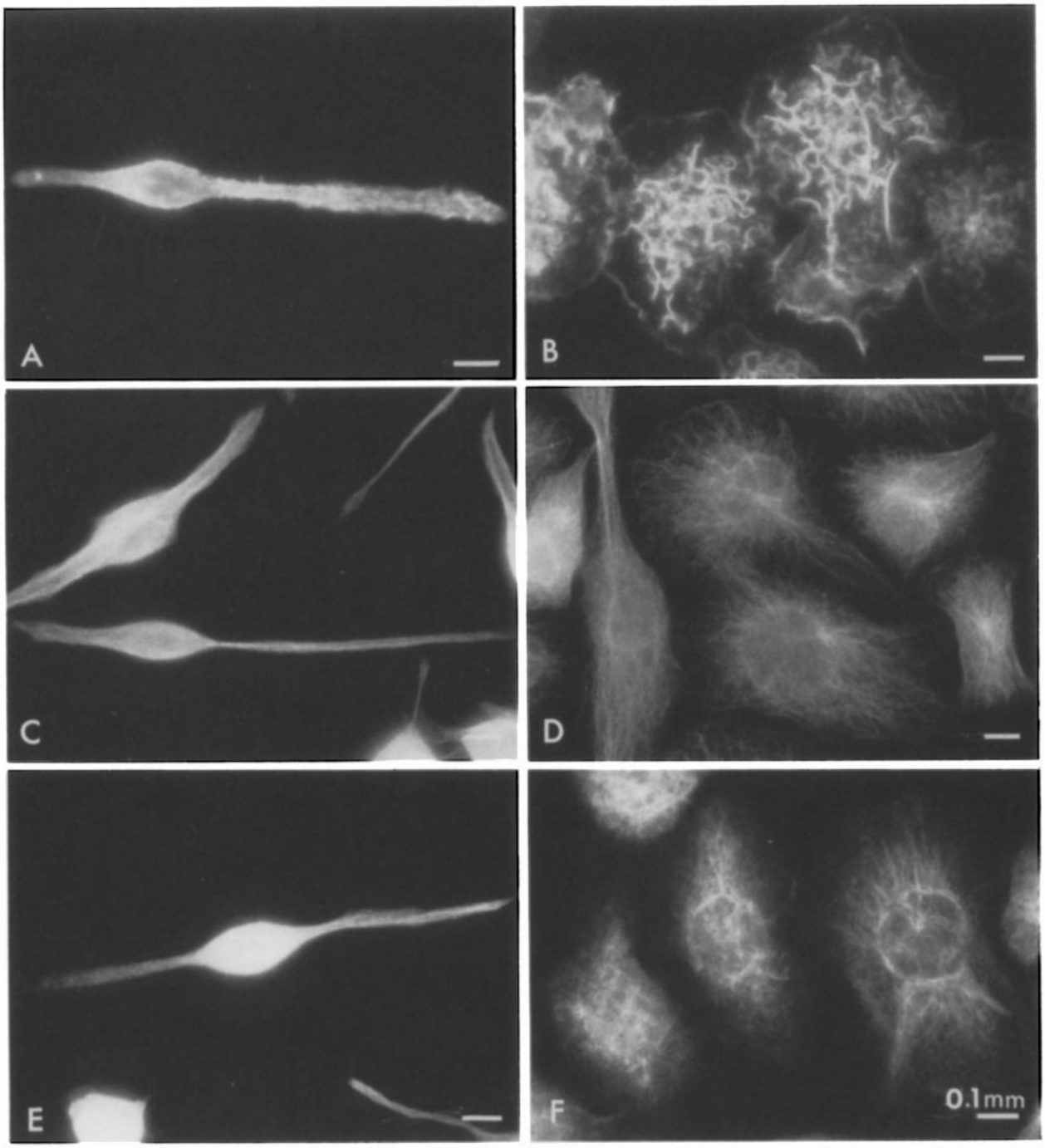


Figure 6. The mAb induces redistribution of cytoskeletal elements. When untreated cells (*A*) are stained with FITC-phalloidin, the staining is primarily associated with the plasma membrane. In cells which have been treated with mAb AA4 for 30 min (*B*), the staining for actin is concentrated in the 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

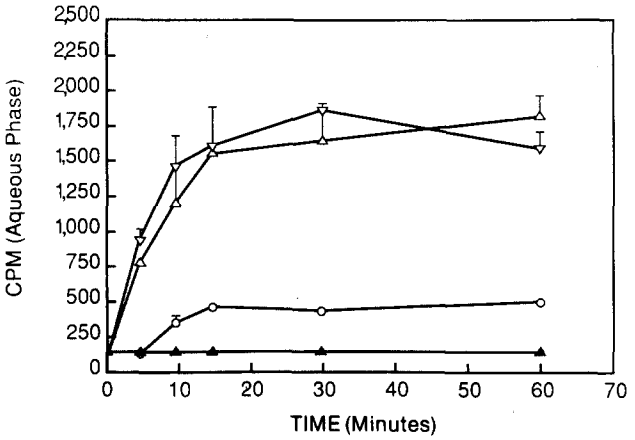


Figure 7. The mAb AA4 induced the hydrolysis of inositol phospholipids. Following stimulation of RBL-2H3 cells with antigen (△) or anti-receptor antibody mAb BC4 (▽), there is an immediate and sustained release of inositol phosphates into the aqueous phase as compared to controls (▲). After exposure to mAb AA4 (○) 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 [^{3}H]myoinositol 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 $p < .001$.

elements and the surface ruffles. Both the tubulin and vimentin were confined to the cell body. Western blots of the cytoskeletal extracts revealed no differences in amount of polymerized tubulin or vimentin between untreated and mAb AA4-treated cells. This cytoskeletal reorganization induced by binding of mAb AA4 is similar to that seen when the FcεRI is activated (39).

These morphological changes induced by binding mAb AA4 are similar to those produced by activation of the FcεRI. Therefore further experiments investigated whether similarities existed between the intracellular signals generated by binding mAb AA4 and those induced following FcεRI activation. When RBL-2H3 cells are stimulated with either antigen or antibody directed against RBL-2H3 cells, there is hydrolysis of the inositol phospholipids (Fig. 7). Treatment of the cells with mAb AA4 also resulted in a significant ($p < .01$) hydrolysis and release of inositol phosphates, although this was considerably less than that seen with antigen or antibody stimulation (Fig. 7). These results suggest that the binding of mAb AA4 caused the hydrolysis of inositol phospholipids and the release of inositol phosphates probably through the activation of phospholipase C.

FcεRI activation with mAb AA4 also results in a rise in the intracellular calcium levels (Fig. 8) in RBL-2H3 cells. Treatment of the cells with mAb AA4 also resulted in a rise in intracellular calcium levels (Fig. 8) as measured by Quin-2 fluorescence. Following the addition of mAb AA4, the resting $[Ca]_i$ levels of 109 ± 6.3 nM increased over 3 min to 149 ± 7.5 nM ($p < .001$). The addition of the anti-receptor mAb BC4 at this point caused a further rise to 402 ± 2.6 nM. The binding of mAb AA4 to the RBL-2H3 cells had little effect on the cells' capacity to respond to stimulation with mAb BC4, and the final intracellular calcium level was not significantly different from when mAb BC4 was used alone. mAb AD1 (29), which binds to a nonreceptor cell surface protein was used as a negative control. mAb AD1 was unable to stimulate an increase in intracellular calcium. In the absence of extracellular calcium in the medium, there was no measurable increase in intracellular calcium following binding of mAb AA4. It is possible that mAb AA4 induces a slight release of intracellular calcium, but that the release is not detectable with the present methods. Therefore, mAb AA4 binding results in a moderate, but significant, increase in the intracellular calcium concentration.

The hydrolysis of phospholipids with the production of diacylglycerol might also result in the activation of PKC as well as producing a rise in intracellular calcium. Phorbol esters, which stimulate PKC, also produce morphological changes similar to those seen with mAb AA4 and like mAb AA4, this stimulation does not result in histamine release. 1-oleoyl-2-acetylglycerol, a synthetic analogue of diacylglycerol, will also produce similar morphologic changes in a concentration dependent manner. Moreover, the mAb AA4 induced changes can be inhibited by 10^{-6} M but not by 10^{-10} M staurosporine, an inhibitor of PKC. Evidence for possible mAb AA4 induced changes in PKC was provided by staining with NBD-dodecanoyl-phorbol acetate (Fig. 9). Changes in fluorescence intensity were observed as early as 5 min in treated cells and reached a maximum at 30 min. No further changes were observed after 30-min exposure to antigen or mAb AA4. In the nonstimulated RBL-2H3 cells (Fig. 9 *A*), the PKC staining is concentrated in the cell body with the highest levels seen in a juxtanuclear position. By 30 min, in antigen stimulated cells (Fig. 9 *B*) or in cells stimulated with antibody directed against the receptor, there is a 48 ± 7.7% drop in average fluorescence intensity after 30 min of stimulation. In contrast, after exposure to mAb AA4 (Fig. 9 *C*) the staining pattern in the cell is similar to that seen in control cells, but there is a 27 ± 2.5% drop in average fluorescence intensity. It appears, therefore, that binding of mAb AA4 did result in significant ($p < .001$) changes in the staining pattern, but these changes were attenuated when compared to the signals induced by activation of the FcεRI. No redistribution of the dye was seen. This may be because of the method used. The dye may not bind to PKC after it is activated. The residual staining may be a result of binding of the dye to unactivated PKC or to other cellular components.

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

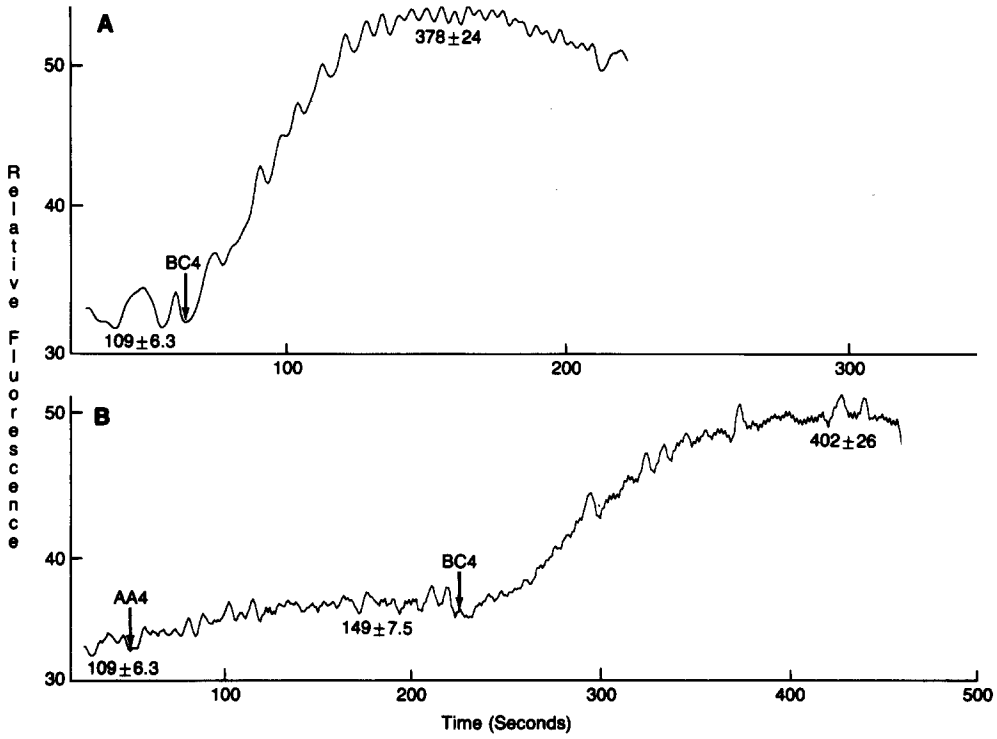


Figure 8. mAb AA4 causes a rise in $[Ca]_i$. Following stimulation of the RBL-2H3 cells with anti-receptor antibody mAb BC4 (*A*), there was an immediate sharp rise in the levels of intracellular calcium from 109 ± 6.3 to 378 ± 24 nM ($p < .001$). After treatment with mAb AA4 (*B*) there was a slow increase in the levels of intracellular calcium to 149 ± 7.5 nM ($p < .01$). The mAb AA4 did not interfere with subsequent stimulation of the cells with mAb BC4, and the levels of intracellular calcium rose to those seen with mAb BC4 alone. Cells were labeled with Quin-2 and $[Ca]_i$ determined as detailed in Materials and Methods. Data are expressed as mean of six experiments ± 1 SD.

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

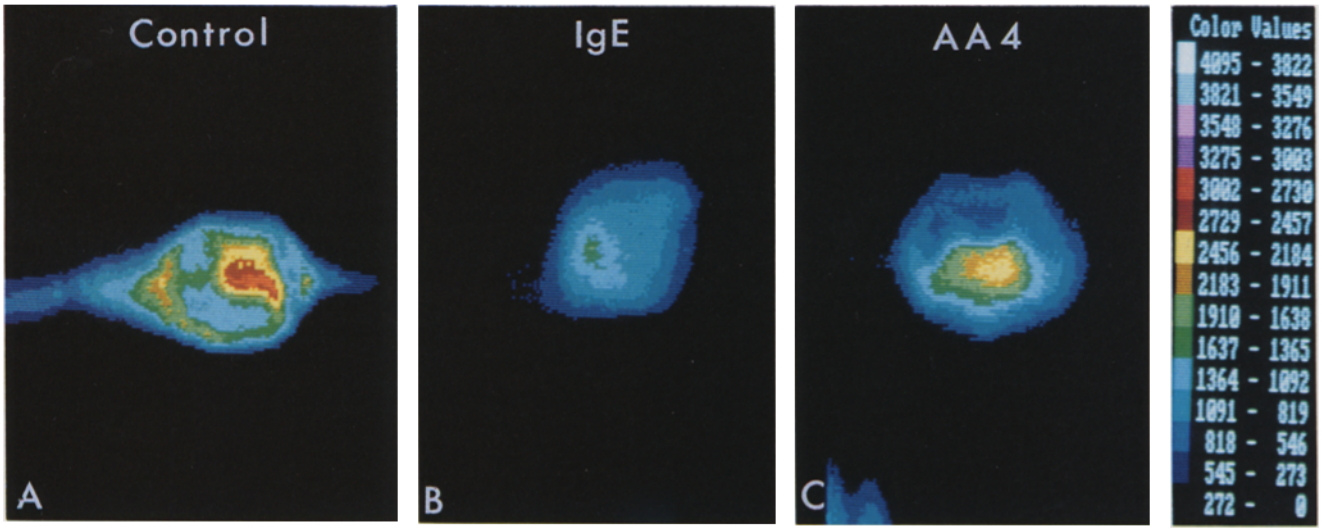


Figure 9. Redistribution of PKC in RBL-2H3 cells. Untreated cells or cells stimulated with antigen or mAb AA4 were stained for PKC with NBD-dodecanoyl-phorbol acetate. In the untreated cells (*A*) the highest intensity of staining is in a juxtanuclear position in the cell body. After FcεRI stimulation with DNP_{44}-HSA for 30 min (*B*), the intensity of staining has dropped dramatically. Following treatment with mAb AA4 for 30 min (*C*), the cells show a drop in fluorescence when compared to untreated cells, but the drop is not as great as that seen in the DNP_{44}-HSA-treated cells.

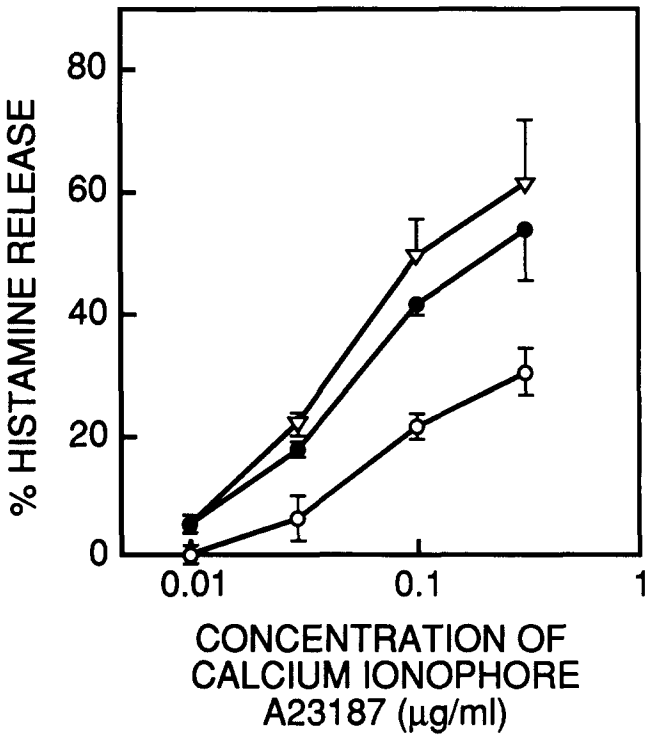


Figure 10. The mAb AA4 enhances the calcium ionophore A23187-induced histamine release. mAb AA4 and the ionophore were added simultaneously to the RBL-2H3 cells. The effect of mAb AA4 in potentiating ionophore induced histamine release is dependent upon the concentration of mAb AA4. Data are expressed 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. (○, no mAb AA4; ●, 1 μg/ml mAb AA4; ▽, 10 μg/ml mAb AA4).

these changes are not accompanied by histamine release, although mAb AA4 does act synergistically with the Ca^{2+} ionophore A23187 to augment histamine release.

The morphological changes in RBL-2H3 induced by mAb AA4 binding are virtually identical to those seen when the FcεRI is activated by antigen or anti-receptor antibodies (39, 45). With the exception of changes induced by either mAb AA4 or the phorbol esters, these changes in the RBL-2H3 cells are strictly associated with secretion. In a survey of 10 variant lines of RBL-2H3 cells, only those cell lines which released histamine following FcεRI stimulation showed any morphological changes (unpublished observations). The changes in the cytoskeleton that accompany cell secretion are early events and precede actual histamine release (45). In RBL-2H3 cells (44), as well as in other cell types (9, 55), receptor-mediated cell activation results in the association of the receptor with cytoskeleton. Studies are now in progress to determine if binding of mAb AA4 to cells also induces an association of the FcεRI with the cytoskeleton.

The biological importance of gangliosides in cellular regulation is well recognized (17, 19, 25, 26). Gangliosides are known to be receptors for bacterial toxins, function in cell–cell and cell–substratum interaction as well as acting as modulators of transmembrane signaling. They can also regulate differentiation and serve as differentiation markers in various cell types. Gangliosides can also modulate receptor function (10, 11, 19). For example, gangliosides inhibit the tyrosine protein kinase activity of the epidermal growth factor receptor, the insulin receptor, and the platelet-derived growth factor receptor. Dimerization or oligomerization of the epidermal growth factor receptor is considered essential for tyrosine kinase activation, and GM_3 will prevent the dimerization of the receptor. Recently, using ganglioside deficient cells, Weis and Davis (54) demonstrated that the autophosphorylation of the receptor is regulated by gangliosides. The ability of antibodies directed against gangliosides to produce biological effects is not as well characterized. Antibodies to gangliosides produce an autoimmune disorder which closely resembles experimental allergic encephalomyelitis (36), inhibit the growth of BALB/3T3 cells and hamster fibroblasts (31), and enhance the response of T lymphocytes to various stimuli (21, 22). Similar to the effects seen with the RBL-2H3 cells, antibodies binding to gangliosides have been shown to increase cGMP levels, activate PKC (21, 22), stimulate calcium influx (15), and inhibit cAMP production (30) in other cell types. However 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 FcεRI 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 (14, 47). The products of phosphatidyl inositol hydrolysis can act directly to exert their effect on the cytoskeleton. In platelets the phosphatidylinositol 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 FcεRI similar to that seen for the epidermal growth factor receptor and G_{M3} (54). The FcεRI receptor and the G_{D1b}-derived gangliosides may function by association with common intracellular components. These gangliosides are close to the FcεRI (5, 16) and the intracellular signals induced by mAb AA4 have similarities to those produced by stimulation of the FcεRI. 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_{D1B}-derived gangliosides. These membrane proteins could be important in transmembrane signaling by mAb AA4 and perhaps in linking the gangliosides to the FcεRI.

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