Short-Term and Long-Term Cytokine Release by Mouse Bone Marrow Mast Cells and the Differentiated KU-812 Cell Line Are Inhibited by Brefeldin A

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_J Immunol_ 1998; 161:2541-2551; http://www.jimmunol.org/content/161/5/2541
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Mast cells and basophils produce a wide range of cytokines, including large amounts of both IL-6 and granulocyte-macrophage CSF (GM-CSF). However, the route by which cytokines are secreted is poorly understood. In the current study, we used two inhibitors of vesicular transport, brefeldin A and monensin, to examine the routes of secretion of IL-6 and GM-CSF in the differentiated KU812 human cell line and cultured mouse bone marrow mast cells (mBMMC). Studies of cytokine production over 6 to 24 h demonstrated that IL-6 and GM-CSF release from both cell types were inhibited by brefeldin A (BFA) following activation with calcium ionophore, A23187. Monensin had similar inhibitory effects to that of BFA on the initial and ongoing IL-6 release from KU812 cells. In contrast, the amount of each cytokine remaining within the cells was significantly enhanced. Similar results were obtained following IgE-mediated activation of mBMMC. BFA significantly inhibited both the constitutive secretion of IL-6 and the immediate ionophore-induced increase in IL-6 release from KU812 cells at 20 min postactivation. However, treatment with these agents did not alter the release of histamine and β-hexaminidase from either mBMMC or KU812 cells. These studies suggest that both the initial 20-min release of IL-6 and secretion of IL-6 and GM-CSF over up to 24 h by mBMMC and differentiated KU812 cells occur predominately through a vesicular transport-dependent mechanism, and that little, if any, IL-6 and GM-CSF is released through degranulation. The Journal of Immunology, 1998, 161; 2541–2551.

Materials and Methods

Mast cell source and activation

The human basophilic cell line, KU812, was maintained in RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 10% FCS, 2 mM l-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 50 μM 2-ME. The cells were passaged twice per week. For further differentiation under conditions very similar to those that have previously been reported to lead to a more differentiated

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*Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; BFA, brefeldin A; mBMMC, bone marrow-derived mast cell; HSA, human serum albumin; mBMMC, mouse bone marrow-derived mast cell.*
phenotype (25) and that lead to enhanced expression of both GM-CSF and IL-6 following stimulation (Marshall et al., unpublished), the cells were cultured in the above medium further supplemented with 0.3 mM sodium butyrate (Sigma, St. Louis, MO) for 3 days, followed by 3 or 4 days of culture in the sodium butyrate-containing medium with the addition of 50 U/ml human rIFN-γ (Genzyme, Cambridge, MA). To verify the differentiation effect, KU812 cells, human mast cell tryptase was measured in the cells using a RIA (Pharmacia, Uppsala, Sweden). After culture, differentiated KU812 cells were washed twice and resuspended in experimental medium before analysis. The experimental medium consisted of RPMI 1640 without phenol red, 5% FCS, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM CaCl2, and 100 μg/ml soybean trypsin inhibitor (Sigma). 

mBMMC were cultured from male C57BL/6 mice, 6 to 8 wk old, housed in the Central Animal Facility at McMaster University (Hamilton, Ontario, Canada). All experimental procedures were approved by Animal Research Ethics Board of McMaster University. The mice were sacrificed by cervical dislocation. Intact femurs and tibias were removed from mice. The marrow cells were harvested by repeated flushing of the bone shaft with endotoxin-free RPMI 1640 medium (Life Technologies). The bone marrow cell culture was established at a concentration of 105/ml in medium consisting of RPMI 1640 (Life Technologies), 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, and 10% WEHI-conditioned medium as a source of IL-3 (26). Nonadherent cells were transferred to fresh culture medium once per week. After 4 to 6 wk of culture, the majority of mast cells from the mouse bone marrow culture was >95%, as confirmed by toluidine blue and Acanth blue/safranin O staining of cytocentrifuge preparations. Before experiments, the mBMMC were washed twice and resuspended in the same experimental medium as used for KU812 cells.

A stock solution of BFA (Sigma) was prepared of 1 mg/ml BFA in ethanol, and a monensin (Sigma) stock solution was made of 10 mM monensin in ethanol, both of which were stored at −20°C until use. Either differentiated KU812 cells or mBMMC at 1 × 105/ml were exposed to BFA, or monensin at the designated concentrations, or medium control for 5 min at 37°C before being activated with calcium ionophore. A23187 (Sigma), at a range of concentrations from 0.1 to 1 μM at 37°C for either 20 min or times up to 24 h (as described within Results for individual experiments), and were then centrifuged at 800 rpm for 10 min. After the supernatant was collected, the pellets were resuspended in the original volume of experimental medium, and disrupted by repeated freezing and thawing, or sonication. Both supernatant and pellet samples were stored at −20°C for later analysis. A23187 induces degranulation of mast cells, and has no significant cytotoxic effect on cells. Differentiated KU812 cells or mBMMC at the levels used in this study, BFA and monensin also have no toxic effects on the two cell lines, as assessed by trypan blue vital staining for cell viability.

IgE-dependent stimulation of mBMMC

mBMMC after 4 to 6 wk in culture and having over 95% purity were incubated for 24 h with culture medium containing DNP-specific mouse IgE (27). The cells were washed four times to remove any unbound IgE, then incubated in medium without DNP for 5 min at 37°C, followed by incubation with DNP-human serum albumin (DNP-HSA; Sigma) at a range of concentrations for 24 h. Supernatant and pellet samples were collected for cytokine assays using a similar methodology to the differentiated KU812 cell studies described above.

B-9 bioassy

IL-6 bioactivity in both mBMMC and differentiated KU812 cell experiments was measured by B-9 hybridoma proliferation assay (28). Briefly, B-9 cells were cultured in MEM F-11 (Life Technologies) supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, and a supernatant source of IL-6. The IL-6 assay was performed in triplicate for each sample and standards in microtiter plates (Nunc Intercon Med, Nunc, Roskilde, Denmark). After a 72-h culture of B-9 cells (2500/well) with samples and standards, 10 μl/well of 0.5% MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium; Sigma) was added, followed by 50 μl/well of 10% Triton/HCl. The plates were read at 570 nm on an ELISA reader. IL-6 values were expressed as U/ml, in which 1 U/ml is equivalent to approximately 0.45 pg/ml IL-6. The limit of detection for IL-6 was 10 U/ml. BFA and soybean trypsin inhibitor were checked for their effects on B-9 cell proliferation in the presence of IL-6 standards. These chemical compounds at the concentrations used in this study had no significant effects on B-9 bioassy. A23187 at the concentrations in this study was previously confirmed to have no significant effects on the IL-6 bioassay (8).

β-hexosaminidase and histamine release assay over 20 min

Briefly, 1 × 106 differentiated KU812 or mBMMC cells/ml in HEPES-Tyrode’s buffer were incubated for 20 min at 37°C in the presence of or absence of BFA or monensin at designated concentrations with or without 1 μM A23187, then the cells were centrifuged at 800 rpm for 10 min at 4°C. After collection of supernatant, the pellets were resuspended in the original volume of medium. Both supernatant and pellets were boiled for 5 min to inactivate histaminase and stored at −20°C for histamine assay. The modified HEPES-Tyrode’s buffer was prepared, as follows: 137 mM NaCl, 2.7 mM KCl, 0.5 mM NaH2PO4, 1 mM CaCl2, 10 mM HEPES, plus 0.1% BSA, pH 7.3. Histamine levels were measured using a radioenzymatic histamine assay (29). Histamine release was expressed as the percentage of the total cellular histamine content calculated by the following formula: histamine in supernatant/histamine in supernatant + histamine in pellet × 100.

β-hexosaminidase assay was conducted using a previously reported method (30). Supernatant and pellet samples obtained as described above were examined without boiling. A total of 50 μl of samples in duplicates was incubated with 50 μl of 1 mM p-nitrophenyl-N-acetyl-β-glucosaminide (Sigma) dissolved in 0.1 M citrate buffer, pH 5, in a 96-well microtiter plate at 37°C for 1 h. The reaction was stopped with 200 μl/well of 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 nm in an ELISA reader. The net percentage of β-hexosaminidase release was calculated, as follows: β-hexosaminidase in supernatant/β-hexosaminidase in supernatant + β-hexosaminidase in pellet) × 100.

GM-CSF and IL-6 ELISA assays

Human GM-CSF levels in supernatant and pellets were measured either using commercially available ELISA kits (R&D Systems, Minneapolis, MN) for experiments examining release of this cytokine over 20 min (minimum detection level 2.8 pg/ml for human and 1 pg/ml for mouse) or with an in-house ELISA assay for studies of the time course of GM-CSF production. Briefly, the in-house ELISA involved coating wells of a 96-well ELISA plate with anti-human GM-CSF Ab (Genzyme) at 1 μg/ml for 16 to 20 h at 4°C. Nonspecific binding to the plates was blocked by a 1% BSA, 0.1% Tween-20 solution in PBS for 1 h at 37°C. A total of 50 μl/well of GM-CSF standards (human rGM-CSF; R&D Systems) and samples was added to the plate and incubated for 18 to 20 h at 4°C. Biotinylated anti-human GM-CSF (0.2 μg/ml) (Endogen, Woburn, MA) was added to each well and incubated for 1 h at 37°C. This was followed by 50 μl/well of a 1/2000 dilution of streptavidin-alkaline phosphatase solution (Life Technologies) for 30 min at room temperature and detection of alkaline phosphatase signal using a commercial ELISA amplification system (Life Technologies), according to manufacturer’s instructions. The minimal detectable dose was 3 pg/ml for human GM-CSF using this system. In the case of differentiated KU812 cells treated with monensin, the IL-6 assay was also conducted with purchased ELISA kits (Amersham Life Science, Buckinghamshire, U.K.), since the growth of B-9 cells was sensitive to monensin inhibition. The minimal detectable dose of IL-6 using this system was 1 pg/ml.

Pulse-chase experiments

Pulse-chase experiments were performed according to an established protocol (31). Briefly, differentiated KU812 cells at a concentration of 1 × 106/ml were starved for 1 h at 37°C in t-leucine-deficient RPMI 1640 medium. The cells were washed once and pulsed for 1 h with 20 μCi [1H]-t-leucine (Amersham, Buckinghamshire, U.K.) in cold t-leucine-free RPMI 1640 medium. BFA at the concentrations stated in Results was present throughout the pulse-chase period. The cells were washed once with cold 0.02% NaN3/0.01% BSA/PBS before precipitation of total cellular protein with cold 10% TCA in PBS for 30 min. The radioactivity associated with the cellular protein was assessed using a scintillation counter.

Semiquantitative RT-PCR

Differenitated KU812 cells at a concentration of 1 × 106/ml were incubated at 37°C with or without 0.1 μg/ml BFA for either 6 or 12 h. Total RNA was extracted from the cells using TRizol reagent (Life Technologies), according to manufacturer’s instructions. RT-PCR procedure was performed according to the method of Eichmann et al. (31) with minor modifications. Primer sequences for human IL-6 and GM-CSF were as reported in the literature (32). β-actin primer sequences were: 5’ primer, ACA TCC GCA AAG ACC TGT ACG, and 3’ primer, TTG CTG ATC CAC ATC TGC TGG. One microgram of total RNA was used as a template for cDNA synthesis in the presence of 5 μM of random hexamer and

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200 U of Moloney Leukemia virus reverse transcriptase (Life Technologies) in a final volume of 20 μl at 37°C for 1 h. PCR was conducted with 5 μl of cDNA product in the presence of 1 U of Taq polymerase (Life Technologies) and 2 μM of specific primers in a final volume of 50 μl, as follows: 94°C for 1 min, 57°C for 2 min, and 72°C for 2 min, with 36 cycles for IL-6, 38 cycles for GM-CSF, and 24 cycles for β-actin in a PTC-100 thermocycler (MJ Research, Watertown, MA). These cycle numbers were determined previously to generate PCR products at the exponential phase of amplification. Ten microliters of PCR products were run on 1.8% agarose gels in the presence of ethidium bromide and visualized with a transilluminator (Doc 1000; Bio-Rad, Hercules, CA). The OD of the PCR products were normalized against that of β-actin in each sample.

**Electron-microscopy examination**
mBMMC with more than 95% purity were washed three times with bone marrow culture medium, then resuspended in experimental medium, followed by the addition of either further experimental medium as control or one of the following: 1 μM A23187, 0.5 μg/ml BFA, or 1 μM A23187 plus 0.5 μg/ml BFA, and incubated at 37°C for 3 h. BFA was added 5 min before A23187. The final cell concentration was 1×10⁶/ml. After incubation, the supernatant was collected for cytokine assays, and the cells were washed three times with the bone marrow culture medium, and finally resuspended in 0.5% glutaraldehyde, and sent to the electron microscopy laboratory at McMaster University for routine processing. Electron-microscopic examination was carried with 1200EX Biosystem (JEOL, Tokyo, Japan).

**Statistical analysis**
The response of samples of the same initial preparations to different treatments was compared using a Student’s t test for IL-6, GM-CSF, β-hexosaminidase, and histamine release.

**Results**

**BFA and monensin inhibit IL-6 release at 20 min postactivation, but have no effect on degranulation of differentiated KU812 cells**

KU812 cells, originally established as a basophilic cell line, have the potential to develop into more differentiated cells with some mast cell characteristics when treated with a variety of agents, including TNF-α or stem cell factor (25, 33). Human mast cell tryptase, a mast cell marker found in very low or undetectable levels in basophils, had been detected in such differentiated cells (33). In this study, tryptase levels were measured in KU812 cells differentiated with sodium butyrate and human rIFN-γ by using a RIA (Amersham). The average concentration was 6 ± 0.6 μg/10⁶ cells (mean ± SEM, n = 8), less than the normal tryptase concentration of human mast cells (10 to 35 μg/10⁶ cells) (34), but greater than the amounts observed in normal basophils (0.04 μg/10⁶ cells) (35). To examine the effects of BFA and monensin on degranulation and cytokine release, the differentiated KU812 cells at a concentration of 1×10⁶/ml were activated with 1 μM A23187 with or without a range of doses of BFA or monensin. Differentiated KU812 cells were treated in parallel with BFA/monensin or medium alone as negative controls. BFA at doses of 0.01 μg/ml to 1 μg/ml alone or in combination with A23187 had no significant effect on either histamine or β-hexosaminidase release from differentiated KU812 cells (Fig. 1A) (p > 0.05). Monensin at the same dosage range as BFA also did not exhibit significant inhibition of differentiated KU812 cell degranulation induced by A23187, e.g., cells treated with 1 μM monensin plus 1 μM...
A23187 had a similar percentage β-hexosaminidase release (34 ± 0.8%) as that of cells treated with 1 μM A23187 alone (36 ± 0.8%, p > 0.05, n = 4).

When cytokine release at 20 min was assessed by B-9 bioassay, A23187 was found to significantly enhance IL-6 release (p < 0.001) from KU812 cells that endogenously produce a substantial amount of IL-6, while BFA alone significantly decreased IL-6 release (p < 0.01) (Fig. 1B). When BFA and A23187 were used in combination, there was a dose-dependent response to BFA with a significant reduction of IL-6 release at the dose of 1 μg/ml (p < 0.001) and 0.1 μg/ml (p < 0.01), but not at 0.01 μg/ml (p > 0.05). There was also a dose-dependent increase in IL-6 remaining within differentiated KU812 cells treated with 0.1 μg/ml and 1 μg/ml BFA in combination with A23187 (p < 0.001, p < 0.05).

Close examination of the data obtained suggested an inhibitory effect of BFA on IL-6 release over the first 20 min postactivation from differentiated KU812 cells. To confirm this, A23187-induced IL-6 release from KU812 cells. Based on the data of Table I, the inhibition of baseline release of IL-6 by BFA was 22%, as calculated from 39% (medium control release) minus 17% (BFA-treated group release). BFA treatment decreased IL-6 release rate from 80%, as seen in cells treated with A23187 alone, to 48%, as seen in cells treated with BFA and A23187.
together; the difference was 32%, significantly more than the inhibition rate (22%) by BFA alone ($p < 0.05$). A similar comparison of the effect of monensin on A23187-induced and baseline IL-6 release suggested that this agent only significantly inhibited the baseline release of IL-6 over 20 min. As shown in Table I, the difference between the amount of IL-6 release from the A23187 group (80%) and that from the monensin and A23187 group (66%) was 16%, which was similar to the inhibition rate by monensin alone (14%), calculated from 39% (medium group) minus 23% (monensin group).

*BFA and monensin have no effect on the initial 20-min release of granule-associated mediators from mBMMC*
mBMMC were treated similarly to differentiated KU812 cells, i.e., the cells were incubated with 1 μg/ml BFA for 5 min, followed by 1 μM A23187 for another 20 min at 37°C. Supernatant and pellets

![Figure 3](http://www.jimmunol.org/DownloadedFrom)
of KU812 cells and mBMMC when histamine release by BMMC was evaluated (data not shown). No IL-6 release or increase in cell-associated IL-6 was detected in any of the groups). No IL-6 release or increase in cell-associated IL-6 was detected in any of the groups. No IL-6 release or increase in cell-associated IL-6 was detected in any of the groups.

These results confirmed that BFA was also an appropriate agent to examine vesicle transport-dependent mechanisms as distinct from degranulation in mBMMC. The alternate inhibitor of vesicular transport, monensin, also did not alter preformed mediator release from BMMC. A mean β-hexosaminidase release of 19.5 ± 0.14% was observed in cells treated with 0.5 μM A23187 alone, while 19.2 ± 0.20% was released in the presence of A23187 plus 1 μM monensin (n = 4/group). Similar results were obtained when histamine release by BMMC was evaluated (data not shown).

BFA treatment changes in IL-6 secretion and increases the amount of cell-associated IL-6 over up to 24 h of treatment of KU812 cells and mBMMC

Differentiated KU812 cells were treated concurrently with 0.1 μg/ml BFA and 0.5 μM A23187, or with either agent alone for up to 24 h; supernatant and pellet samples were collected at different time intervals; and IL-6 and GM-CSF were measured. BFA was shown to significantly inhibit IL-6 release from differentiated KU812 cells in the presence or absence of A23187 activation at all of the time intervals (Fig. 2A). In contrast, BFA alone or in combination with A23187 significantly increased IL-6 in KU812 cell pellets taken over the full 24-h time course (Fig. 2B).

mBMMC were treated with 0.1 μM A23187 and 1 μg/ml BFA in the same fashion as with differentiated KU812 cells. While there was no detectable IL-6 release or cell-associated IL-6 observed in inactivated cells, BFA significantly inhibited IL-6 release from mBMMC activated with A23187 (Fig. 3A). Analysis of pellet samples demonstrated that BFA significantly enhanced IL-6 accumulation in the BFA-treated mBMMC with or without A23187 activation (Fig. 3B).

Monensin inhibits IL-6 release from KU812 cells over 24 h

To confirm that the inhibitory effect of BFA on the 24-h release of mast cell cytokines was the result of vesicular transport blockade, monensin was used in place of BFA on KU812 cells. Differentiated KU812 cells were treated with 0.1 μg/ml BFA or 0.1 μM monensin alone or in combination with 0.5 μM A23187 at 37°C for 24 h. BFA and monensin alone significantly inhibited IL-6 release from KU812 cells (p < 0.001 and p < 0.01, respectively), and increased cell-associated IL-6 (p < 0.001 and p < 0.05, respectively). BFA and monensin also significantly inhibited IL-6 release (p < 0.001 and p < 0.01, respectively), and increased cell-associated IL-6 by A23187-activated differentiated KU812 cells (p < 0.01 and p < 0.05, respectively, Table II).

BFA blocks Ag-induced IL-6 release from mBMMC

Immunologic activation of mast cells induces a number of cellular signaling processes not observed following ionophore treatment. To examine the effects of BFA on cells activated through IgE cross-linking, mBMMC were incubated with DNP-specific IgE for 24 h, then activated by incubating with DNP-HSA at 37°C for 24 h. Without stimulation, sensitized mBMMC produced very little IL-6, but they produced up to 40,000 U of IL-6/10^6 cells following stimulation with 10 ng/ml DNP-HSA. BFA at a dose of 1 μg/ml, which was not toxic to these cells, significantly inhibited IL-6 release (p < 0.01) and increased the amount of IL-6 that remained cell associated (p < 0.01) in mBMMC activated with DNP-HSA (Fig. 4).

BFA inhibits the release of GM-CSF from differentiated KU812 cells and mBMMC

We examined the ability of BFA to alter GM-CSF secretion over 20 min from both mBMMC and differentiated KU812 cells. Neither BFA nor A23187 treatment had any significant effects on the extremely small amount of GM-CSF release or storage by differentiated KU812 cells (on average less than 5 pg/10^6 cells in either supernatant or pellets was found in any of the groups).

Very low levels (1.5–4.5 pg/ml) of GM-CSF were detected in both supernatant and pellets of mBMMC either cultured in medium or treated with A23187 and BFA alone or in combination for 20 min. Again, there was no significant difference in GM-CSF levels among different treatment groups. Given the lack of a detectable GM-CSF response to ionophore treatment over the initial 20-min activation, we could not assess the effects of BFA for this cytokine over this time period.

After a 24-h incubation of differentiated KU812 cells, low levels of GM-CSF were detected in both supernatant and pellet samples of control cells or cells exposed to 0.1 μg/ml BFA; the difference between the two groups of cells in GM-CSF release or storage was not significant. There was a striking increase in GM-CSF release from the A23187-treated cells (p < 0.01). BFA significantly decreased GM-CSF release (p < 0.01) and increased the amount of cell-associated GM-CSF (p < 0.05) in differentiated KU812 cells stimulated with A23187 (Fig. 5).

mBMMC were also found to produce low levels of GM-CSF after a 24-h incubation with or without 1 μg/ml BFA. A quantity
amounting to 0.1 μM A23187 significantly increased GM-CSF release up to 9.8 ± 1.9 pg/10⁶ cells compared with 1.8 ± 1 pg/10⁶ cells exposed to medium (p < 0.05), while BFA was able to significantly inhibit GM-CSF release from sensitized mBMMC activated immunologically with DNP-HSA (56 ± 6 versus 149 ± 29 pg/10⁶ cells, p < 0.05, Fig. 6).

The effect of BFA on protein synthesis
BFA previously has been shown to inhibit cellular protein secretion while having little or no effect on protein synthesis. In the present study, the effects of BFA on total protein synthesis by KU812 cells were investigated using pulse-chase experiments. Differentiated KU812 cells were treated with or without 0.1 μg/ml BFA for 6 or 12 h, respectively, and were pulse labeled with L-leucine for 1 h. The BFA dose used was the same as that used in the other time-course experiments. At a 6-h time point, BFA had no significant inhibitory effect on total protein synthesis in terms of L-leucine incorporation (Table III), although substantial inhibition of cytokine secretion was observed at this time point (Figs. 2 and 5). At 12 h, significant inhibition of protein synthesis was observed in the presence of BFA (mean 36% inhibition) (Table III), although not sufficient to account for the profound inhibition of cytokine secretion observed at this time point (mean inhibition 98% for IL-6, and >99% for GM-CSF).

Effects of BFA on IL-6 and GM-CSF mRNA levels in differentiated KU812 cells
Semiquantitative RT-PCR was conducted to examine IL-6 and GM-CSF gene transcription by differentiated KU812 cells treated with BFA alone or in combination with A23187 for either 6 or 12 h. Cells were treated with BFA alone (0.1 μg/ml), A23187 alone (5 × 10⁻⁷ M), BFA plus A23187 or media alone for 12 h, and the mRNA from all conditions was isolated and subjected to RT-PCR for IL-6, GM-CSF, and β-actin in parallel (Fig. 7, B and C). To assess the effects of BFA on gene transcription, the ratio of mean pixel density of PCR bands on electrophoresis for each of the
cytokines compared with the bands from β-actin was determined under each condition over a time course up to 24 h postactivation. BFA had no significant inhibitory effects on cytokine mRNA levels, assessed using this method (Fig. 7A). Figure 7, B and C, illustrates the results obtained from four separate experiments after a 12-h incubation of cells in the presence or absence of BFA or A23187. Again, no significant inhibition of mRNA levels was observed in BFA-treated cells compared with controls. Significant (p < 0.05, n = 3) inhibition of both GM-CSF and IL-6 mRNA levels was observed when cells were treated with actinomycin D (10−6 M) in place of BFA (data not shown).

Ultrastructural analysis confirms that BFA does not induce degranulation

Electron-microscopic examination of mBMMC demonstrated that mBMMC cultured in medium alone contained a large number of mature and immature granules, and smaller-sized vesicles in the cytoplasm, with an intact Golgi apparatus. The Golgi apparatus was not visible in mBMMC treated with BFA, as has been reported by other groups working with the same compound (36). The degree of granulation in BFA-treated cells was similar to that of untreated cells (Fig. 8, A and C).

<table>
<thead>
<tr>
<th>Duration of Treatment Prior to Pulse-Chase</th>
<th>Medium (μg/ml)</th>
<th>BFA (0.1 μg/ml)</th>
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<tbody>
<tr>
<td>6 h (n = 8)</td>
<td>12.7 ± 0.70</td>
<td>11.7 ± 0.99</td>
</tr>
<tr>
<td>12 h (n = 12)</td>
<td>16.6 ± 2.19</td>
<td>10.6 ± 1.20***</td>
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*Mean values ± SEM. ††† p < 0.001 compared with medium incubated cells.

A23187 induced marked degranulation, but had little effect on the appearance of Golgi apparatus, as few granules were present in mBMMC following treatment with A23187, while intact Golgi complexes were readily observed in these cells. The Golgi apparatus and the majority of granules were not observable in mBMMC treated with both BFA and A23187 (Fig. 8D).

Discussion

There are two classical pathways for protein secretion from cells, the constitutive and regulated pathways (37, 38). The constitutive
pathway is spontaneous, characterized by continuous and low level protein secretion in a distinct set of small vesicles delivered from the Golgi apparatus to the cell surface. The regulated pathway is induced by secretagogues, featured by immediate and massive release of proteins stored in granules, which after storage in the cell cytoplasm for a period of time, quickly move to fuse with the cytoplasmic membrane and release the granule contents outside of the cell in response to stimulation.

Mast cells and basophils have long been recognized for their characteristic regulated secretion pathway, known as degranulation, by which the cells release preformed mediators, such as histamine, proteoglycans, and a variety of enzymes following activation. In contrast, little is known about the vesicle-dependent secretory pathway of mast cells and basophils, and even less about how cytokines are secreted, although it has been known for many years that these cells are the source for a number of cytokines (3, 4).

To avoid confusion of the term “constitutive production” with what has been known as the “constitutive secretory pathway,” we will use “vesicle-dependent pathway” to refer to the Golgi-mediated vesicular transport-dependent cytokine release from mast cells, and “degranulation-related pathway” to describe the cytokine release due to degranulation and release of stored cytokines from stimulated mast cells.

The main goal for this study was to determine whether the cytokines, IL-6 and GM-CSF, are secreted from mBMMC and differentiated KU812 cells through a degranulation-related pathway or a vesicle-dependent pathway. We found that the differentiated KU812 cells without any stimulation consistently produced and released most of their IL-6 without substantial cytokine storage. The observations that very little IL-6 is normally cell associated and that this cytokine is spontaneously released indicate that IL-6 secretion is unlikely to be due to release by a degranulation-related pathway. However, since ongoing degranulation processes such as piecemeal degranulation have been described (39), it is important to examine the cytokine secretion routes more specifically.

BFA and monensin are fungal metabolites able to rapidly and reversibly block intracellular vesicle transport from endoplasmic reticulum to Golgi apparatus, and therefore, inhibit protein secretion with minimal effect on protein synthesis (23, 24). Both BFA and monensin are Golgi-specific agents, but the former mainly affects the proximal compartments, the latter the distal compartments of the Golgi apparatus. BFA causes disassociation of the coat protein, β-COP from Golgi membranes, which then are re-distributed into endoplasmic reticulum, while monensin, as a Na+ ionophore, disrupts the ion gradients in the intracellular compartments (23, 40). It has been demonstrated that BFA blocks the release of newly synthesized protein, but has no effects on pre-formed granule-bound proteins (41). Although BFA and monensin have been widely used as inhibitors of protein secretion, the information concerning their effects on cytokine release, especially from granulated cells, is scarce. It has been reported previously that BFA inhibited the release of TNF-α and TGF-β from the rat mast cell line, RBL-2H3 (12, 42). In these studies, only the effect of BFA on long-term cytokine release was determined.

Given the well-characterized properties of BFA and monensin, it is reasonable to conclude that a cytokine is secreted through the vesicle transport pathway if its release from the cell is inhibited by BFA or monensin, while a cytokine is more likely to be secreted via a degranulation-related pathway if its immediate or long-term release is not blocked by BFA or monensin, and is temporally related to the release of histamine or β-hexosaminidase, two well-known mediators stored within the mast cell and basophil granules.

Initially, we examined the effect of BFA on degranulation by KU812 cells and mBMMC. There was no significant inhibition of the initial 20-min release of the granule-bound mediators, histamine and β-hexosaminidase, from either KU812 cells or mBMMC. These findings are in agreement with early reports that BFA would not interfere with the degranulation by RBL-2H3 cells (12, 42). However, BFA was shown to significantly inhibit the initial 20-min release of IL-6 from differentiated KU812 cells treated with or without A23187. This suggested that the increased initial 20-min release of IL-6 in response to A23187, which we could measure in differentiated KU812 cells, was not the result of degranulation, but rather due to a vesicular transport-dependent pathway initiated rapidly upon cell activation that could be inhibited by BFA.

Twenty-four-hour activation of mBMMC and differentiated KU812 cells in the presence or absence of BFA revealed that BFA alone or in combination with A23187 significantly inhibited IL-6 release. At the same time, BFA significantly increased the amount of IL-6 remaining inside these cells. The secretion of GM-CSF was inhibited significantly in the similar fashion by BFA. These data strongly suggest that vesicle transport-dependent mechanism is responsible for the ongoing 24-h secretion of these cytokines by mast cells.

To further confirm the effects of BFA on cytokine release from KU812 cells, we treated them with monensin in parallel with BFA for 20 min and for time points up to 24 h. We demonstrated that monensin, like BFA, did not inhibit β-hexosaminidase release.

**FIGURE 8.** Typical ultrastructural changes in murine BMMC incubated for 3 h under four different conditions: A, medium alone; B, treated with 1 μM A23187 alone; C, treated with 0.5 μg/ml BFA alone; and D, treated with 0.5 μg/ml BFA plus 1 μM A23187. Intact Golgi apparatus (arrow) and large numbers of high-density granules are located in untreated KU812 cells (A). BFA treatment resulted in the disappearance of classic Golgi structure without significant effect on the granulation of the cells (C). A23187-treated cells lost most of their granules, while they retained the Golgi apparatus (B). No Golgi apparatus was visible, and dramatically decreased granulation was observed in the cells treated with both BFA and A23187 (D).
from differentiated KU812 cells induced by calcium ionophore, A23187, but it significantly inhibited both 20-min and 24-h IL-6 release from KU812, providing further evidence that IL-6 is secreted via a vesicle-dependent pathway.

Although BFA and monensin had similar effects on mast cell degranulation and cytokine release, there were some differences between the two agents, e.g., BFA was a more efficient inhibitor of initial 20-min IL-6 release from differentiated KU812 cells, and it significantly inhibited both constitutive and ionophore-induced initial 20-min release of IL-6 from the mast cells, while monensin significantly inhibited only constitutive, but not ionophore-induced IL-6 release. These different effects of BFA and monensin may be due to the fact that BFA disturbs intracellular transport between the endoplasmic reticulum and the Golgi apparatus, while monensin acts later, between the medial and trans cisternae of the Golgi complex (23, 40). A23187-induced IL-6 release in mast cells may occur at the more proximal compartments of Golgi apparatus.

In most systems studied, secretion of proteins through the vesicle-dependent pathway is a spontaneous process, but our data indicate that this process can be promoted, if not initiated, by A23187. This observation could have important implications in that it suggests another level of regulation of the vesicle-dependent secretion pathway in differentiated KU812 cells. It is generally believed that the vesicle-dependent secretion (constitutive pathway) is regulated primarily at the synthetic level (43), but our data suggest that regulation of the secretion pathway used for the initial 20-min release of IL-6 by mast cells may also occur at the vesicular transport level, as has been demonstrated in pancreatic exocrine cells (43) and colonic epithelial cells (44).

It has sometimes been assumed that the short-term (within 20 min) induced release of mediators, e.g., cytokines, by basophils and mast cells is caused by degranulation. Depending on the mediator being studied, however, such early mediator production could represent a combination of newly synthesized and preformed granule-associated mediators. Our observations suggest that the initial 20-min release of IL-6 induced by A23187 treatment of differentiated KU812 cells is not predominately from granule-associated source. This observation is consistent with a recent report that the rate of 35S-labeled glycosaminoglycan transport from the Golgi apparatus to the cell surface was increased by IgE receptor stimulation in rat basophilic cells (45).

The data from our observations with mBMMC show that BFA not only blocked A23187-induced, but also Ag/IgE-induced release of IL-6 and GM-CSF over 24 h. This suggests that similar effects of BFA are observed with cells activated by other physiologic stimuli, such as IgE receptor cross-linking, as with ionophore activation.

Another interesting point emerging from the experiments with mBMMC is that the results dispute the common assumption that elevating intracellular Ca2+ level would have similar effects on mast cells as those induced by cross-linking IgE receptors. Although both A23187 and treatment of IgE-sensitized cells with IgE-specific Ag, DNP-HSA, significantly increased the production and release of IL-6 and GM-CSF by mBMMC, stimulation via the FceRI pathway was greater than 10-fold stronger than the calcium ionophore in inducing the release and production of either cytokine.

To confirm that decreased IL-6 and GM-CSF secretion from BFA-treated differentiated KU812 cells are due to an effect of BFA on protein secretion rather than on protein synthesis or mRNA regulation, we performed pulse-chase experiments with L-leucine and semiquantitative RT-PCR analysis. Our data demonstrate a nonsignificant 8% inhibition of overall protein synthesis in cells treated with BFA for 6 h and a significant 36% inhibition at 12 h. While the latter result might suggest some overall inhibitory effect of long-term BFA treatment, this could not account for the more than 98% inhibition of GM-CSF production and 94% inhibition of IL-6 production observed in A23187-activated cells in the presence of BFA. It should also be noted that more than 80% of the secreted IL-6 or GM-CSF response to ionophore activation alone in differentiated KU812 cells was complete after 6 h of treatment, further reducing any possibility that inhibition of protein synthesis at later time points could account for our observations. The RT-PCR analysis of both IL-6 and GM-CSF mRNA levels did not demonstrate any significant inhibition of cytokine expression in BFA-treated cells at any of the time points examined up to 24 h. Notably, GM-CSF mRNA levels, but not IL-6 mRNA levels, were increased in cells treated with A23187 compared with control cells. This finding is in keeping with previous observations from Northern blot analysis of rat peritoneal mast cells, which suggest a high degree of posttranscriptional regulation of expression of IL-6 (10). Taken together, the results from the pulse-chase experiments and mRNA analysis confirm that, as demonstrated in many other systems, the BFA appears to be acting mainly at the level of protein secretion blockade to inhibit the release of GM-CSF and IL-6.

Our electron-microscopic examination of mBMMC further provided morphologic evidence to support the selective effects of BFA on the intracellular transport system. The Golgi apparatus was not detectable in the cells treated with BFA in the presence or absence of A23187. This is in agreement with the observation by other groups working with BFA (36). Notably, the electron micrographs confirm that BFA does not inhibit the release of granules from mast cells.

Besides the two classical secretion pathways, a third secretion pathway has been proposed by Dvorak et al., termed piecemeal degranulation (39). This unique secretion model has been observed in basophilic and mast cells from a variety of species based on morphologic evidence that there is decreased condensation of the granules in basophilic and mast cells (39, 46), and on the more recent evidence that indicated that histamine and granule-associated Charcot-Leyden crystal protein were found within the small vesicles in activated basophils (47, 48). Unlike the vesicle-dependent secretion pathway, piecemeal degranulation is induced by stimulants, such as phorbol ester. In contrast to the explosive release of the granule contents in the event of typical mast cell degranulation, piecemeal degranulation is a slow and often incomplete release of the granule contents. Since BFA appears to have no effect on the release of preformed granule mediators at either 20 min postactivation or at later time points, it is unlikely that piecemeal degranulation is responsible for the observed cytokine release; however, without formal studies of the effect of BFA on the piecemeal degranulation process, we cannot exclude the possibility of some components of cytokine secretion through this pathway. Although it has been suggested that each secretory pathway carries different products (37, 40), a small proportion of IL-6 in KU812 cells may possibly be secreted via nonvesicle-dependent pathways, as IL-6 release was not completely inhibited by either BFA or monensin treatment.

In summary, we have demonstrated that initial 20-min release of IL-6 from differentiated KU812 cells was inhibited by BFA and monensin, two protein secretion blockers that had no effect on degranulation, suggesting that a mechanism exists for an initial 20-min induced release of IL-6 that is not degranulation dependent. We have also shown that the ongoing up to 24 h release of both GM-CSF and IL-6 from a human mast cell/basophil cell line and murine mast cells was inhibited by BFA and monensin, suggesting that the major secretion route for these two cytokines is a vesicular transport-dependent pathway.
Overall, it appears that mast cells release IL-6 and GM-CSF mainly through a vesicular transport-dependent pathway, the traditional protein secretion route, rather than through the degranulation-related pathway. The present work further supports the growing evidence that mast cell release of cytokines is under complex regulation, and that regulation of such cytokine expression in disease will require a different approach from the regulation of such cytokine expression in mast cell degranulation.

Acknowledgments
We thank Michael G. Blennerhassett for his advice on β-hexosaminidase assay; Laurie Nielsen, Ursula Kadel-Stolarz, and Anna Zgianzie for their excellent technical assistance; and Mary Kirikopoulos for her excellent secretarial assistance.

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