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QUERCETIN-INDUCED EXPRESSION OF RAT MAST CELL PROTEASE II AND ACCUMULATION OF SECRETORY GRANULES IN RAT BASOPHILIC LEUKEMIA CELLS

JAN TRNOVSKY, RICHARD LETOURNEAU, EMAN HAGGAG, WILLIAM BOUCHER and THEOHARIS C. THEOHARIDES*

Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA 02111, U.S.A.

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Abstracts—Rat basophilic leukemia (RBL) cells are considered to be similar to bone-marrow derived mast cells and to mucosal mast cells (MMC), the latter of which may be involved in inflammatory bowel diseases. RBL cells are not able to accumulate histamine and secretory granules under regular growing conditions. Here we show that the flavonoid quercetin, which inhibits mast cell secretion of histamine, also inhibited RBL cell proliferation and constitutive histamine release while it induced synthesis of rat mast cell protease (RMCP) II and triggered processes leading to accumulation of secretory granules. Cell viability was also retained in the presence of quercetin, whereas untreated cells did not survive past 6 days of growth. Quercetin did not affect the expression of mRNA for α-subunit of immunoglobulin E (IgE) receptor, but led to increased expression of mRNA for, and synthesis of RMCP II, which is a marker protein for MMC. Many of these granules showed metachromasia with toluidine blue after 3 days of growth, stained red with alcian blue counterstained with safranin after 8 days of growth, and contained electron dense material. Our results suggest that RBL cells have the capacity to progress to a more mature state and may lend themselves to further analysis of a growth regulator(s) with action similar to that of quercetin.

Key words: basophils, differentiation, histamine, mast cells, maturation, protease, quercetin, secretion

Basophils and mast cells are a primary source of histamine and other mediators, such as proteoglycans and proteases, stored in their secretory granules [1, 2]. The content of these granules can be released when the cells are triggered with immunoglobulin E (IgE)† and specific antigen [3, 4], or by a variety of non-immunologic stimuli [5, 6]. Both basophils and mast cells derive from the bone marrow, but while basophils are found fully differentiated in blood, mast cells enter the tissues as undifferentiated cells where they mature under the influence of factors such as interleukin 3 [7]. Connective tissue mast cells (CTMC) are found primarily in skin and lungs, and contain rat mast cell protease I (RMCPI I), while mucosal mast cells (MMC) are located in mucosa and contain RMCP II [7–9]. Rat basophilic leukemia (RBL) cells were originally developed in blood of rats after oral feeding with β-chlorethylamine [10], and have been adapted to culture. These cells have been shown to bind rat IgE with high affinity [11, 12]. Various sublines of RBL cells have been cloned and the 2H3 variant was selected for further work as a good histamine releaser upon stimulation with IgE and antigen [13]. RBL cells are considered immortalized MMC-like cells because of their low content of histamine and presence of chondroitin sulfate di-B proteoglycan and RMCP II rather than heparin and RMCP I [14].

Quercetin is a bioflavonoid known to inhibit histamine release from basophils and mast cells [15–17]. Here we report the effect of the long-term presence of quercetin on growing RBL cells. We have studied morphological changes, histamine content and RBL ability to release it, together with the expression of mRNA for the α-chain receptor of IgE and for RMCP II, as well as synthesis of RMCP II, to obtain some insight about RBL cell differentiation.

MATERIALS AND METHODS

Quercetin dihydrate. This compound was obtained from Sigma (St. Louis, MO), was dissolved in propylene glycol, filter-sterilized, and diluted in filter-sterilized propylene glycol so that 50 μL to 50 mL growth medium was added to obtain the concentrations indicated.

Cells. RBL-2H3 cells, provided by Dr. H. Metzger (NIH, U.S.A.), were grown in S-MEM medium (GIBCO, Grand Island, NY) supplemented with 15% fetal bovine serum, 100 U/mL penicillin, 0.1% streptomycin (GIBCO) and 0.5 mM calcium chloride either in 25 cm^2 tissue culture dishes (Corning, NY) or in six-well tissue culture dishes (Costar,

* Corresponding author: T. C. Theoharides, Ph.D., M.D., Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Tel. (617) 956-6866; FAX (617) 956-6456.

† Abbreviations: BSA, bovine serum albumin; CTMC, connective tissue mast cells; DMSO, dimethyl sulfoxide; IgE, immunoglobulin E; FcεRI, immunoglobulin E receptor; MMC, mucosal mast cells; RBL, rat basophilic leukemia; and RMCP, rat mast cell protease.

Cambridge, MA). Cells were plated at a density of 0.2×10^6 cells/mL taken from the 3-day-old culture grown under the same conditions. Cells were growing in an atmosphere of 5% CO_2 95% O_2 at 37°.

Determination of cell viability. Two methods were used for determination of cell viability: trypan blue exclusion and the ability of RBL cells to grow in fresh medium after treatment with quercetin. Cells either treated or not treated with quercetin were washed, trypsinized and pelleted by centrifugation for 5 min at 400 *g*. Cells were then resuspended in S-MEM medium, 0.1% trypan blue solution was added, and cells were counted under the microscope. Viability is expressed as the percentage of cells that did not take up trypan blue.

Alternatively, RBL cells from 3-day-old cultures with quercetin were diluted to a density of 0.2×10^6/mL with fresh medium and grown for 24 hr. At that point, the cells were counted and compared to cells grown similarly without quercetin which constituted a 100% viability. Counts were taken in triplicate from triplicate cultures.

Histamine measurement. Histamine in cells or histamine released to growth medium was estimated by a radioenzymatic method, essentially a modification of the method described by Kaplan *et al.* [18].

RBL cells growing attached were washed, trypsinized and harvested by centrifugation at 400 *g* for 5 min in 1.5-mL Eppendorf tubes. Cells were then resuspended in water (5×10^6/0.2 mL). This suspension was sonicated in a Branson 1200 ultrasound device for 10 min, vortexed for 5 min, and pelleted by 10-min centrifugation at top speed in an Eppendorf microfuge. Histamine in growth medium taken from growing cells or from cells treated with IgE and antigen was estimated after pelleting the cells by centrifugation at 400 *g* for 5 min. To 20 μL of supernatant taken from disrupted cells or to 20 μL of growth medium 2 μL of 20% perchloric acid, 6 μL 1 N NaOH and 100 μL of 0.5 M sodium phosphate, pH 7.4, were added. The precipitate was pelleted by 5-min centrifugation at top speed in an Eppendorf microfuge, and aliquots were taken from the supernatant for further histamine assay. In 50 μL of reaction mixture for histamine assay 5–25 μL sample, 5 μL rat kidney histamine methyl transferase and *S*-[*methyl*-^{3}H]-adenosyl-L methionine (73.8 Ci/mmol, Amersham) were present. Isotope and enzyme were diluted with 0.05 M sodium phosphate, pH 7.4, for optimal conditions. The reaction mixture was incubated for 1 hr at 37°, following which 20 μL of 1.5 M perchloric acid, 20 μL of 10 M NaOH and 500 μL of freshly prepared toluene–isoamyl alcohol (4:1, v/v) were added, and the mixture was shaken for 10 min. After a 10-min centrifugation at 2000 *g*, 0.3 mL was taken from the upper phase and 4 mL of Aquasol (New England Nuclear, Boston, MA) scintillation fluid was added. Radioactivity was measured in a beta counter, and the concentration of histamine was computed by using a standard curve. Samples were always performed in duplicate from duplicate cultures.

Histamine release upon stimulation with IgE and antigen. Cells (1×10^6) in a six-well tissue culture dish were washed with S-MEM containing 1 mg/mL bovine serum albumin (BSA) but without CaCl to reduce spontaneous secretion. They were then sensitized in the same medium for 30 min at 37° with 2 mL of mouse monoclonal anti-DNP IgE (500 ng/mL). After sensitization, the cells were washed again and treated for 30 min at 37° with 2 mL of DNP-BSA (1 μg/mL) in the same medium, but now supplemented with 0.5 mM calcium to permit secretion. Control samples without IgE were run simultaneously in the presence of 0.5 mM Ca^{2+}, and these values represented nonspecific release and were subtracted from the overall histamine release. Histamine released in the medium was estimated as described earlier in Materials and Methods. Mouse monoclonal anti-DNP-IgE and DNP-BSA were provided by Dr. Fu-Tong Liu, Scripps Clinic, La Jolla, CA.

Measurement of RMCP II. The presence of this enzyme in RBL cells was identified by using an ELISA kit (Moredun, Edinburgh, U.K.) on whole cell homogenates obtained at different days of growth in the presence of quercetin.

Measurement of RNA synthesis in the presence of quercetin. RBL cells (5×10^5) were seeded in 2.5 mL growth medium, 2.5 μL quercetin was added, and cells were grown in six-well tissue culture dishes for 20 hr. [5,6-^{3}H]Uridine (10 μCi, 36.5 Ci/mmol, New England Nuclear) was added, and cells were grown for an additional 4 hr, washed, trypsinized and precipitated with 10% trichloroacetic acid (TCA). The precipitate was collected on glass Millipore filters and washed with 5% TCA and with 70% ethanol. Radioactivity of the precipitate was then determined in a liquid scintilllation counter. Samples were performed in triplicate.

Staining of RBL cells. Cells were grown on chamber slides (Lab-Tek, NUNC, Naperville). After washing with phosphate-buffered saline, cells were fixed for 5 min with 10% formaldehyde, 10% acetic acid (pH 2.5). In one chamber, cells were stained for 5 min with 0.5% alcian blue in 10% acetic acid (pH 2.5), washed with phosphate-buffered saline for 5 min, and were counterstained with 0.1% safranin in 10% acetic acid (pH 2.5). In the second chamber, cells were stained for 5 min with 0.5% toluidine blue at pH 2.5. Cells were then photographed using a Nikon inverted microscope.

Electron microscopy of RBL cells. RBL cells growing attached were washed at 4° with a solution containing 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 1 mg/mL BSA, pH 7.0, and fixed for 1 hr at room temperature with 3% glutaraldehyde in 0.1 M cacodylate-HCl buffer (pH 7.2). At the end of this fixation period, cells were scraped and pelleted at 2000 rpm in a Sorvall Clinical Table Top centrifuge. Cells were then washed with cacodylate buffer and pelleted two more times. Cells were then postfixed with 1% OsO_4 in cacodylate buffer for 1 hr at room temperature, washed with cacodylate buffer and pelleted. Cells were then infiltrated with two 10-min changes of 100% propylene oxide followed by overnight exposure to a 1:1 mixture of propylene oxide and DMP-30. The next day cells were embedded in Epon with DMP-30. Embedded cells were placed in a 56° oven to polymerize for 48 hr.

Samples were thick and thin sectioned on Sorvall MT-1 and MT-2B equipped with glass and diamond knives, respectively. Sections (1000 Å) were picked up on 300 mesh copper grids and stained with both uranium and lead salts. Then the sections were examined and photographed using a JEOL JEM-100s transmission electron microscope operated at an accelerating voltage of 80 kV.

Northern blot analysis. Rapid isolation of total RNA from RBL cells treated with quercetin was done after washing with ice-cold phosphate-buffered saline according to a procedure described previously ([19], Chapter 7.10). RNA was separated on a 1% agarose gel containing formaldehyde and transferred to a nitrocellulose membrane by northern blotting according to standard procedures described in Ref. 19 (Chapters 7.43 and 7.46). RNA was probed with three probes: probe "A" was an antisense oligonucleotide coding for the first 17 amino acids from the amino-terminal part of RMCP II [20]; probe "B" was generated by T7 polymerase transcription of plasmid Edp16 linearized by *Sca* I. The plasmid has the full length of the immunoglobulin E receptor fragment for rat α-chain IgE receptors (FcεRI) inserted into the *Eco*RI site of pGEM-3zf(−) and was provided by Dr. J. P. Kinet (NIH) [21]. Transcription of Edp 16 was done in 50 μL of reaction mixture containing chemicals from a Promega kit, 8 μL of 5× transcription buffer, 4 μL of 100 mM dithiothreitol, 1.6 μL RNasin (40,000 U/mL), 4 μL [α-^{32}P]UTP (800 Ci/mmol), 4 μL (2 μg) linearized plasmid DNA and 1 μL T7 polymerase (20,000 U/mL). After a 1-hr incubation at 37°, 1.2 μL of DNase Q (1000 U/mL) was added and the mixture was incubated for 15 min at 37°. The phenol-extracted probe was purified on a Nensorb (New England Nuclear) column; probe "C" was antisense oligonucleotide coding for amino acids 72–88 of RMCP II [20]. Oligonucleotides were prepared by Oligos Etc. Inc. (CT) and were labeled with [γ-^{32}P]ATP (>5000 Ci/mmol, Amersham) and T4 polynucleotide kinase (IBI 10 U/1 μL) as described previously [19]. Oligonucleotides were purified also on a Nensorb column, but 20% *n*-propanol instead of 50% methanol was used as an elution buffer. Filters were prehybridized for 2 hr at 40° in a solution containing 10× SSC (SSC = 0.15 M sodium chloride + 0.015 M sodium citrate), 50% formamide, 1× Denhardt solution, 0.5 mg/mL denatured salmon sperm DNA, 0.25 mg/mL yeast tRNA and 10% dextran sulfate. Hybridization with radioactively labeled probes was done in the same solution for 16 hr at 40°. Northern blots were washed for 30 min at 24° in 0.2× SSC, 3 min at 60° in 2× SSC and

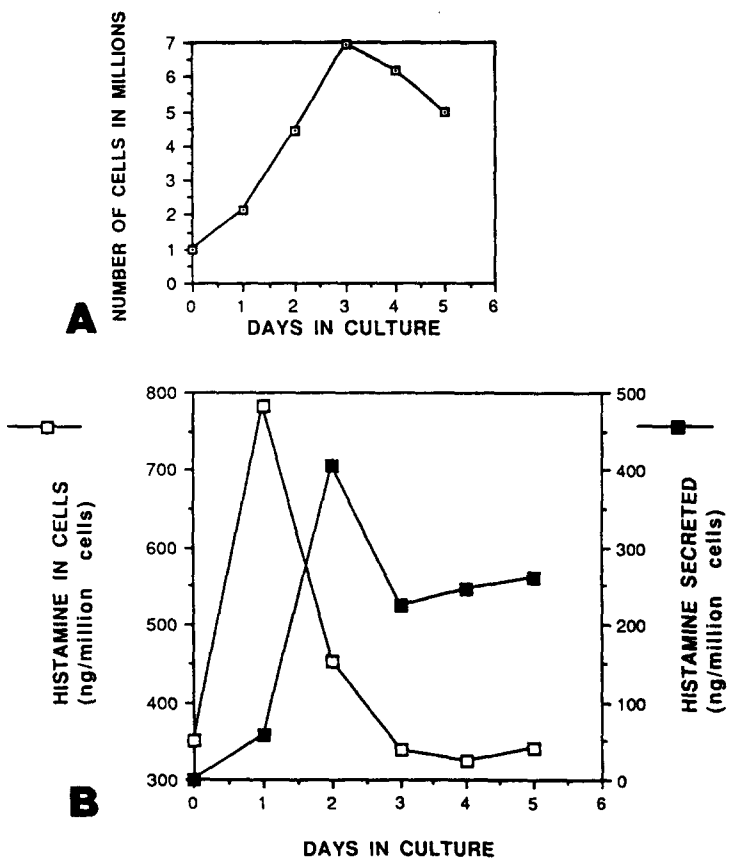

Fig. 1. Synthesis and constitutive release of histamine from RBL cells. Only attached cells were counted and were analyzed for histamine content. (A) Growth curve (N = 4). (B) Synthesis and release of histamine during growth (N = 2).

Table 1. RMCP II content in RBL cells

Days in culture	RMCP II content (ng/mL)	
	Control	Quercetin (100 μM)
1	0.49 ± 0.04	0.90 ± 0.25
2	0.50 ± 0.08	0.98 ± 0.19
3	0.36 ± 0.05	1.60 ± 0.70
4	0.54 ± 0.08	1.59 ± 1.00
5	0.60 ± 0.17	3.80 ± 0.28
6	0.54 ± 0.30	3.82 ± 0.25

Results are means ± SD (N = 3).

30 min at 24° in 0.2× SSC. The blot probed with RNA generated from plasmid DNA was treated with RNase after the first wash. Details of this procedure are described elsewhere [19].

For the northern blot analysis, the same RNA was used for each blot, and transfer of RNA for all three blots was done simultaneously from the same agarose gel; the efficiency of the transfer was determined by staining RNA on the blotting membrane with ethidium bromide. Two separate experiments for each growth period were done with similar results. A fresh blot was used for every probe because in our hands reprobing leads to the loss of RNA from the blot.

RESULTS

Synthesis of histamine and RMCP II from RBL cells. Figure 1A shows the growth of RBL cells over 5 days. Figure 1B shows that histamine was synthesized during the early exponential phase of growth and was released from RBL cells constitutively during late exponential and stationary phases of growth. RMCP II was barely detectable during any of these days (Table 1).

Release of histamine upon the stimulation of RBL cells with IgE and antigen. Histamine content and the ability of RBL cells to release it upon the stimulation with IgE and antigen are shown in Table 2. Even though 1-day-old cells not treated with quercetin had high histamine content, they had poor ability to release it upon stimulation with IgE and antigen. It was evident that RBL cells acquired the best ability to release histamine upon stimulation with IgE and antigen when treated with 100 μM quercetin for 6 days.

Table 2. Histamine content and release from RBL cells

Conditions	Histamine content (ng/10^6 cells)	Histamine released* (% total)
1-Day-old not treated	785 ± 145	7.5 ± 2.5
2-Day-old not treated	450 ± 150	5.0 ± 2.5
3-Day-old not treated	350 ± 65	40.0 ± 4.8
6-Day-old plus 100 μM quercetin	920 ± 170	78.5 ± 5.0

Results are means ± SD (N = 6).
* RBL cells were stimulated with IgE and antigen as described in Materials and Methods.

Effect of quercetin on the growth and viability of RBL cells. Quercetin added at the beginning of growth prevented RBL proliferation in a concentration-dependent manner (Fig. 2A). RMCP II became detectable (3.82 ± 0.25 ng/mL) at day 6 of culture in the presence of quercetin (Table 1). Even though 80 μM quercetin did not fully stop cell proliferation after 6 days of growth, 100 μM quercetin did (data not shown). Consequently, 100 μM was used in experiments requiring the long-term presence of quercetin. The presence of quercetin during 24 hr of growth resulted in a concentration-dependent inhibition of histamine secretion into the medium and led to its concomitant accumulation in RBL cells (Fig. 2B).

The viability of RBL cells treated with 100 μM quercetin for 6 days determined by the trypan blue exclusion method was 96 ± 2.5% (N = 6) and compared with that of 6-day-old cells not treated with quercetin (6.5 ± 2.5%, N = 6). When cells treated with 100 μM quercetin for 6 days were replated at a density of 0.2 × 10^6/mL and grown for 24 hr in fresh medium, viability was 98 ± 1.8% (N = 6), indicating that there was no significant decrease in the number of cells. The viability of these replated cells after 24 hr of growth was 96 ± 1.8% (N = 4) by trypan blue exclusion.

Analysis of RNA synthesized in RBL cells growing in the presence of quercetin. Quercetin up to 20 μM suppressed RNA synthesis during the first 24 hr of growth, but increasing its concentration permitted new RNA synthesis (Fig. 3). Because histamine accumulated in RBL cells during growth in the presence of quercetin, the possibility that differentiation specific genes were expressed as a result of new RNA synthesis was examined further. The mRNAs for IgE receptor and for RMCP II were chosen as possible differentiation specific genes [20, 22, 23].

RNA synthesized in RBL cells grown in the absence and presence of quercetin was analyzed by northern blots. Figure 4-II shows that mRNA for the α-chain of IgE receptor was synthesized steadily both in the absence (lanes a, b, c, d, e) and presence (f, g, h) of quercetin, but apparently had a higher expression rate in proliferative cells grown without quercetin. In contrast, mRNA for RMCP II was maximally expressed at day 6 of growth in the presence of 100 μM quercetin, whether probed with an oligonucleotide taken from the amino-terminal part (lane h in Fig. 4-I) or from the middle of RMCP II gene (lane h in Fig. 4-III). Because the same amounts of total RNA taken from different phases of cell growth were used for northern blot analysis, our results suggest that at day 6 of growth in the presence of quercetin mRNA for RMCP II is either specifically expressed or synthesized at a higher rate compared with other types of mRNA. These findings indicate that quercetin induces proliferative

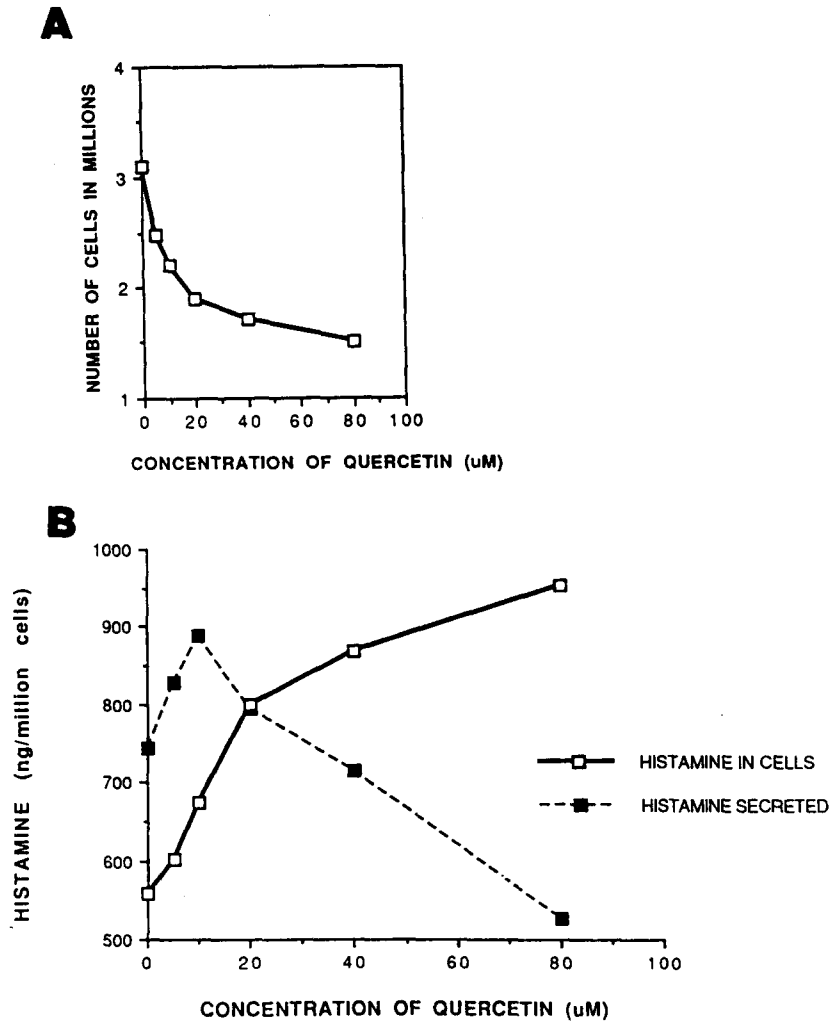

Fig. 2. Effect of quercetin on the growth of RBL cells. The number of cells (A) and the amount of histamine (B) were measured after 24 hr of growth. Quercetin was dissolved in propylene glycol and 2.5 μL per 2.5 mL of medium was used to obtain the concentrations indicated (N = 2).

quiescence in RBL cells during which synthesis of mRNA is not stopped completely, but continues at least for some proteins, presumably specific for growth-arrested cells.

Morphology of RBL cells grown in the absence and presence of quercetin. When 2-day-old RBL cells grown in the absence of quercetin were stained with alcian blue and counterstained with safranin, approximately 30% of the cells contained a small number of granules that only stained blue with alcian blue, but did not stain red with safranin (Fig. 5A). These cells did not contain granules stainable with toluidine blue (data not shown).

Three-day-old cells not treated with quercetin contained more granules, some of which stained with toluidine blue (Fig. 5B). However, no more granules were apparent during further culture; in fact, RBL cells lost viability and floated after 3 days of growth unless quercetin was present. RBL cells growing for 6 days in the presence of 100 μM quercetin contained (approximately 50% of the cells) a large number of granules heavily stained with toluidine blue (Fig. 5C), but still not with safranin. When the cells were grown for 8 days in the presence of 100 μM quercetin, about 10% of the cells contained granules that stained red with alcian blue, counterstained with safranin (Fig. 5, D and E).

Figure 6A is an electron micrograph of a typical 6-day-old RBL cell grown in the absence of quercetin. It contained a large, not segmented nucleus, and a small number of granules, the majority of which did not contain electron dense material (Fig. 6A). On the other hand, cells grown for 6 days in the presence of 100 μM quercetin showed many more granules containing fine electron dense material (Fig. 6B). The degree of granule maturation in such cells varied, as can be seen in Fig. 6C, where the granules contain strong electron dense material. The difference between cells grown in the absence and presence of quercetin can be appreciated at high

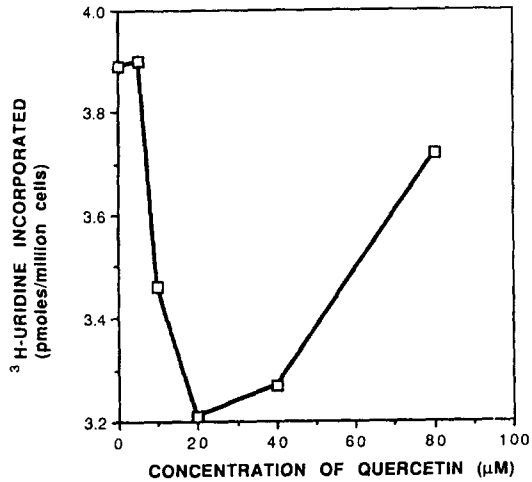

Fig. 3. Effect of quercetin on RNA synthesis in RBL cells. Cells were labeled for 4 hr prior to the end of a 24-hr growth period as described in Materials and Methods (N = 2).

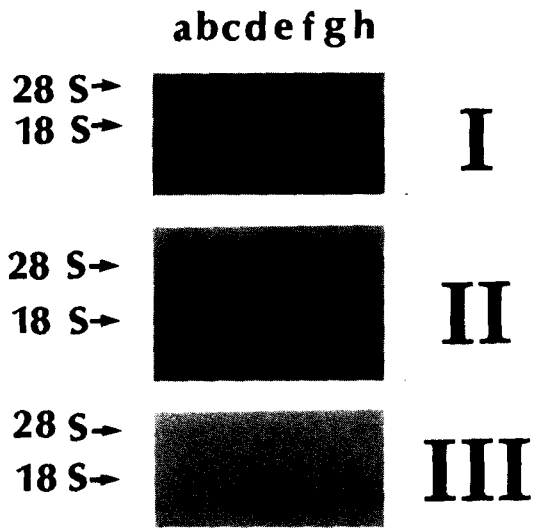

Fig. 4. Northern blot analysis of RBL RNA. Cells were grown for 1 (a), 2 (b), 3 (c), 4 (d) and 5 (e) days in the absence and 2 (f), 4 (g) and 6 (h) days in the presence of 100 μM quercetin. In blot I, 4 μg of total RNA was probed with oligonucleotide 5-ATG/GGC/CAT/GTA/AGG/-GCG/GGA/GTG/AGG/AAT/AGA/CTC/CAC/ACC/-ACC/GAT/AAT-3 (probe "A") and in blot III with oligonucleotide 5-ATG/AAG/ATT/GGG/AAC/GGA/-GTT/GTA/ACT/TTC/GTG/AAT/GAT/TTG/TTT/-TTC/GAC-3 (probe "C"). In blot II, 14 μg of total RNA was hybridized with RNA probe "B" coding for rat α-chain, FcεRI receptors. The same RNA samples were used for each blot. Transfer of RNA for all three blots was done simultaneously from one agarose gel. The specific activities of probes A and C were 1.6 and 1.2×10^8 cpm/μg, respectively. The specific activity of probe B was 3.5×10^7 cpm/μg. During hybridization procedures 220 million counts of probes A and C and 40 million counts of probe B were present (details in Materials and Methods) (N = 2).

magnification in panels D and E of Fig. 6, where secretory granules are absent and present, respectively.

DISCUSSION

RBL cells have long been used as a model for studying IgE-mediated processes leading to the secretion of histamine. However, RBL cells are not able to accumulate histamine and secretory granules, which seems to be a characteristic of differentiation, even at stages expressing increased membrane receptors for IgE [22]. In accordance with previous findings [22], we observed a large constitutive leakage of histamine during the cell growth of RBL cells (Fig. 2B). In fact, during growth arrest in the late stationary phase of growth, where the majority of cells are in the G_1 phase [22] and should express features of differentiation, the level of histamine dropped to the lowest possible value. After the growth arrest at day 3, cells slowly detached from the support of the tissue culture dish. Attached cells from day 3 contained only a small number of granules, the majority of which were not stainable with toluidine blue (Fig. 5B). Floating cells from day 3 contained large toluidine blue stainable granules (data not shown), but were not suitable for further study because of low viability (only 60%). The viability dropped even further by day 6 to 6.5%, making such cells useless for any comparative studies. To be able to study changes associated with maturation differentiation processes in RBL cells, quercetin was chosen as a possible differentiation-triggering agent because of its property to block histamine release from basophils and mast cells [15–17]. It was not known however, if such an event could be permanent in growing cells and if this could lead to histamine accumulation, which seems to be a prerequisite for differentiation in mast cells [8]. Here we show that quercetin, if added at the beginning of growth (Fig. 2B) and left in medium for 6 days, stopped constitutive release of histamine and permitted cells to increase histamine content by 17% (Table 2), synthesize RMCP II and accumulate electron dense granules. These results extend previous findings indicating that quercetin can inhibit stimulated histamine release from MMC [24, 25].

Long-term presence of 100 μM quercetin was not cytotoxic for RBL cells. This finding is in accord with that of Mitsumori *et al.* [26]. Those authors showed that quercetin blocks progression of human gastric cancer cells from G_1 to the S phase of growth and after removal of quercetin, cells moved through the subsequent S, G_2, and M phases as a synchronized cell population. In all previous publications, dimethyl sulfoxide (DMSO) was used as a solvent for quercetin, but DMSO alone can cause differentiation changes in human promyelocytic leukemia cells [27]. Therefore, propylene glycol was substituted as a solvent and in the concentration used had no influence on the proliferation of RBL cells (results not shown).

It has been shown that quercetin and other flavonoids have the ability to inhibit many enzymatic systems *in vitro* (reviewed in Ref. 28), but it is not known what is the primary target for quercetin in

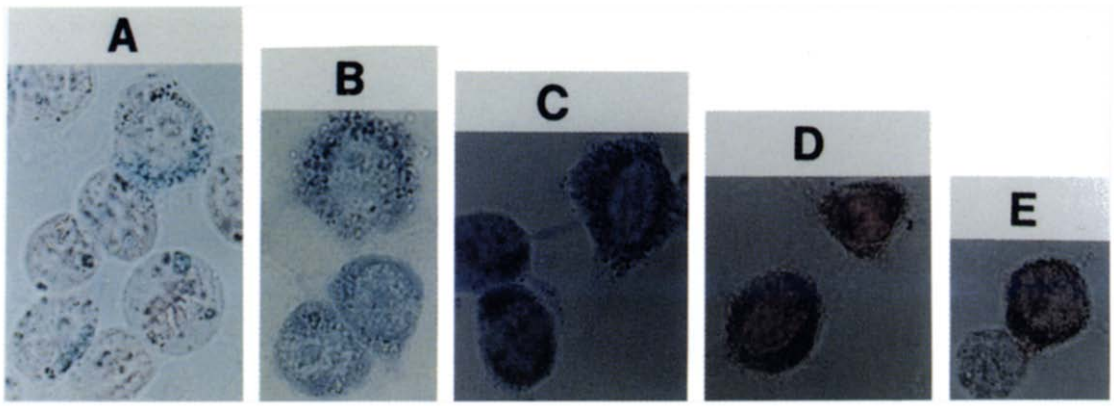

Fig. 5. Light micrographs of RBL cells grown in the absence and presence of quercetin. (A) Two-day-old cells grown in the absence of quercetin, (B) three-day-old cells grown in the absence of quercetin, (C) six-day-old cells grown in the presence of quercetin (100 μM), and (D and E) eight-day-old cells grown in the presence of quercetin (100 μM). A, D and E: RBL cells were stained with alcian blue and counterstained with safranin, while in B and C they were stained with toluidine blue (N = 8). Magnification: 100×.

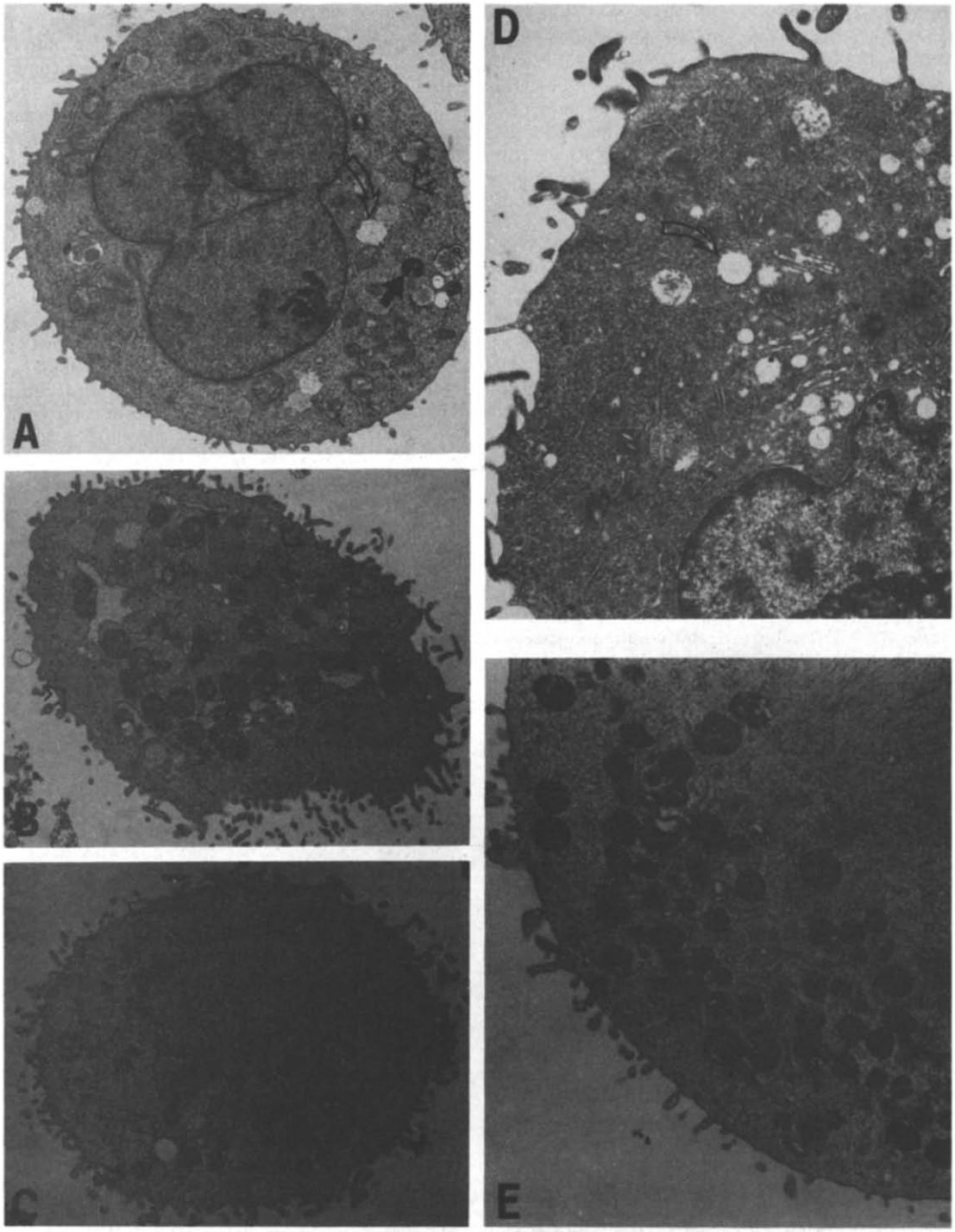

Fig. 6. Electron micrographs of RBL cells grown in the absence and presence of quercetin. (A) Six-day-old cell grown in the absence of quercetin; the nucleus of this cell is in the process of division; note abundance of mitochondria. A few granules are present: one entirely empty (curved open arrow), one filled with fine material (open arrow head), while another is completely filled with electron dense material (solid arrow). Magnification: 10,000×. (B) Six-day-old cells grown in the presence of quercetin (100 μM); note numerous granules filled with fine, faintly electron dense material (open arrow heads). Magnification: 10,000×. (C) Six-day-old cell grown in the presence of quercetin. Note that most of the granules are filled with electron dense material (solid arrow), while one (open arrow head) has fine material and one (curved open arrow) is empty. Magnification: 10,000×. (D) Part of the cytoplasm of a six-day-old cell grown in the absence of quercetin; note active Golgi apparatus and numerous empty (curved open arrow) granules. Magnification: 17,000×. (E) Part of the cytoplasm of a six-day-old cell grown in the presence of quercetin. Note numerous granules at different stages of filling with electron dense material, such as one containing fine material (open arrow head) and one almost completely filled with electron dense material (solid arrow). Magnification: 17,000×.

living cells. Because quercetin is able to intercalate into model membranes [29], it is reasonable to believe that the first targets would be membrane proteins or enzymatic systems. Because of well known inhibition of protein kinase C by quercetin [30, 31], this enzyme may be the target for quercetin because of its membrane localization and its involvement in regulation of secretion [32]. Quercetin has also been shown to bind to type II estrogen binding sites leading to inhibition of growth of ovarian tumor cells [33]. However, a putative Ca^{2+}-dependent protoplast ATPase could also be the primary target, since quercetin has been shown to inhibit membrane ATPases [34, 35]. Interestingly, quercetin is able to bind to ATP binding sites of different enzymes and at low concentrations is able to replace the nucleotide and may activate the reaction [35]. This could explain why quercetin up to 10 μM induced histamine secretion in growing RBL cells (Fig. 1B).

Our study indicates that quercetin in concentrations up to 20 μM inhibited RNA; however, at higher concentrations (20–100 μM), there was an increase of RNA synthesis during the first 24-hr growth period. These data could be explained assuming that in order for qualitatively new RNA to be synthesized in quercetin-treated cells cessation of the previously synthesized RNA is necessary. Quercetin-induced RNA synthesis could be differentiation-specific because mRNA for the differentiation-specific protein (RMCP II) was overexpressed. Others have shown that RBL cells contain a very low level of RMCP II, hardly detectable with antibody [36]. Our experiments suggest that the presence of quercetin or other differentiation-inducing agents may be necessary for inducing higher levels of RMCP II.

At the present time, we are not able to explain the presence of two strong signals for RMCP II in northern blot analysis, both in the proliferative stage (Fig. 4, Ib) and in cells growth-arrested by quercetin (Fig. 4, Ih and IIIh). It has been reported that RMCP I and at least six distinct serine proteases are expressed in various combinations in different mast cell populations [37]. It has also been suggested that RMCP II belongs to the family of rat serine proteases one of which has been partially sequenced from the genomic clone of RBL cells [20]; however, only the amino-terminal part from the coding region was sequenced and this part is highly homologous to RMCP II (only 4 amino acids from 17 are changed). In this region, two stretches, 15 and 30 bases long, are identical to the oligonucleotide probe selected for hybridization in the northern blot shown on Fig. 4-I; therefore, the possibility that this protease is expressed in proliferative RBL cells cannot be excluded. This or another serine protease(s) expressed in proliferative cells may be indispensible for proliferation as it has been shown for the serine protease myeloblastin [38, 39]. Down-regulation of myeloblastin causes growth arrest and differentiation of promyelocytic leukemia cells. Because of high homology between serine proteases in mast cells (compare sequences in 20, 37, 40 and 41), other genes or regulatory sequences in serine proteases should be used in the future as differentiation-specific marker genes.

A higher histamine level and higher expression rate for RMCP II during the long-term presence of quercetin were accompanied with other differentiation-specific events in RBL cells, like accumulation of granules stainable with toluidine blue and safranin, as well as accumulation of electron dense material. The appearance of cells containing mature granules stainable red with safranin suggests that RBL cells could have the potential for acquiring the phenotype of CTMC. Trans-differentiation between mouse MMC and CTMC was proved possible *in vivo* [39]. Our results suggest that this process would be nonproliferative and *in vivo* would require the presence of factors with action similar to that of quercetin. Formation and accumulation of mast cell granules in heat-sensitive, but not in cold-sensitive growth-arrested mutants isolated from an undifferentiated mastocytoma cell line [42] indicate that gene products are involved in the transition from the proliferative to a quiescent, presumably more mature state. However, it was shown recently that granule maturation, as judged by histochemistry, did not necessarily correspond to the full complement of mature granule content in bone-marrow derived mast cells grown in the presence of interleukin 3 of recombinant c-*kit* ligand [43]. Consequently, analyses of gene activation, gene product expression and granule ultrastructure are required in the study of mast cell differentiation/maturation.

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